SZÖVETI FEJLŐDÉST, REGENERÁCIÓT, ÖREGEDÉST ÉS PATOLÓGIÁS ELVÁLTOZÁSOKAT SZABÁLYOZÓ INTRA- ÉS INTERCELLULÁRIS JELÁTVITELI FOLYAMATOK VIZSGÁLATA EGÉR ÉS HUMÁN IMMUNSZÖVETI MODELLEKBEN

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TARTALOMJEGYZÉK

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1. BEVEZETÉS

A biológiai folyamatokat bonyolult sejten belüli és sejten kívüli jelátviteli hálózatok szabályozzák. Úgy az embrionális fejlődést, mint a felnőtt szövetek napi működését, szöveti regenerációját és öregedését ezek az összetett jeltovábbítási hálózatok irányítják. Nem meglepő tehát, ha a jelátviteli utak hibái patológiás elváltozásokhoz vezetnek. Ezért a jelátvitel megértése fejlődési rendellenességek és felnőttkori betegségek mechanizmusának feltérképezéséhez nélkülözhetetlen. Ez a tudás szolgál alapul a terápiás célok azonosításához, farmakológiai hatóanyagok bevizsgálásához és új terápiás módszerek kidolgozásához, illetve akár az öregedés folyamatának lelassításához is.

Ennek ellenére, évtizedeken keresztül még a sejten belüli és sejten kívüli kommunikáció alapját képező molekuláris kapcsolatok biokémiai és genetikai feltérképezése is nehézségekbe ütközött. Amikor végül a technikai problémák megoldódni látszottak, a felhalmozott információ sokszor ellentmondásosnak bizonyult és a kialakult jelátviteli dogmák nem magyarázták a fiziológiásan megfigyelhető folyamatokat. Ekkor vált nyilvánvalóvá, hogy a jelátvitel vizsgálata, a jelátvitel biológiai rendszerekben történő modellezése és a kísérleti eredmények értelmezése alapos átgondolást igényel.

Munkám fő irányvonala együtt evolválódott a jelátvitel megértéséhez nélkülözhetetlen kísérletes megközelítési módokkal és értelmezési koncepciókkal. Kollégáimmal a fenti meggondolások figyelembevételével végeztük jelátviteli kísérleteinket, hogy ez a nagyon bonyolult, és meglepő módon, nagy pontossággal működő biokémiai hálózat fiziológiás szinten történő megértéséhez hozzájáruljunk.

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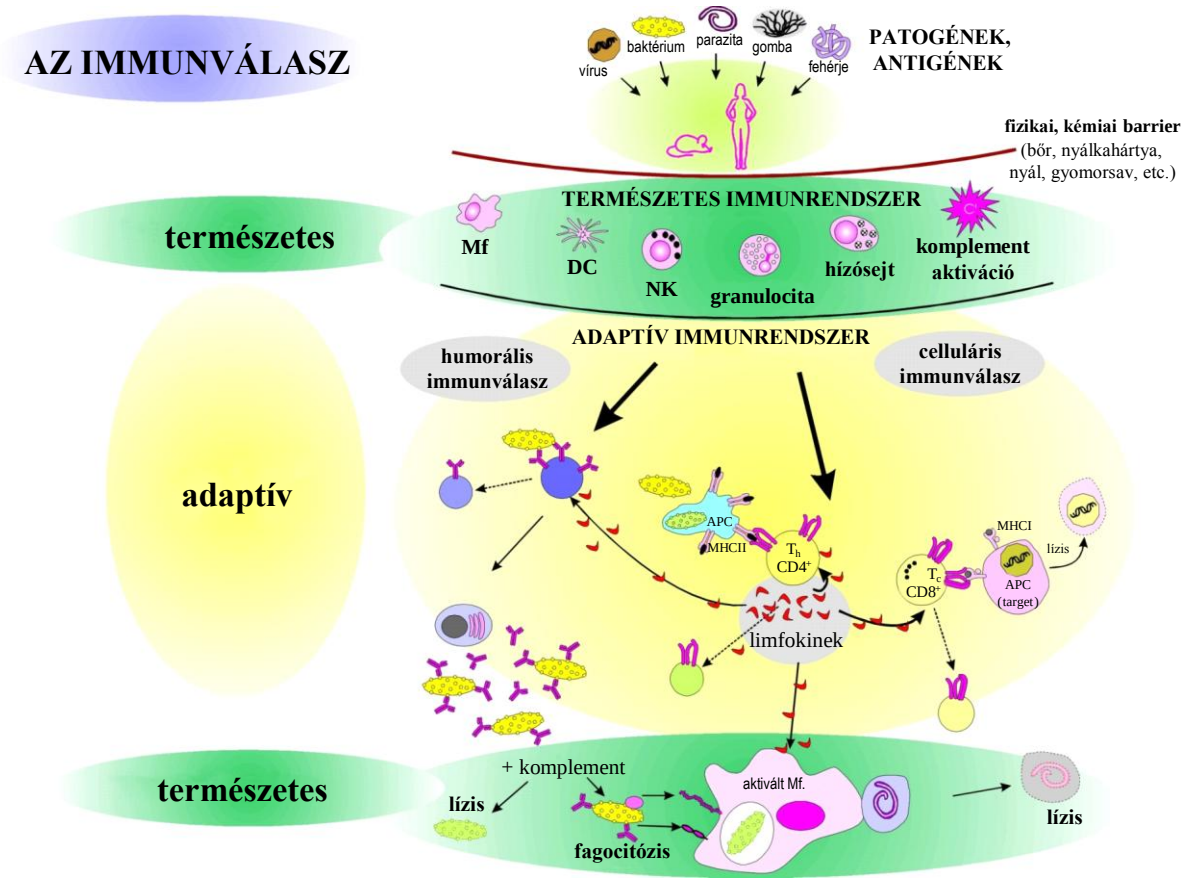
2. IRODALMI ÁTTEKINTÉS

Az értekezésemben elsősorban azoknak a vizsgálatainknak az eredményeit ismertetem, amelyek során a protein kináz C és a Wnt jelátvitel főbb tulajdonságait és fiziológiás jelentőségét tanulmányoztuk sejtvonalakon, normál tímusz-szöveti modelleken, öregedés során és az immunrendszer patológiás elváltozásaiban.

2.1. Az immunrendszer

2.1.1. Az immunrendszer sejtjei és szerepük

A szervezet védekezési folyamatait az immunrendszer irányítja. Az immunrendszer központi szövetei termelik az immunsejteket és irányítják azok fejlődését, majd érését. A kifejlett immunsejtek általános és specifikus antigénfelismerő képessége biztosítja a fertőzések, illetve daganatos betegségek elleni védelmet. Az immunrendszer fő sejtes elemeit a természetes vagy veleszületett, illetve a szerzett immunitásban résztvevő kategóriába soroljuk (1. ábra). Mind a természetes, mind a szerzett immunitás sejtes elemei speciális szöveti mikrokörnyezetben fejlődnek ki, ahol a környezetből származó sejten kívüli jeleket a sejten belüli jelátviteli rendszereik segítségével fordítják le és hasznosítják fejlődésük, differenciálódásuk és aktivációs mechanizmusaik megvalósításához.



1. ábra: Az immunrendszer és funkciója

Az immunsejtek fejlődéséhez a nyirokszervek biztosítják a szöveti mikrokörnyezetet, melyek között megkülönböztetünk elsődleges és másodlagos nyirokszerveket. Az elsődleges nyirokszerv a csontokban található vörös csontvelő, valamint a szegycsont alatt elhelyezkedő csecsemőmirigy. A vörös csontvelőben jönnek létre az immunsejtek őssejtjei, majd fejlődnek ki a természetes immunitás sejtjei, mint a makrofágok, granulociták és dendritikus sejtek, továbbá a B limfociták, illetve a T limfociták prekurzorai, melyek közül a T limfociták a tímuszban (csecsemőmirigy) érik el teljes fejlettségi szintjüket. A másodlagos nyirokszervek: lép, mandulák, nyirokcsomók biztosítják az antigén-specifikus immunválasz és az antigén-specifikus immunsejtek aktiválódásának helyszínét. Immunrendszerünk sejtjei naponta millió számra termelődnek, differenciálódnak és szelektálódnak, illetve lépnek aktív állapotba szervezetünk integritásának védelmében. Nem meglepő tehát, hogy az immunsejtek kifejlődésében vagy aktiválódásában fellépő zavarok súlyos következményekkel járnak. Az osztódási zavarok proliferációs betegségek – pl. akut vagy krónikus mieloid leukémia, limfoblasztos leukémia stb.- kialakulását idézik elő. A specifikus szelekciós folyamatokban fellépő zavarok az immunrendszer saját struktúráival szemben kialakult toleranciájának

megszűnéséhez vezetnek, amely autoimmun kórképek kialakulását eredményezi. Az immunrendszer fokozott „érzékenysége” is a specifikus immunitás zavaraira vezethető vissza, amely minimális környezeti ingerekre fokozott válaszadás kialakulását eredményezheti és allergiás megbetegedések kialakulásához vezet. Az immunrendszer válaszadási képességének teljes vagy részleges megszűnése pedig a fertőzések megállíthatatlan ismétlődésével vezet a beteg korai halálához.

Annak érdekében, hogy hatékony terápiás módszerek, specifikus gyógyszerek előállítására lehetőség nyíljon, az immunszövetekben zajló folyamatok megértése, mind szöveti, mind sejtek közötti, mind sejten belüli jelátviteli szinten nagy fontossággal bír.

2.1.2. Az immunrendszer öregedése

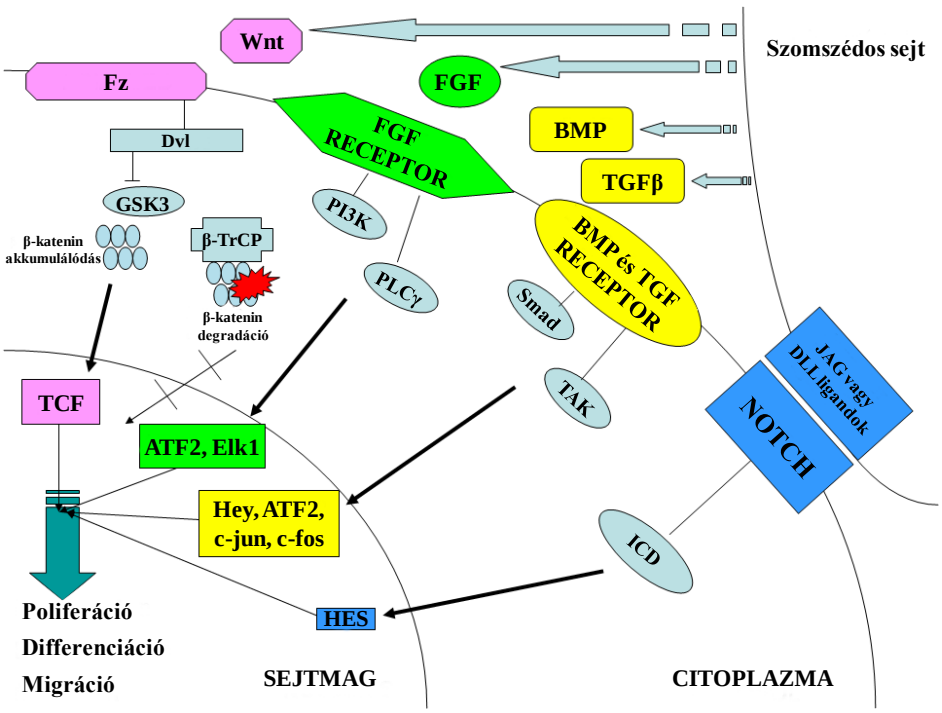
Az immunrendszer öregedése a szervezetben zajló fiziológiás öregedési folyamatokkal párhuzamosan jelenik meg és vezet különböző szintű immunhiányos állapotok kialakulásához. Az egyedi immunológiai kompetenciát a primer limfoid szervekben fejlődő specializált érett limfociták és a másodlagos nyirokszervekben vagy a fertőzés helyén funkciójukat beteljesítő érett nyiroksejtek jelentik. Érthető módon, ha a szöveti mikrokörnyezet megváltozik, az döntő módon befolyásoja mind a primer, mind a szekunder immunválaszt. Az immunszövetek öregedésének vizsgálata során már kimutatásra került, hogy mind a B, mind a T limfociták szöveti környezete öregszik. Az öregedési folyamatok jellemzője pl a limfoid memória sejtek túléléséhez szükséges szöveti kapcsolatokból származó jelek hiánya (Aydar, Balogh et al. 2004) (Aydar, Balogh et al. 2003), avagy a T limfociták termelésének lecsökkenése a tímusz szövet atrofiájának következtében. Összességében az öregedés következtében bekövetkező változások autoimmun betegségek kialakulásához és fertőzésekkel szembeni válaszképtelenséghez vezethetnek.

2.2. A szöveti fejlődést és differenciációt szabályozó molekulacsaládok

2.2.1. Jelátviteli molekulacsaládok

Mind az embrionális fejlődést, mind a felnőtt szövetek homeosztázisát olyan sejten belüli jelátviteli útvonalak szabályozzák, melyeket közvetlen és közvetett sejt-sejt közötti

kölcsönhatások kontrollálnak. Kezdeti kutatásokban a jelátvitelt lineáris, molekuláról molekulára történő folyamatnak feltételezték, melynek végén jól körülhatárolható válaszreakció áll. Azonban a kísérleti eredmények arra engednek következtetni, hogy igen kiterjedt jelátviteli hálózatokról van szó, melyek komplex módon gátolják, illetve erősítik a sejtet érő behatásokat. A nem csak direkt sejt-sejt közötti kontaktus, hanem szekretált faktorok és ligandok által is kiváltott jelek mennyiségi viszonyai döntő fontossággal bírnak a sejt-válasz meghatározásában. Noha egy-egy sejt válaszadó képessége igen széles skálán mozog, a fejlődést, regenerálódást és az öregedés folyamatát csak néhány, fő jelátviteli molekula, illetve molekulacsalád szabályozza. Köztük a csont morfogén fehérje, azaz „bone morphogenic protein" (BMP), a transzformáló növekedési faktor, azaz a „transforming growth factor β" (TGFβ), a fibroblaszt növekedési faktor, azaz „fibroblast growth factor" (FGF), a Notch és a Wnt gliko-lipoprotein család (**2**. ábra) játsza a legfontosabb szerepet.



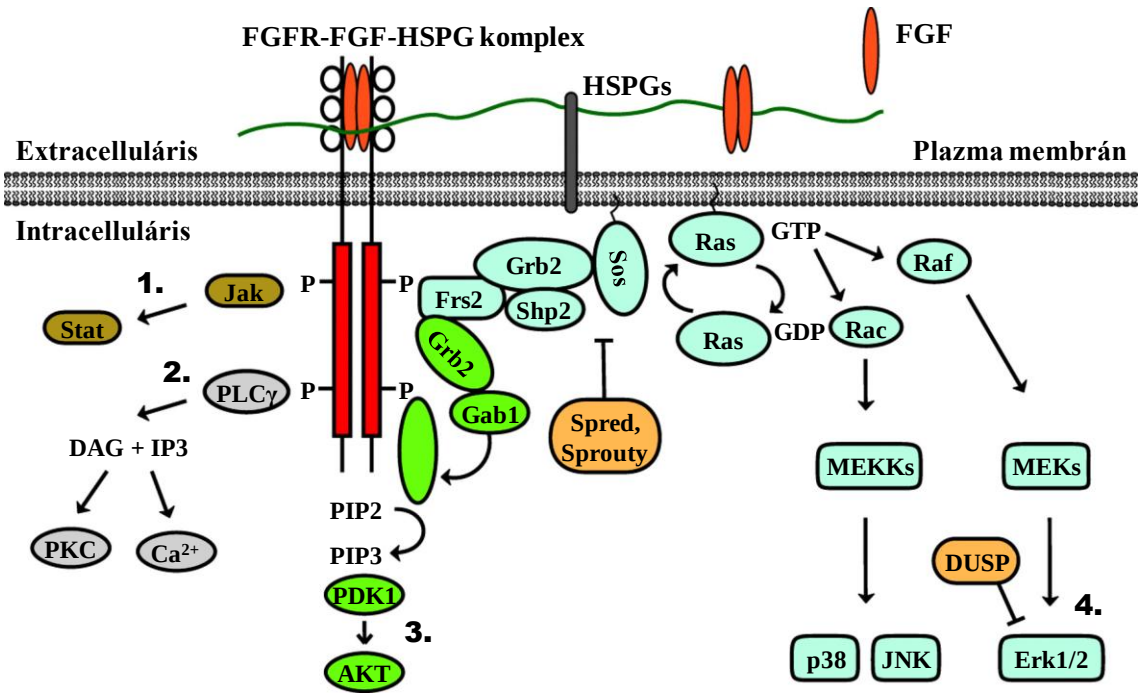
2. ábra: A fejlődést és regenerációt szabályozó fő jelátviteli útvonalak és receptoraik (Molnar és Pongracz 2010)

A négy fő jelátviteli molekulacsalád fő receptorait és célgénjeit demonstrálja az ábra. A fent említett molekulacsaládok folyamatos szöveti jelenlétének kombinációi olyan intracelluláris jelátviteli útvonalakat képesek aktiválni, melyek nem csak kontrollálják a receptorok, ligandok és jelátviteli molekulák expresszióját, de a jelek modulálásán keresztül szabályozzák a sejtek funkcióit is. Ezek a jelátviteli hálózatok egyaránt fontosak embrionális fejlődésben, normál szöveti homeosztázis és regeneráció fenntartásában, vagy sérülést és műtétet követő szövetképződésben és funkció-specifikus differenciálódásban. Természetesen a fent említett molekulák, illetve jelátviteli útvonalaik mutációi patológiás elváltozásokhoz vezethetnek. Ezért az általuk irányított folyamatok megértése kiemelt fontossággal bír az orvosbiológiai kutatások szempontjából.

2.2.2. Szövet specifikus jelátviteli interakciók

2.2.2.1. FGF jelátvitel (Lanner és Rossant 2010)

Az FGF fehérje család 22 tagú. Az FGF ligandok négy tirozin kináz függő receptoron (FGFR-k) keresztül indukálnak proliferációt, differenciációt, migrációt és túléléshez vezető sejtfiziológiai változásokat. A szervek szöveteinek egészséges fejlődéséhez elengedhetetlen a szövetek folyamatos és összerendezett egymásra hatása, amelyben az FGF fehérje család ligandjai és receptorai központi szerepet játszanak. A szöveti interakció reciprokális szabályozása megfigyelhető a csecsemőmirigy fejlődése és szöveti differenciációja során is. A mezenhimális eredetű FGF7 és FGF10 a csecsemőmirigy epiteliális hálózatának kialakításában elengedhetetlen. FGF hiányában az epiteliális sejtek osztódása erősen lecsökken, ezzel gátolva a normál méretű tímusz kialakulását (Jenkinson, Jenkinson et al. 2003). Az FGF jelátvitelben részvevő intracelluláris molekula rendszerek fő elemeit a 3. ábra foglalja össze.



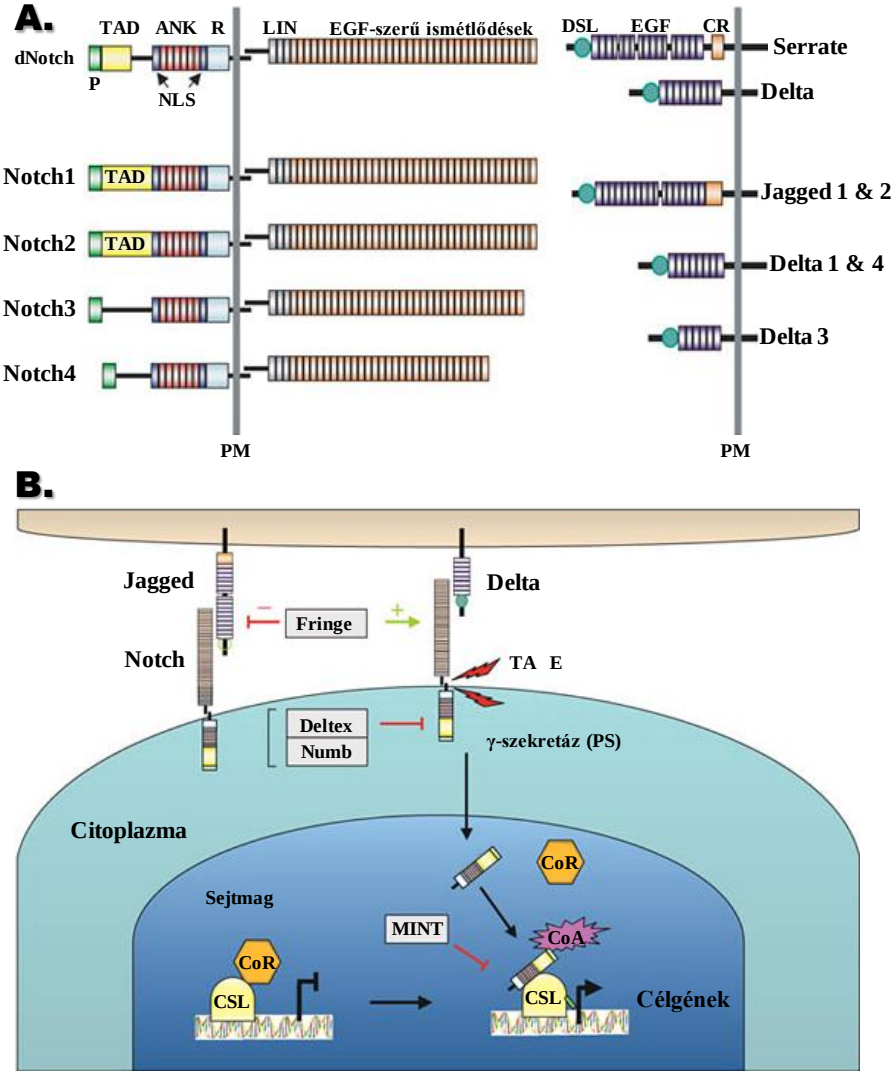
3. ábra: FGF receptorokból induló jelátviteli folyamatok

A jelátvitel megindulásához FGFR-ral trimert kell alkosson az FGF és a heparán szulfát proteoglikán (HSPG). A négy fő jelátviteli útvonal a receptor aktiválást követően szabályozza a génátíródáshoz vezető jeleket. Az egyik a Janus kináz és Jak/Stat aktivátor (1, barna színnel jelölve), foszfoinozitid-foszfolipáz C (PLCγ; 2, szürke színnel), foszfatidil-inozitol 3-kináz (PI3K; 3, zöld színnel) és a mitogén-aktiválta protein kináz/extracelluláris szignál regulálta kináz (MAPK/Erk; 4, kék színnel jelölve). A „dual specificity phosphatase”-ok (DUSP), Spred és Sprouty proteinek (narancssárga) negatív visszacsatolási mechanizmussal szabályozzák az FGF-ek által indított jelátviteli folyamatokat.

2.2.2.2. Notch jelátvitel (Radtke és Raj 2003)

A Notch gén, amelyet 1980-ban sikerült megklónozni, olyan receptort kódol, amely egy transzmembrán doménnel rendelkezik. Noha egy prekurzor fehérjeként szintetizálódik, a Notch molekula kettéhasítva transzportálódik és heterodimer receptorként jelenik meg a sejtfelszínen. A receptor extracelluláris részén epidermális növekedési faktor (epidermal growth factor) (EGF)-szerű szekvenciák találhatók, melyeket három, ciszteinben gazdag Notch/Lin12 (LN) szekvencia követ. Az ismétlődő EGF-szerű szakaszok a ligand kötésért felelősek, míg az LN szekvenciák a ligand hiányában megakadályozzák a jeltovábbítást. A receptor citoplazmikus meghosszabbítása juttatja a jeleket a sejtmagba. A receptornak ez a része tartalmazza a RAM vagy hat ankirin domént, hordoz két nukleáris lokalizációs szignált, transzkripciós transzaktivációs domént (TAD) és a PEST szekvenciát. Emlősökben négy Notch receptort (Notch1-4) és öt ligandot (Delta-like azaz DLL1, DLL3, DLL4, illetve a szerát (Ser)-szerű ligandokat Jagged (JAG1 és JAG2) sikerült eddig azonosítani.

A Notch jeltovábbítást a két sejt között létrejövő receptor–ligand interakció inicializálja, mely a Notch receptor citoplazmikus végének proteolitikus hasításához és annak felszabadításához vezet. A Notch-IC ezután a membránból a sejtmagba jutva a CSL transzkripciós faktorhoz kapcsolódik, majd ko-aktivátorokat vonz a komplexbe, mint például a Mastermind nevű molekulát és a hiszton acetil-transzferázokat, amelyek a CSL-t transzkripciós represszorból transzkripciós aktivátorrá alakítják. A Notch jelátvitelnek csak néhány transzkripciós célmolekulája ismert, egyikük a „hairy/enhancer of split” avagy HES (Iso, Kedes et al. 2003).



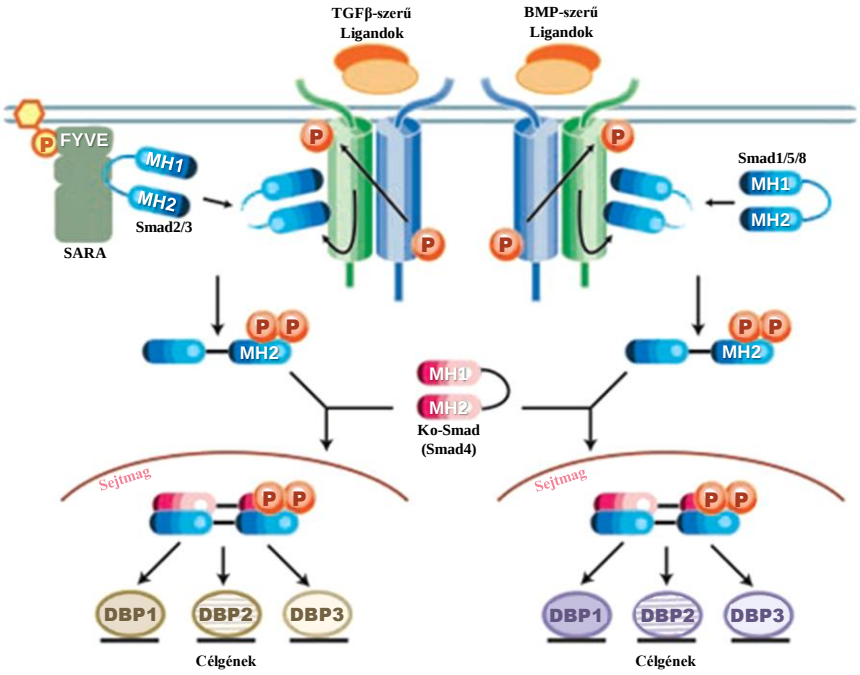
4. ábra: A Notch receptor/ligand család és Notch jelátvitel

A Notch jelátvitel megindulásához Notch receptor és ligand kapcsolódás szükséges. A receptorok és ligandok szerkezetét az ábrán a felső panel mutatja be (A), míg a receptorból induló jelátvitel folyamán lezajló folyamatokat a (B) panel összegzi.

2.2.2.3. BMP-TGFβ jelátvitel (Miyazono, Maeda et al. 2005)

BMP molekulák a TGFβ szupercsaládhoz tartoznak, amely magában foglalja a TGFβ-n kívül az aktivinokat és inhibineket, Nodal-t, miosztatint és AMH-t, azaz az „anti-Müllerian-hormone”-t. A TGFβ szupercsalád ligandjai szerin/treonin kináz receptorokhoz kapcsolódnak, amelyek Smad-függő és -független mechanizmusokon keresztül továbbítják a receptorból származó jeleket. Eddig több mint 20 BMP-jellegű fehérjét sikerült azonosítani, melyeket további alcsoportokba sorolnak szerkezetük és funkciójuk alapján. BMP-2 és BMP-4 alkotják az első, míg BMP-5, -6, -7, -8 a második, végül BMP-1, -12, 13 a harmadik csoportot. A

BMP család térben és időben jellegzetes expressziós mintázata szabályozza a sejtfiziológiás változásokat és ezeken keresztül a szöveti funkciókat.



5. ábra: Jelátvitel a TGFβ és BMP receptorokból

TGF-β és BMP ligandok jól körülhatárolható receptorokból indítanak intracelluláris jeleket. A receptor először foszforizálódik, majd meghatározott SMAD molekulákat foszforizál, melyek heteromer komplexeket alkotnak Smad4-gyel, majd a sejtmagba transzlokálódnak. A különböző Smad kombinációk más-más DNS-kötő proteint (DBP) ismernek fel, mely végül differenciált célgén átíródáshoz vezet.

2.2.2.4. Wnt jelátvitel (Pongracz JE és Stockley RA 2006)

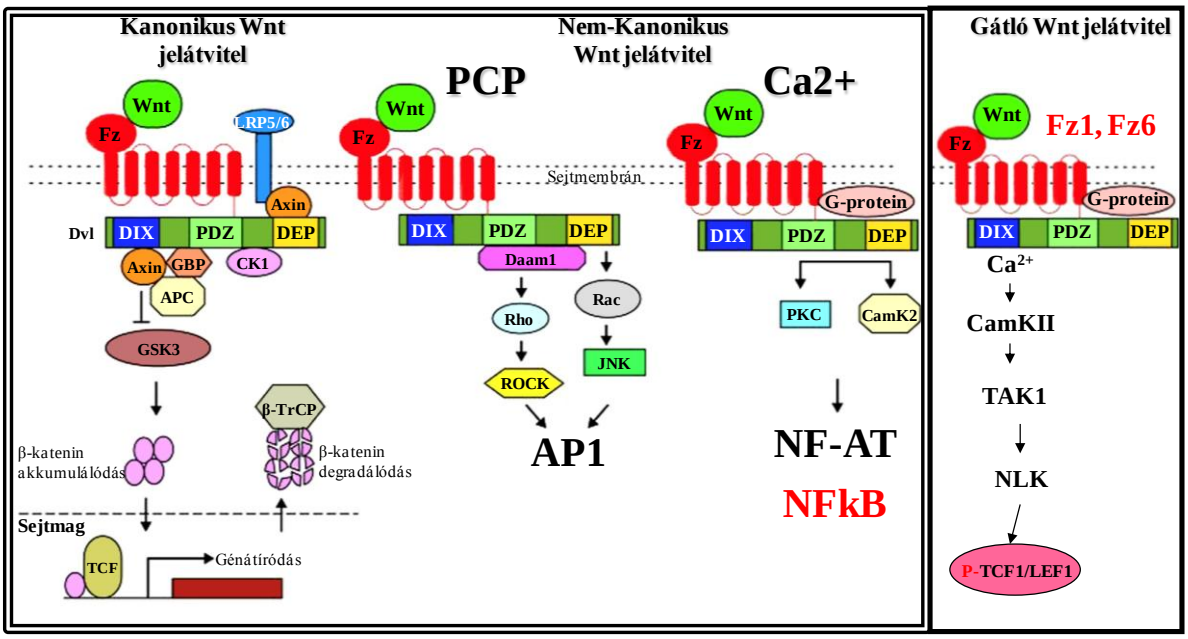
A Wnt molekulacsalád elemei esszenciálisak az embrionális fejlődés, a felnőtt szövetek folyamatos regenerációja és őssejt állományának fenntartása szempontjából. A Wnt jelátvitel hibás szabályozása daganatok, fibrózis és perzisztáló gyulladásos folyamatok kialakulásához, illetve felgyorsult öregedéshez vezet (Nateri, Spencer-Dene et al. 2005; Liu, Fergusson et al. 2007). Mivel kutatásaim jelentős részét a Wnt jelátvitel vizsgálatával töltöttem, ezért a Wnt jelátvitelt részleteiben mutatom be.

2.3. Wnt jelátvitel

2.3.1. A Wnt jelátvitel általános jellemzői

Maga a Wnt elnevezés a Drosophila „wingless” (szárnyatlan) és annak gerinces homológja az „Int” összevonásából keletkezett. A gerincesek 19 Wnt fehérjéje bizonyítottan központi szerepet játszik a sejtdifferenciálódás, sejtosztódás, sejtmigráció és sejtpolaritás szabályozásában. A Wnt-ok ciszteinben gazdag, szekretált gliko-lipoproteinek, amelyek jeltovábbítása két fő és ezeken belül is több alcsoportot alkotó, bonyolult jeltovábbítási mechanismusra osztható. A Wnt fehérjék a Ryk (Kim, Her et al. 2008), illetve a Frizzled (Fz) elnevezésű receptorokhoz kötődve indítják el a jelátvitelt. A 9 gén által kódolt 10 Fz fehérje az LRP5/6 (low density lipoprotein related protein) ko-receptorral alkot aktív receptor komplexet (Pinson 2000).

A Wnt-ok két fő jeltovábbítási rendszer aktiválásával szabályozzák a génátíródást. Az egyik a klasszikus, avagy kanonikus jelátviteli út, amely β-katenin függő. A másik, a nem-kanonikus út, mely további két jelátviteli útra osztható: a PCP (polar cell polarity) avagy a c-Jun N-terminális kináz (JNK) függő és a protein kináz C (PKC) függő jelátviteli utakra. Attól függően, hogy kanonikus vagy nem-kanonikus jeltovábbítási rendszereket aktiválnak, a Wnt fehérjék két csoportra oszthatók: a kanonikus (Wnt1, Wnt3, Wnt7b, Wnt10b, stb) és a nem-kanonikus (Wnt4, Wnt5a, Wnt11) Wnt-okra. A Wnt rendszer bonyolultságát tovább növeli, hogy a Wnt molekulák receptor specificitása nem abszolút, és így több jeltovábbítási rendszert is aktiválhat egy fajta Wnt fehérje, ami a Wnt-rendszerektől függő celluláris folyamatok vizsgálatát igencsak megnehezíti.



6. ábra: A Wnt jelátviteli útvonalak összefoglaló ábrázolása

A Wnt jelátvitel három fő jelátviteli útra osztható. Kettő aktiválja a génátíródást, míg a harmadik gátolja a TCF/LEF transzkripciós faktorok által aktivált génátíródást.

2.3.2. Kanonikus Wnt jeltovábbítás

A kanonikus vagy β-katenin/TCF függő Wnt jeltovábbítási út (6. ábra) a leginkább vizsgált, következésképpen a legismertebb. A legújabb eredményeket gyakori összefoglaló cikkek igyekeznek átláthatóbbá tenni (Moon, Kohn et al. 2004; Reya és Clevers 2005). Röviden: Wnt-ok hiányában a glikogén szintáz kináz β (GSK-3β) aktiválódik és foszforilálja a β-katenin-t az „adenomatous polyposis coli" (APC) és axin alkotta fehérje komplexben, ahol a β-katenin a foszforilálás hatására inaktívvá válik (Ikeda 1998; Yamamoto 1999) és a proteoszómákban degradálódik (Moon, Bowerman et al. 2002), ezzel csökkentve a citoszolban a β-katenin szintet (Aberle 1997).

A Wnt molekulák jelenlétében a Fz receptorból meginduló jelátvitel a GSK-3β inaktiválásához, majd ennek következtében a β-katenin akkumulálódásához vezet. A β-katenin ezután a sejtmagba transzlokálódik, ahol a T sejt faktor azaz „T cell factor" (TCF) családba tartozó transzkripciós faktorokkal (TCF1-4, LEF1) alkot aktív transzkripciós komplexet (Young 1998; van Noort és Clevers 2002) a CBP/p300 transzkripciós ko-aktivátor (Labalette, Renard et al. 2004) jelenlétében. A klasszikus Wnt jeltovábbítási út számos célgén aktiválását eredményezheti, köztük mátrix metalloproteináz (MMP2, MMP3, MMP7 és MMP9)(Tamamura, Otani et al. 2005), cyclin D1 (Shtutman, Zhurinsky et al. 1999), Cox-2

(Longo, Kennell et al. 2002), c-myc (He, Sparks et al. 1998), c-jun (Mann, Gelos et al. 1999), Fra-1 (Mann, Gelos et al. 1999), stb., amely gének nagy fontossággal bírnak minden szövetátrendeződési folyamatban.

2.3.3. Nem-kanonikus Wnt jeltovábbítás

A két nem kanonikus jeltovábbítási út egyike (6. ábra) a JNK-függő PCP, a másik a kalcium/PKC-függő jeltovábbítási út (Kuhl, Sheldahl et al. 2000; Pésur, Maurus et al. 2002). A nem kanonikus jeltovábbítási utak aktiválása a kanonikushoz hasonlóan G-protein függő (Malbon, Wang et al. 2001), de ezután más-más jeltovábbítási molekulák aktiválása útján különböző transzkripciós faktorok, és ennek következtében más-más gének aktiválásához vezetnek. Míg a JNK-függő Wnt jelátviteli rendszer NFAT/AP1 aktiváláson keresztül cyclin D1, MMP3, c-Jun, GMCSF stb gének átírását vonja maga után, addig a PKC-függő Wnt jeltovábbítási útvonal NFAT és NFkB aktiválás után a gyulladási folyamatok szabályozásában igen fontos szerepet játszó gének transzkripciójához vezet, többek között IL6, IL8, IL15 (Sen 2005).

2.3.4. A Wnt jeltovábbítás szabályozása

Mivel a Wnt jeltovábbítási rendszerek igen szerteágazó celluláris funkciókat szabályoznak, a Wnt-ok szabályozó mechanizmusai is igen összetettek. Ezekben úgy sejten belüli (Yan, Wallingford et al. 2001), mint sejten kívüli (Mao 2001), szekretált szabályozó molekulák, illetve jeltovábbító rendszerek vesznek részt. A két fő Wnt jeltovábbítási rendszer egymás aktiválását is képes szabályozni (Kuhl, Geis et al. 2001). Ezen túlmenően a sejten belüli inhibítor molekulák az ICAT (β-katenin TCF kötődését gátló molekula) (Tago, Nakamurea et al. 2000) és a Naked (Nkd1 és Nkd2) (Yan, Wallingford et al. 2001) a kanonikus jeltovábbítás gátlásában vesznek részt. A Nkd-ek (Yan, Wallingford et al. 2001) a kanonikus rendszer gátlásán túlmenően a nem-kanonikus rendszer egyidejű aktiválására képesek úgy, hogy a bejövő Wnt jeleket a nem-kanonikus rendszer felé irányítják, a Dishevelled (Dvl) membrán-közeli jeltovábbítási molekula szintjén.

A Fz6 receptorból szintén indulnak gátló jelek (Golan, Yaniv et al. 2004), amelyek megakadályozzák a TCF függő génátíródást úgy, hogy a Nemo-like-kináz (NLK) és a TGFβ aktiválta kináz (TAK1) aktiválásának (Ishitani, Kishida et al. 2003) eredményeként a TCF

transzkripciós faktort foszforilálják, amelynek következtében a TCF olyan szerkezeti változásokon megy át, hogy alkalmatlanná válik a β-katenin körül kialakuló transzkripciós komplexbe történő kapcsolódásra (6. ábra).

2.3.5. A Wnt jeltovábbítás legutóbb felfedezett aktivációs mechanizmusai

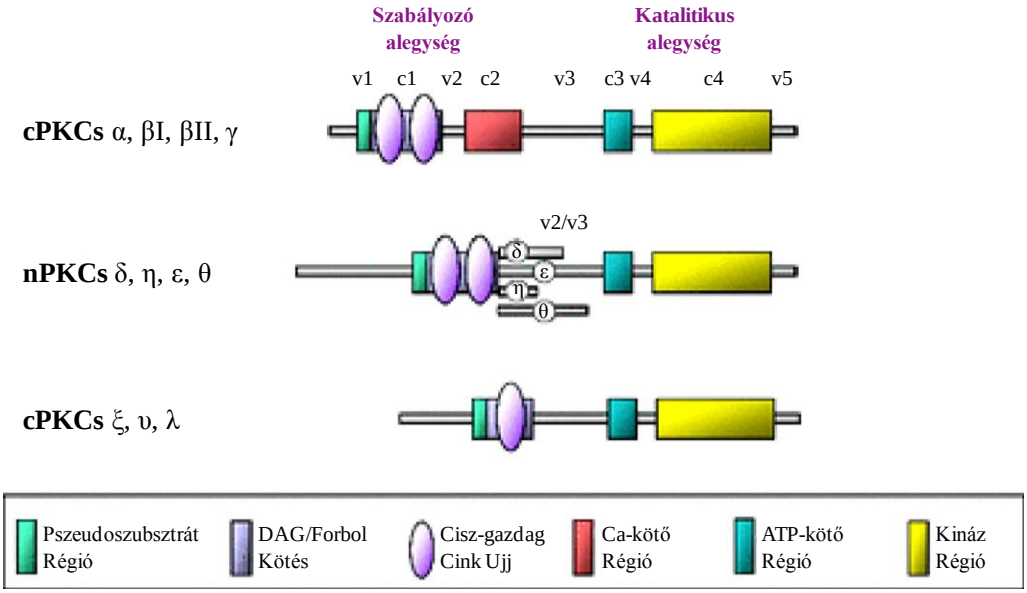
Noha már az eddigi kutatási eredmények is egy igen komplex jelátviteli rendszer képét vázolták fel, napjaink kutatásai a Wnt jelátviteli rendszer további bonyolultságára derítettek fényt. Kiderült ugyanis, hogy az intracelluláris Wnt jelátvitel aktiválásához sok esetben a Wnt ligandot megkötő Fz receptor internalizációjára is szükség van (Blitzer és Nusse 2006), amely folyamat sok esetben más Wnt kötő receptorok, például Ryk (Kim, Her et al. 2008) aktív közreműködésétől függ. Noha az internalizáció jelátvitelben betöltött jelentősége és pontos mechanizmusa sem ismert, annyi nyilvánvaló, hogy heparán szulfát-függő folyamat (Morita, Kawabe et al. 2004).

2.4. Protein kináz C enzimcsalád

2.4.1. A PKC család fehérje szerkezete és főbb jellemzői

A protein kináz C (PKC) szerin/treonin kináz család prototípusát először Nishizuka kutatócsoportja írta le (Takai, Kishimoto et al. 1979). Elsőként bizonyították, hogy a PKC-kat diacilglicerol (DAG) aktiválja, amely a foszfatidilinozitol természetes bomlásterméke (Kishimoto, Takai et al. 1980). További kutatások arra is fényt derítettek, hogy a tíz izoformából álló PKC család (Ono, Fujii et al. 1988) (7. ábra) a tumorképződést indukáló forbolészterek intracelluláris receptora.

Amíg PKC ι és λ humán/egér ortológokat reprezentálnak, a korábban „PKC μ”-ként ismert PKC valójában nem tartozik a C protein kinázok közé, hanem egy jól elkülöníthető másik, a D protein kináz (PKD) család tagja. A PKC-k számos celluláris funkciót irányító jelátviteli útvonal aktív résztvevői, melyek a sejtmigrációt, polaritást, proliferációt, differenciációt és sejthalált irányítják.



7. ábra: A protein kináz család szerkezeti megoszlása

A PKC izoformák három fő csoportba oszthatók struktúrájuk és ko-faktor szükségletük alapján: a klasszikus vagy cPKC (α, βI, βII, és γ), a novel vagy nPKC (δ, ε, η, θ), és az atipikus vagy aPKC (ζ és ι/λ) csoportba. Az aktivációhoz a PKC család minden tagjának szüksége van foszfatidilszerinre.

A PKC család minden tagja nagyon hasonló szerkezettel bír. Szabályozó domének találhatók az N-terminálison, míg katalitikus domének a C terminálison. A különböző régiókat konzervált régiókként (C1–C4) és variábilis régiókként kategorizálták (V1–V5). Az utóbbiak, azaz a variábilis régiók az izoformák között sokféleséget mutatnak (Ono, Fujii et al. 1988). A PKC családra jellemző, hogy mindegyik izoforma szerkezetében fellelhető a pszeudoszubsztrát domén jelenléte a szabályozó régióban. A pszeudoszubsztrát domén hasonlít az enzimek szubsztrát felismerő helyére, de fő funkciója az, hogy blokkolja a szubsztrát felismerő helyet, ezzel akadályozva a nem kívánt aktivációt.

A PKC izoformák három fő csoportba oszthatók struktúrájuk és ko-faktor szükségletük alapján (7. ábra): a klasszikus vagy cPKC (α, βI, βII, és γ), a novel vagy nPKC (δ, ε, η, θ), és az atipikus vagy aPKC (ζ és ι/λ) csoportba. Az aktivációhoz a PKC család minden tagjának szüksége van foszfatidilszerinre. A cPKC-k ezen túlmenően érzékenyek a kálcium (Ca^{2+}) szintre és szükségük van még DAG-ra vagy forbolészterre (pl 12-tetradecanoyl-13-phorbol acetate, azaz TPA) az aktivációhoz. Az nPKC-k függetlenek a Ca^{2+} -tól, de DAG vagy forbol-észter jelenléte nélkül nem aktiválhatók. Az aPKC-knak pedig elegendő a foszfatidilszerin a maximális aktiválási szint eléréséhez. Az izoformák jellemző domén szerkezete lehetővé teszi, hogy a C kinázok működését aktiváló és gátló molekulák széles skálája szabályozhassa (Mellor és Parker 1998).

2.4.2. Szubsztrát specificitás

A PKC izoformák szubsztrát specificitása is jellemző egy adott izoformára. Például a PKC α, β, és γ erősen foszforilálja a hiszton, a „myelin basic protein" (MBP), és a protamin fehérjéket, míg a PKC δ, ε, és η ezeket a szubsztrátokat nem képes foszforilálni. A szubsztrát felismerése szempontjából elengedhetetlen, hogy a szubsztrátot felismerő régióból eltávolításra kerüljön a gátló funkciót ellátó pszeudoszubsztrát (Mellor és Parker 1998). Noha a PKC-knek számos szubsztrájuk ismert, a legtöbb PKC izoforma hasonló szekvenciát foszforilál. Ezért nyilvánvaló, hogy számos mechanizmus együttes működésére van szükség ahhoz, hogy a különféle PKC izoformák specifikusan működhessenek egy-egy jelátviteli útvonalban.

2.4.3. A tirozin foszforiláció jelentlősége

A PKC izoformák szerin/treonin kináz aktivitással bírnak, de saját aktiválásukhoz is szerin/treonin foszforiláció szükséges (Le Good és Brindley 2004). Ezen túlmenően, aktivitásuk tirozin foszforiláción keresztül is szabályozódik, melyet először a PKC δ izoforma gátlásánál mutattak ki. Más tirozin foszforilációs helyek foszforilációs szintjének változása azonban pozitívan is szabályozhatja a PKC aktivitást úgy PKC δ, mint más PKC α, β, ε, és ζ izoformák esetében akár sejthalál, akár bizonyos növekedési faktorok által indukált jelek átvitele kapcsán.

2.4.4. Intracelluláris eloszlás

Másik fontos jellemzője a PKC aktiválási folyamatnak, hogy a PKC izoformák foszfolipidekkel asszociálódnak és képeznek stabil membrán komplexeket (Ron és Kazanietz 1999). Ez az alapja a klasszikus transzlokációs vizsgálatoknak a PKC izoformák aktiválódásának bizonyítására (Altman, Villalba et al. 2003). Ezen túlmenően, bizonyos PKC izoformák jól körülhatárolható celluláris kompartmentekbe lokalizálódhatnak. Például a PKC α citoszkeletális, „tight junction", „caveolae" és dezmoszóma komplexeket, a PKC γ Golgi apparátus, és a PKC η perinukleáris endoplazmatikus retikulum (RER) komplexeket képez. Továbbá, az aktivált PKC-k specifikus sejten belüli lokalizációja meghatározza a PKC funkcionális szerepét. Igy például a PKC α, β, δ, ε, és ζ transzlokációja a mitokondriumba, a Golgi apparátusba, a nukleáris vagy perinukleáris régióba szabályozza a mitózis, apoptózis és

sejttúlélési jelátviteli útvonalakat. PKC aktiváció a plazmamembránban szerin foszforilációhoz és transzmembrán proteinek és receptorok endocitózisához vezet. Így például PKC α a plazmamembránban a fibroblaszt növekedési faktor receptor, azaz a „fibroblast growth factor receptor" (FGFR), míg PKC δ az inzulin receptor (IR) internalizációjához vezet. PKC δ az inzulin receptor szubsztrát-1-t (IRS-1) is foszforilálja inzulin hatására, és ezzel, mint a negatív visszacsatolási rendszer része, szabályozza az IR funkcióját. Továbbá, az extracelluláris matrix (ECM) receptor, integrin és citoszkeletális fehérjék foszforilációjában is számos PKC családtag vesz részt, pl. a PKC α, PKC δ és PKC ε. Egy másik mechanizmus is létezik a PKC-k citoszolból egy meghatározott sejt kompartmentbe való transzlokációjára. Ebben a folyamatban a „receptors for activated/inactive kinases" (RACKs/RICK) szekvenciák fontos szerepet töltenek be. Mivel ezek a szekvenciák meghatározott PKC izoformák specifikus szekvenciáihoz kapcsolódnak, a RACK-ok segítségével meghatározhatóvá vált a PKC-k aktivációs állapota.

2.4.5. Szöveti megoszlás

PKC izoformák általánosan elterjedtek a különféle szövetekben. Amíg azonban némelyik izoforma (PKC α, δ, és ζ) széles szöveti megoszlást mutat, addig más izoformák szövet-specifikusak (Mellor és Parker 1998). PKC γ és a PKC ζ rövid transzkriptjének PKM-nak expressziója például az agyra és egyéb neurális szövetre jellemző (Cardell, Léssend et al. 1998; Hernésez, Blace et al. 2003). Korábbi kutatások kimutatták, hogy PKC ι a herékre és inzulint szekretáló sejtekre jellemző (Selbie, Schmitz-Peiffer et al. 1993), míg PKC θ a szkeletális izomban és T-sejtekben expresszálódik (Berry és Nishizuka 1990). Legújabb eredmények viszont rámutattak, hogy mindkét izoforma megtalálható az epidermiszben; ahol a PKC ι a „tight junction"-ok formálásában vesz részt (Helfrich, Schmitz et al. 2007). Szöveti megoszlástól függően a PKC δ-ról már kimutatták, hogy különféle sejtmodellekben kontrollálhat mind proliferációt, mind apoptózist (Pongracz, Johnson et al. 1994; Shen, Alt et al. 2001). Továbbá, a „klasszikus" inzulinra reagáló szövetekben vagy sejtekben, mint például az izom, máj és adipociták, a PKC δ a glukóz transzportot és metabolizmust szabályozza (Braiman, Alt et al. 1999; Shen, Alt et al. 2001).

2.5. PKC-k a Wnt jelátvitelben

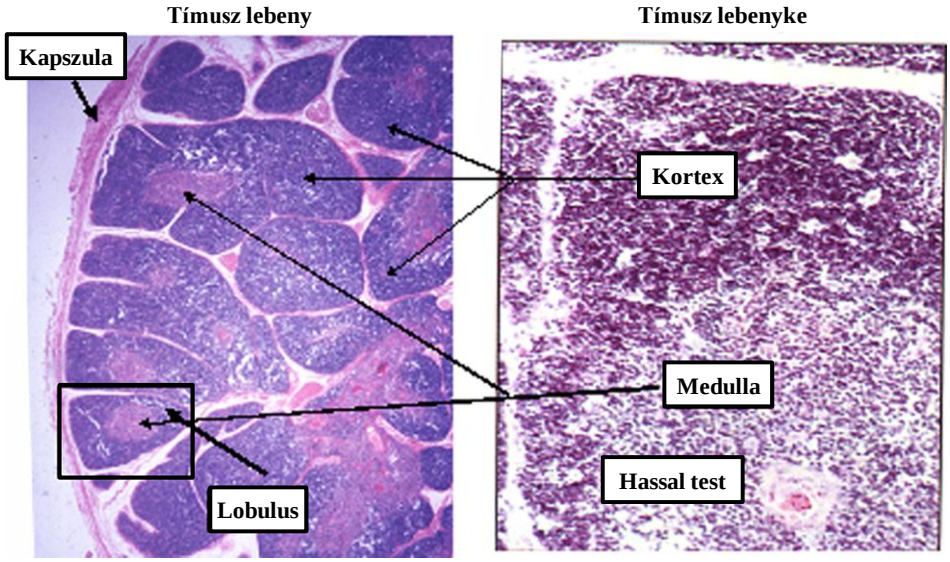
A Wnt molekulák két, már korábban részletezett jelátviteli útvonalon a β-katenin függő kanonikus, és a JNK és PKC függő nem-kanonikus jelátviteli úton keresztül szabályozzák a génátíródást és ezáltal a különféle sejtfunkciókat. Ezen felosztás arra az időre vezethető vissza, amikor még a mainál is kevesebb ismerettel rendelkeztünk a Wnt-ok jelátviteléről. Azóta nyilvánvalóvá vált, hogy a Wnt eredetű jelátvitel komplex szabályozásának része a jelátviteli utak hálózatszerű összekapcsolódása és az, hogy bizonyos jelátviteli molekulacsaládok nem rendelhetők kizárólagosan egyik vagy másik Wnt által aktivált jelátviteli úthoz. A kálcium függő és a JNK planáris cell polaritási kaszkádok kapcsolódási pontjaként már sikerült meghatározni a PKC aktiválta cdc42 (Schlessinger, McManus et al. 2007) molekulát, mely a Wnt jelátviteli rendszerek szoros kapcsolatát bizonyítja. Eddig konkrétan a PKC α (Kuhl, Geis et al. 2001), PKC ζ (Ossipova, Bardeesy et al. 2003), és PKC δ (Kinoshita, Iioka et al. 2003) izoformák specifikus szerepét sikerült bizonyítani. A PKC δ-ról kiderült, hogy azon túlmenően, hogy Wnt5a stimulus hatására a plazmamembránba transzlokálódik, a Dvl foszforilációjában és ezért a Wnt receptorból való jelátvitelben fontos szerepet játszik. A PKC ζ viszont a GSK-3β foszforiláció-függő aktiválásában vesz részt. GSK-3β aktivációja a β-katenin proteoszomális degradációját (Moon, Bowerman et al. 2002) szabályozza, és ezzel a kanonikus Wnt jelátvitel gátlását eredményezi. A mieloma plazma sejtek inváziójának és migrációjának vizsgálata során derült fény arra, hogy a Wnt molekulák képesek a β-katenint megkerülve is szabályozni a RhoA migrációt indukáló aktivitását a PKC α, β, és μ izoenzimeken keresztül (Qiang, Walsh et al. 2005).

2.6. A tímusz fiziológiája és a Wnt-ok

2.6.1. A tímusz anatómiája és fő funkciói

A tímusz páros lebenyes szerv, a szegycsont alatt helyezkedik el és a harmadik garattasakból fejlődik ki (Manley 2000). Egér embriókban a tímusz-kezdemény már a 10. embrionális nap körül (E10.5) láthatóvá válik. Feltételezések szerint a szerv kifejlődésében mindhárom csírasejt-réteg részt vesz. A folyamat két szakaszra osztható: a korai stádiumban az epitéliális- és mezenhimális szövetek kölcsönhatása, míg a második szakaszban az epitéliális sejtek és a fejlődő T-sejtek közötti kapcsolat a döntő. A tímusz organogenezisét számos szekretált

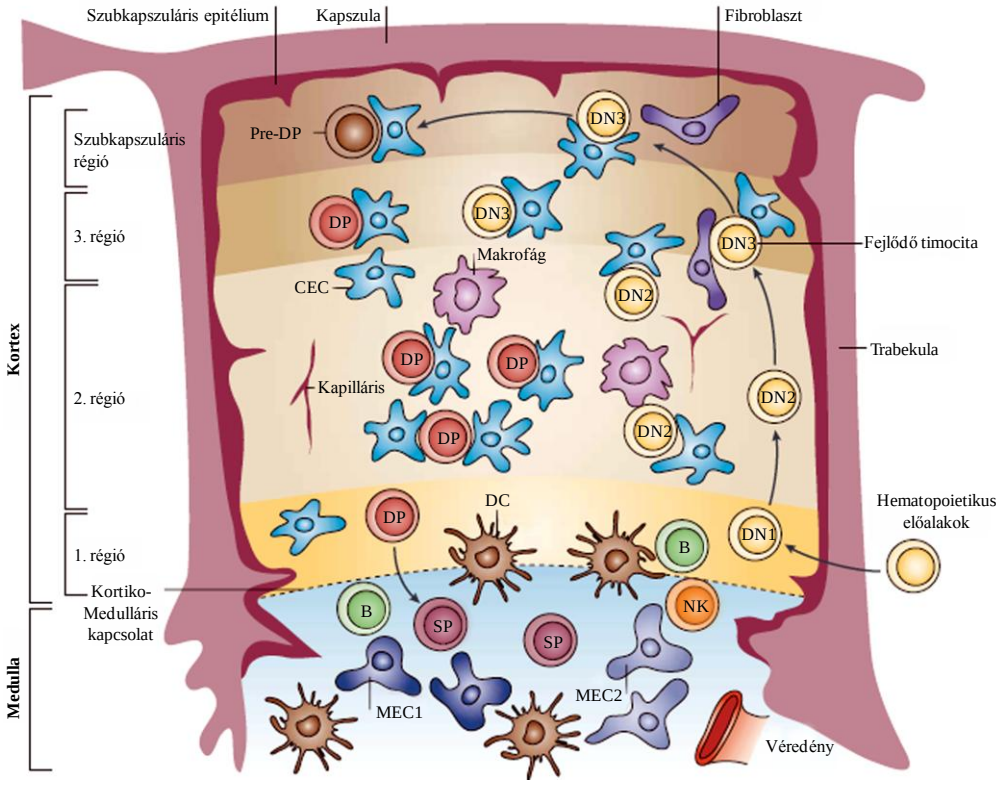
molekulacsalád szabályozza. Míg az epitéliális sejtek osztódásához elengedhetetlenek az FGF (Jenkinson, Jenkinson et al. 2003), addig a tímusz epitéliális karakter kialakításához a Wnt család tagjai szükségesek (Balciunaite, Keller et al. 2002). A tímusz fő szerkezeti egységei a lebenykék, melyeken belül a tímusz epitéliális sejtek hálózata alkotja a kortexet és a medullát (8. ábra).



8. ábra: A tímusz szerkezete

A tímusz fő szerkezeti egységei. A lebenykéken belül az epitéliális sejtek hálózata alkotja a kortexet és a medullát, mely hálózaton belül fejlődnek a T-sejtek.

A tímusz epitéliális sejtek által alkotott hálózaton belül a limfoid progenitorokból fejlődnek ki a T-sejtek (9. ábra), melyek a tímuszt elhagyva, a perifériás immunszövetekben fejezik be fejlődésüket és érnek funkcionálisan aktív immunsejtekké.



9. ábra: T-sejtek fejlődése a tímuszban (Blackburn és Manley 2004)

Timociták fejlődési alakjai: DN (double negative), azaz $CD4^-8^-$, DP (double positive), azaz $CD4^+8^+$; SP (single positive) $CD4^+8^-$, $CD4^-8^+$.

A humán tímuszban – az egéréhez hasonlóan - az epitéliális sejtek igen fontos szerepet töltenek be a timociták fejlődésének szabályozásában. Kemokineket, mint pl CCL21 és CCL25 termelnek, melyek a csontvelőből a tímuszba vonzzák a limfoid progenitorokat (Liu, Ueno et al. 2005). Citokineket is szekretálnak, mint pl IL7 (Chantry, Turner et al. 1989; Zamisch, Moore-Scott et al. 2005) és IGF (Kecha, Brilot et al. 2000), melyek esszenciálisak a fejlődő T-sejtek túléléséhez és osztódásához (Anderson, Owen et al. 1994; Anderson és Jenkinson 1995). A T-sejt progenitorok avagy előalakok a tímuszba lépve több lépcsős fejlődésen esnek át, amikor még nem expresszálnak CD4 és CD8 koreceptorokat, azaz kettősen (double) negatívak (DN). A korai fejlődési állapotban lévő timociták CD44 és CD25 markerek expressziós változásai alapján különíthetők el és oszthatók négy jól definiálható fejlődési csoportba (DN1-DN4). A proliferációs és differenciációs lépéseket követően $CD4^+8^+$, azaz „double" pozitív (DP) timocitákban az átrendeződött T sejt receptor β és α láncok génjei antigén felismerő receptorokká alakulnak, majd a sejtek TCR-k antigén felismerőképessége alapján szelekciós lépéseken esnek át. A funkcióképes TCR alapján a kortikális és medulláris epitéliális sejtek, illetve a dendritikus sejtek a fejlődő T-sejtek közül

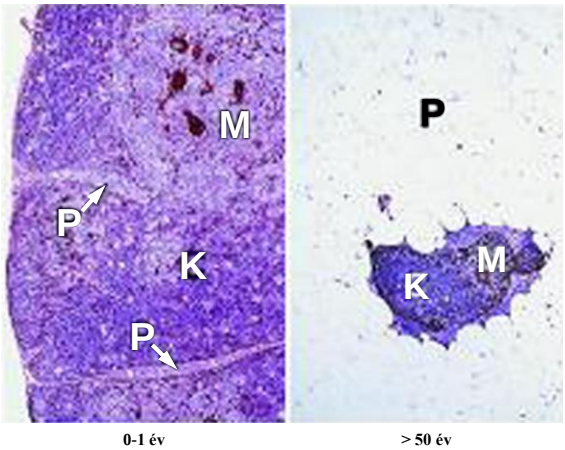
eliminálják a funkcióképtelen, illetve a potenciálisan autoreaktív sejteket (Anderson MS, Venanzi ES et al. 2005).

2.6.2. Wnt-ok a tímuszban

A Wnt jelátvitel vizsgálata kezdetben a T-sejtek fejlődésére koncentrálódott. Kezdeti kísérletek bizonyították, hogy a tímuszban a szolubilis Fz receptorokkal manipulált Wnt jelátvitel (Staal 1999; Mulroy 2002) a T-sejtek fejlődésében drasztikus változásokat okozott, mind túlélés, mind differenciálódás terén. Nem meglepő módon, hiszen a fejlődő timociták DN3-ból DN4 fejlődési állapotba való lépéséhez β-katenin függő, kanonikus Wnt-ok által aktivált, TCF/LEF transzkripciós faktorok által szabályozott génátíródásra van szükség (Verbeek 1995; Schilham 1998). Későbbi kutatások kiderítették azonban, hogy nem csak a timociták igénylik a Wnt jelátvitelt, hanem a timociták fejlődéséhez elengedhetetlen tímusz epiteliális sejtek is, hiszen Wnt jelátvitel szabályozza a tímusz epitélium karakterét meghatározó FoxN1 transzkripciós faktor expresszióját (Balciunaite, Keller et al. 2002).

2.6.3. A tímusz szövet öregedés során bekövetkező változásai

A tímusz mérete és szerkezete egyéb szerveinkkel ellentétben drasztikusan változik az életkorral (10. ábra).



10. ábra: Tímusz szerkezete változik az öregedéssel

Születés után kb egy éves korig a tímusz epitéliális hálózata teljes, majd megindul az atrófia, amelynek kezdeti nyomai esetenként már három éves kor körül felfedezhetők. Ötven éves korra viszont a tímusz szöveti állománya elzsírosodik, és T-sejt termelése lecsökken. Ott azonban, ahol még szigetekben fellelhető a tímusz epitélium, a szövet változatlanul kortexre (K) és medullára (M) tagolódik és képes a de-novo T-sejt szelekcióra.

Ötven éves korra már a tímusz szöveti állományának 80%-a adiopoid szövet (Marinova 2005). Ezzel párhuzamosan a még meglévő epitéliális sejtekben csökken a tímusz karaktert fenntartó FoxN1 expresszió. A tímusz epitéliális szöveti állományának elvesztése következtében a korábbi de novo T-sejt-termelési és szelekciós kapacitása lecsökken, amely lehetővé teszi autoimmun kórképek gyakoribb kialakulását és a fertőző, pl. virális eredetű betegségek elleni ellenállóképesség lecsökkenését (Grubeck-Loebenstein 2010).

2.6.4. Wnt-ok az öregedésben

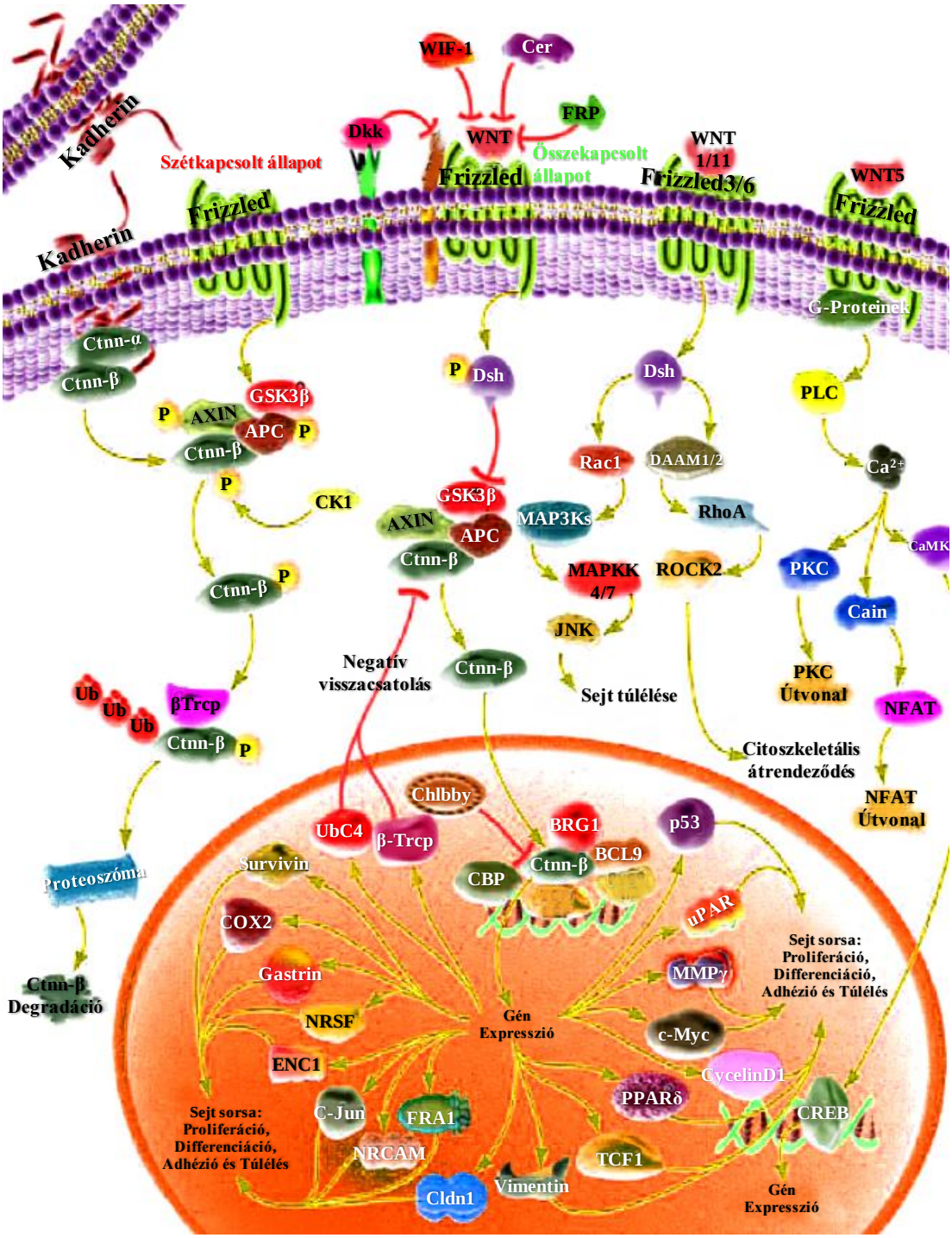
Az őssejtek túlélésének és differenciálódásának fontos szabályozói a Wnt-ok. A legtöbb vizsgálat arra mutatott rá, hogy a drasztikusan lecsökkent Wnt szintek felgyorsult öregedéshez vezetnek, feltehetően az őssejtek depléciójának következtében. Érdekes módon az ellenkező hatás is bizonyítást nyert, amely szerint a Wnt jelátvitel gátlását blokkoló inhibítort, a „KLOTHO”-t túlexpresszáló egerek szövetei ugyancsak felgyorsult öregedési folyamaton estek át (Kuro-o, Matsumura et al. 1997; Liu, Fergusson et al. 2007). Ennek feltehetően az az oka, hogy az őssejt állomány a folyamatos osztódás miatt kimerül és ezért a regenerációs folyamatokhoz szükséges sejtforrás kiapad.

2.6.5. Szteroidok hatása a tímusz öregedésre

Kezdetben a fiziológiás, szex szteroidok termelésének megindulását feltételezték a tímusz atrófia kiváltó okaként, hiszen korábbi kutatások az öregedés látható jeleit a pubertást követően azonosították. Ezt a feltételezést látszott alátámasztani, hogy akár sebészeti, akár kémiai kasztrálás eredményeként a tímusz atrófiája drasztikusan csökkent. A szteroid hatás kizárólagosságának látszik ellentmondani azonban, hogy a mostanában végzett vizsgálatok már jóval a pubertás megkezdése előtt behatároltak öregedésre utaló jeleket (LGuatente, Partridge et al. 2008). A szteroidok tímusz öregedésre kifejtett hatása azonban mégis valószínűsíthető, mivel terápiás szteroidok alkalmazásáról is kimutatható, hogy felgyorsult tímusz atrófiához vezet (Blomgren és Andersson 1970; Boersma, Betel et al. 1979; Fletcher, Lowen et al. 2009).

2.7. Jelátviteli hálózatok

A kutatásokból kiderült, hogy a Wnt jelátvitel extracelluláris és intracelluláris aktiváló és gátló molekulák jelenlététől és a párhuzamosan fellépő egyéb jelátviteli rendszerek aktiválásától függ, melynek komplexitását a 11. ábra szemlélteti. A sejten belüli jelátviteli utak, amelyekben a PKC család tagjai is aktívan részt vesznek, olyan összetett módon szabályozott hálózatot alkotnak (Kestler és Kühl 2008), melyek felderítése elengedhetetlen a fiziológiás folyamatok megértéséhez és az immunválaszt befolyásoló hatékony gén-, immun-, és farmakoterápiák kidolgozásához.



11. ábra: Wnt jelátviteli hálózatok

A Wnt jelátviteli utak és a jeltovábbításukban résztvevő intracelluláris jelátviteli molekulák és a köztük bizonyított kapcsolatok hálózata.

3. CÉLKITŰZÉSEK

Az értekezésemben kitűzött munka célja az immunrendszer és az immunrendszer kifejlődését támogató sejtekben folyó jelátviteli rendszerek megismerése volt. A jelátviteli folyamatok vizsgálata igen nagy jelentőséggel bír, hiszen az immunrendszer sejtes állományának kifejlődése és aktivitásának manipulálása ígéretes terápiás célpontok azonosítására nyújt lehetőséget. Munkám során a PKC-k mieloid sejtek proliferációs és apoptotikus folyamataiban betöltött szerepét vizsgáltam, illetve a TCR-ból, a Notch és Wnt receptorokból származó jelek T-sejtek fejlődésében betöltött szerepének tisztázására állítottam fel különféle kísérleti rendszereket. Munkám további célja volt, hogy a tímusz epiteliális mikrokörnyezetében az extra- és intracelluláris jelek változásainak a tímusz fiziológiájára kifejtett hatását vizsgáljam az öregedés folyamatában.

3.1. A PKC izoformák aktivitásának vizsgálata primer humán immunsejtekben és sejtvonalakon

3.1.1. PKC izoformák aktiválásának és gátlásának módosítási lehetőségei

3.1.2. PKC izoformák neutrofilek aktiválásában és apoptotikus sejthalálában betöltött szerepének megismerése

3.2. Jelátvitel vizsgálata a fejlődő tímusz sejtjeiben egér modellen

3.2.1. TCR-ből származó jelek hatása a T-sejtek fejlődésére

3.2.2. Wnt jelátvitel hatása a T-sejtek fejlődésére a tímuszban

3.3. A Wnt jelátvitel szerepének megismerése a tímusz adipoid involúciója során

3.3.1. A tímusz adipoid degenerációs mechanizmusainak vizsgálata

3.3.2. A PKC-k szerepének vizsgálata az tímusz involúciója során

4. ANYAGOK ÉS MÓDSZEREK

Kísérleteim során olyan szerteágazó módszertani és technikai repertoárt alkalmaztam, melyek részletezésére nincs mód a jelen keretek között. A leglényegesebbekről teszek említést, a hivatkozások megjelölésével.

4.1. Sejtek és szövetrendszerek

4.1.1. A kísérletekben felhasznált szövetek forrásai

4.1.1.1. Humán sejtek

Egészséges felnőtt donorok vénás vére szolgált az emberi sejtek forrásául. Az emberi vérből származó neutrofilek kísérletben történő felhasználásához etikai engedéllyel rendelkeztünk (University of Birmingham).

4.1.1.2. Egér törzsek és fenntartásuk

A kísérletek egyes csoportjaihoz meghatározott korú embriókra volt szükség, melyeket időzített terhességgel állítottunk elő, a pároztatás napját véve az embrionális fejlődés 0. napjának. Az állatok tenyésztése patogénmentes környezetben történt. „Ad libitum" tápanyag és víz adagolása mellett. A korosodásra váró egereket is a fenti körülmények között tartottuk, miközben semmilyen kezelésben nem részesültek.

Egér törzsek: Balb/c; kettősen (double) MHC I &II -/- (H-2^d) MHC deficiens egér (Grusby, Auchincloss et al. 1993), azaz DK, Bcl2 transzgén, Balb/c-GFP (Kvell, Czömpöly et al. 2010). Az állatok kísérletben történő felhasználása az etikai szabályok és engedélyek (University of Birmingham illetve a Pécsi Tudományegyetem) figyelembevételével történt.

4.1.2. Egér és humán sejtvonalak és primer sejtek

4.1.2.1. Sejtvonalak

Egér sejtvonalak: Tep1, Tep1-GFP, Tep1-Wnt4-GFP

Humán sejtvonalak: 293T vese epitélium, U937 mielomonocita leukémia (diffúz hisztiocitikus limfóma), HL60 promieloid (akut mieloid leukémia)

4.1.2.2. Primer sejtek

Egér: timociták (double negative (DN), double positive (DP), és single positive (SP) fejlődési alakjai); T-sejt progenitorok, Bcl2 transzgén egerek és MHC I& II -/- ($H\text{-}2^d$) timocitái, tímusz epitéliális sejtek (TEC)

Humán: Neutrofil granulociták

4.1.3. Primer humán és egér szövetek

4.1.3.1. Primer humán szövet

Egészséges felnőttek véréből Percoll grádiens centrifugálással (Jepsen és Skottun 1982) neutrofilokat szeparáltunk kísérletes aktiválásra és apoptotikus sejthalál manipulálására.

4.1.3.2. Primer egér szövetek

Embrionális (E12, E14, E15), újszülött (NB), 1, 3, 6, 9, 12, 18 hónapos Balb/c ($H\text{-}2^d$) és Balb/c-GFP egerek tímuszát, illetve májszövetét használtuk a kísérletekben.

4.2. Sejt- és szövettenyészetek

4.2.1. Emberi és egér sejt- és szövetkultúrák fenntartásához alkalmazott tápfolyadék

4.2.1.1. Emberi sejtkultúrák tápfolyadék összetétele

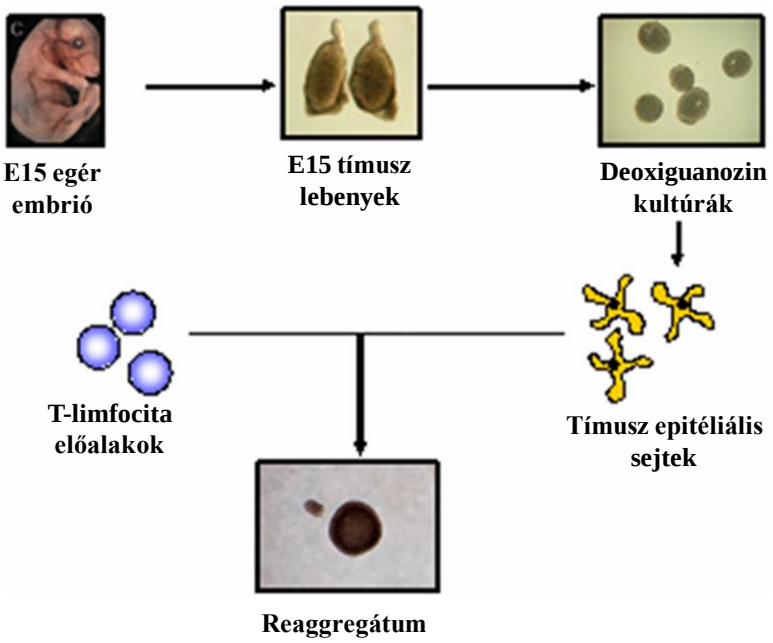
A HL60, U293 sejtvonalakat és a neutrofil inkubációkat 10% FCS-t (foetal calf serum) és 100 μg/ml penicillint, streptomicint és β-merkapto-etanolt ($5x10^{-5}$ M) tartalmazó RPMI 1640-ben tenyésztettük, 37°C-on, 5% CO_2 atmoszférában.

4.2.1.2. Egér sejt- és szövetkultúrák tápfolyadék összetétele

TEP1 (Tanaka, Mamalaki et al. 1993) és 293T sejtvonalakat DMEM médiumban tenyésztettük 10% FCS (foetal calf serum) és 100 μg/ml penicillin, streptomycin és β-merkapto-etanol ($5x10^{-5}$ M) jelenlétében, 37°C-on, 5% CO_2 atmoszférában. A szövettenyészeteket is a fenti médiumban inkubáltuk a kísérletben meghatározott ideig.

4.2.2. Komplex tímusz szövetkultúrák

A tímusz szöveti állománya kollagenázos emésztéssel elemeire bontható, majd újra összeállítható. Az epiteliális sejtek T-sejt fejlődést és szelekciót támogató képessége nem sérül, amennyiben folyamatosan háromdimenziós szöveti kultúrában tartjuk az epitéliumot. A szerv és reaggregátum szervkultúrák legkönnyebben embrionális tímusz szövetekkel állíthatók elő, de felnőtt tímusz szövetek is felhasználhatók az elkészítésükhöz. A sejtes elemeire szétválasztott, majd különféle kombinációban újra összeállított háromdimenziós aggregátumokban megmarad a tímusz epitélium T-sejtek fejlődését támogató képessége. Ez a technika azért is különösen alkalmas a jelátviteli folyamatok vizsgálatára, mert a sejtekben a gén- és fehérje-expresszió módosítása könnyen kivitelezhető a tímusz sejtes elemeinek re-aggregálása előtt vagy alatt.



12. ábra: Tímusz szerv reaggregátum készítése

Időzített terhességből származó egér embriók tímusz lebenyei eltávolításra kerültek. A lebenykéket ezután vagy deoxiguanozin jelenlétében inkubáltuk, amely a timociták depléciójához vezetett – ekkor az epiteliális sejtek könnyebben tisztíthatók és szabadon felhasználhatók kísérletekben –, vagy a lebenyeket timocita forrásként használtuk fel a reaggregátumok készítéséhez.

4.2.2.1. Tímusz sejtek szeparálása és dúsítása

Tímusz lebenyeket 1mg/ml kollagenázzal 30 percen keresztül emésztettük, majd 10% FCS-t tartalmazó DMEM-mel mostuk. Az epitélium tisztításához a sejtszuszpenziót anti-EpCAM1-FITC (G8.8-as klón) epiteliális marker ellen termeltetett ellenanyaggal inkubáltuk, majd vagy mágneses MACS oszlopon anti-FITC mikrogyöngy (Miltenyi Biotec) felhasználásával, vagy MoFlo sejtszorterrel tisztítottuk (Hare, Pongracz J et al. 2003).

Timocita alpopulációkat anti-CD44, anti-CD25 (Anderson, Anderson et al. 1997), anti-CD8, anti-CD4, anti-CD45, anti-CD69 (Hare, Pongrácz et al. 2002) elsődleges ellenanyagokkal és a megfelelő kombinációban alkalmazott másodlagos jelzéssel (FITC, PE vagy APC) Dynalbeads (mágneses elválasztás) vagy MoFlo sejtszorter alkalmazásával választottuk el. Amikor csak timocitákra volt szükség, akkor a tímusz lebenyekből mechanikai úton, a lebenyek felszeletelésével szabadítottuk ki a limfoid sejteket.

4.2.2.2. Limfoid sejteket nem tartalmazó tímusz lebenyek előállítása

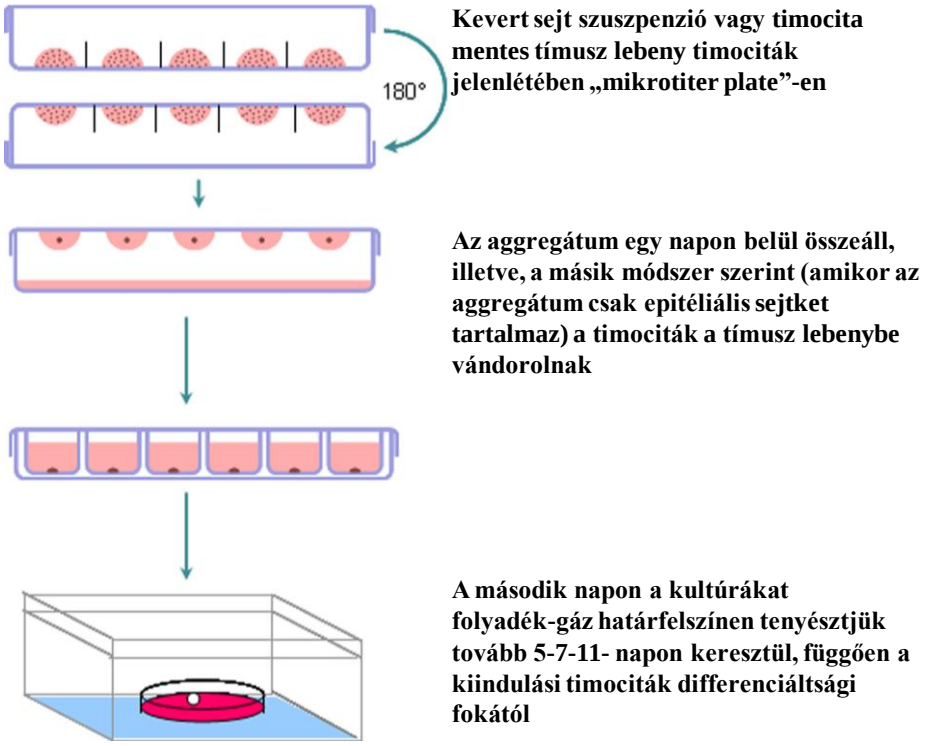
A E14 illetve E15 napos tímusz lebenyeket szövetkultúrákban inkubáltuk 2-deoxiguanozin jelenlétében (Jenkinson, Anderson et al. 1992). Mivel a 2-deoxiguanozin gátolja a sejtosztódást, a jelenlétében történő inkubálás a gyorsan osztódó timociták depléciójához vezet a 4-5 napos inkubálási periódust követően. Az így előkezelt tímusz lebenyekből az epiteliális és egyéb tímusz stróma sejtek tisztítása CD45-ös depléciót követően igen nagy hatásfokú.

4.2.2.3. Tímusz reaggregátum kultúrák

A tisztított sejtkultúrákból 1:4 (timocita:epitélium) arányú keveréket készítettünk, majd vagy centrifugálással (100xg, 5 perc, 4°C, majd 150xg, 5 perc, szobahőn) aggregáltuk (Anderson és Jenkinson 2007), vagy 20 µl-nyi folyadékban függő csepp (12. és13. ábra) kultúrát készítettünk. A 12-24 órás inkubálását követően az aggregált tímusz kultúrák stabilizálódtak és 5 ml-nyi médiumban szivacsra helyezett Millipore filteren 5–11 napon keresztül inkubálhatók voltak, amikor is a timociták differenciálódási markereit áramlási citométerrel ellenőrizni lehetett (Pongracz JE, Parnell SM et al. 2006).

4.2.2.4. Függő csepp (hanging-drop) kultúrák

Mikrogravitációs vagy függő csepp kultúrákat (13. ábra) steril műanyagból készült plate-eken (Terasaki plate) állítottuk össze. A Terasaki plate-ek mind 60-lyukú, mind 72-lyukú formátumban készülnek, orvosi tisztaságú polisztirénből. Minden egyes lyuk térfogata kb.10 µl, ezért lyukanként 20 µl-nyi folyadékkal fejjel lefelé fordítva a plate-eket kialakulnak a függő-cseppek, melyeket a kapillárishatás tart a műanyaghoz rögzítve. Egy ilyen folyadékcsepp-ben a szuszpenzióban levő sejtek lassan leülepednek, és aggregátumot képeznek. Mivel a kicsi térfogatban a tápanyag mennyisége limitált, amint az aggregátum kialakult, a mikroszövetet nagyobb szövettenyésztő edénybe helyeztük át (Pongracz JE, Parnell SM et al. 2006; Anderson és Jenkinson 2007).



13. ábra: Függő csepp kultúra

A függő csepp kultúra megkönnyíti a limitált sejtszámú populációkból szöveti aggregátumok készítését, mivel igen kevés sejtszám esetén is használható és manipulálható eljárás.

4.3. Sejt specifikus molekulák detektálása

4.3.1. Citospin

Kezelt vagy kezeletlen neutrofilekből, timocitákból vagy sejtvonalakból (TEP1, U937, HL60) 1×10^6/ml sejtszuszpenziót készítettünk, majd 100 µl-nyi sejtet vittünk a citocentrifuga sejttartályaiba (300 rpm, 3 perc, 20°C). Centrifugálást, majd szárítást követően a festési eljáráshoz szükség szerint optimalizált fixálási (aceton, metanol, etanol, paraformaldehid) és permeabilizálási (Tween20, vagy szaponin) eljárásnak vetettük alá a sejteket. Ezt követően fehérjespecifikus ellenanyaggal jelöltük a kísérlet szempontjából fontos fehérjéket. Immunfluoreszcens és konfokális mikroszkópiával végeztük az analízist. Az apoptotikus neutrofileket hematoxilin-eozinnal festett sejtszuszpenzióból mikroszkopikus analízissel morfológia alapján azonosítottuk (Afford, Pongracz et al. 1992).

4.3.2. Szöveti metszetek

Fagyasztott primer tímuszból vagy reaggregátum kultúrából 8-9 μm vastag metszetek készültek, amelyeket hideg acetonban vagy 4%-os paraformaldehidben fixáltunk (Talaber, Kvell et al. 2011). A metszetek szárítását követően 5%-os „bovine serum albumin"-nal blokkoltuk (BSA PBS-ben, 20 perc) mielőtt a megfelelő specifikus ellenanyaggal (anti-Ly51-PE (clone 6C3), anti-EpCAM-FITC (clone G8.8), jeleztük a fehérjéket. Amennyiben intracelluláris fehérjék (pl anti-PKC δ) expressziós vagy lokalizációs analízisét végeztük, a metszeteket paraformaldehides fixálás után szaponinos kezeléssel permeabilizáltuk. A sejtmagot DAPI festéssel, míg a zsírszövetet LipidTOX Red festéssel tettük láthatóvá. A metszeteket Olympus BX61 mikroszkóppal és AnalySIS szoftverrel analizáltuk (Kvell, Varecza et al. 2010).

4.3.3. Áramlási citometria és szortolás

A sejtfelszíni markerek vagy GFP expresszió alapján határoztuk meg az egyes sejtpopulációk jelenlétét illetve arányait (Pongracz JE, Parnell SM et al. 2006). A sejtfelszíni markerekre specifikus primer ellenanyagok lézerrel gerjeszthető festékkel direkt konjugált formáit (anti-Ly51-PE, anti-EpCAM-FITC) vagy másodlagos ellenanyagok konjugáltjait alkalmaztuk. A sejteket jégen inkubáltuk az ellenanyagok jelenlétében 30 percig, majd PBS-ben történt mosási lépéseket követően további 30 percig inkubáltuk másodlagos ellenanyag jelenlétében. A sejteket PBS-es mosást követően 0,5%-os paraformaldehidben fixáltuk az áramlási citometria megkezdése előtt. Amennyiben életképes sejtpopulációra volt szükség a további kísérletekhez, úgy a fixálási lépés elmaradt.

4.4. Apoptózis detektálása

4.4.1. Korai apoptózis detektálása

A korai apoptózist annexin festéssel detektáltuk, mivel az annexin V nagy affinitással kötődik a membrán foszfolipid foszfatidil-szerinhez, amely a belső membránból a külső membránba transzlokálódik még a DNS fragmentáció megindulása előtt. A sejteket szuszpenzióban direkt FITC-konjugált annexin V-tel inkubáltuk (végső koncentráció: 1 μg/ml), majd áramlási citométerrel 488 nm-nél analizáltuk (Pongracz, Parnell et al. 2003).

4.4.2. Késői apoptózis detektálása

Az apoptózis későbbi stádiumait, amikor a membrán áteresztőképessége fokozódott, propidium jodiddal teszteltük és áramlási citométerrel mértük. A DNS fragmentáció meghatározásához DNS-t tisztítottunk, majd a DNS létrát (apoptotikus fragmentumokat) agaróz gélben szeparáltuk, majd etídium bromiddal, UV fényben tettük láthatóvá. Neutrofil sejtszuszpenziók esetében az apoptotikus morfológiát mutató sejtek kvantitálása érdekében citospint készítettünk, majd a sejteket hematoxilin-eozin festést követően mikroszkopikus úton analizáltuk (Pongracz, Parnell et al. 2003).

4.5. RNS izolálás, cDNS készítés, RT-PCR és Q-RT-PCR

4.5.1. RNS izolálás, cDNS készítés és végpont RT-PCR

A szöveti vagy sejtes mintákból „NucleoSpin RNA clean-up kit" felhasználásával került sor RNS izolálásra. A kinyert RNS-ből „SuperScript II RNaseH-reverse transcriptase kit" (Invitrogen) felhasználásával készült cDNS. Az így kapott cDNS-ből szekvencia specifikus primerek (szekvenciák a közleményekben találhatók) és „ReddyMix PCR Master Mix" felhasználásával végeztük. A reakcióterméket etídium-bromid jelenlétében, 1%-1,5%-os agaróz gélben szeparáltuk. A gél UV fény alatti elemzése Bio-Rad image analizátor segítségével történt. Belső kontrollként általában β-aktin-t használtunk (Pongracz, Hare et al. 2003).

4.5.2. Q-RT-PCR

A kvantitatív PCR reakciókhoz vagy SYBR green vagy TaqMan módszert alkalmaztunk. A „master mix"-hez génspecifikus primereket adtunk, melyek szekvenciája a csatolt publikációkban megtalálhatók. A kvantitatív RT-PCR adatok analíziséhez a delta Ct (dCt) és a Relative Quantity (RQ) módszereket alkalmaztuk. Minden minta triplikátumokban került mérésre. A PCR reakciót a disszociációs görbe analízisével ellenőriztük (Kvell, Varecza et al. 2010).

4.6. Immunprecipitáció és Western blot

4.6.1. Immunprecipitáció

A megfelelő kezelést követően a kísérleti sejttípusokból szuszpenziót készítettünk, majd lízis pufferben (lásd Western blot), jégen inkubáltuk 20 percen keresztül. A kísérlet által megkövetelt ellenanyagot (pl. anti-foszfo-szerin, anti-Bcl2, anti-PKC δ) és protein A Sepharose-t adtunk a lizátumhoz, majd folyamatos rotáció mellett végzett egész éjszakás inkubálást (O/N, 4°C-on) követően a Sepharose gyöngyöket gyors centrifugálással (5", 4°C, 14,000 rpm) összegyűjtöttük, jéghideg TBS (10 mM Tris/HCl, pH 7,8) pufferben mostuk, majd újra szuszpendáltuk 50 µl TBS-ben +25 µl 2xSDS-minta pufferben, aztán 3 percig forraltuk, mielőtt centrifugáltuk és SDS-PAGE gélben szeparáltuk. Amennyiben enzimaktivitást kívántunk mérni, úgy a TBS puffer helyett az enzimaktivitás méréséhez szükséges összetételű pufferben szuszpendáltuk fel a Sepharose gyöngyöket (Pongracz, Parnell et al. 2003; Varecza, Kvell et al. 2011).

4.6.2. Western blot

A megfelelő kezelést követően a kísérleti sejttípusokból szuszpenziót készítettünk, majd lízis pufferben (20 mM HEPES pH7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$ 137 mM NaCl, 50 mM β-glicerofoszfát, 1% Triton X100 kiegészítve 1mM DTT, 2mM PMSF, 2µg/ml leupeptin, 1µg/ml aprotinin, 2 mM Na_3VO_4 , 0,1 M Na-pirofoszfát, 1 µM β-mikrocisztin és10 mM NaF) jégen inkubáltuk 20 percig. Az inkubálást követően a mintákat folyékony nitrogénben lefagyasztottuk és -70°C-on tároltuk felhasználásig. Mielőtt 10% SDS-PAGE-n szeparáltuk a fehérjéket, a mintákat 2xSDS, minta pufferben felforraltuk. A géleket PVDF (Immobilon)-membránra blottoltuk, majd 1%BSA-val blokkoltuk. Ezt követően specifikus ellenanyagokkal megjelöltük a kísérletünk célfehérjéjét (pl. anti-foszfo-Akt, anti-foszfo-Bad, anti-Bad, anti-Bcl2, anti-β-katenin, anti-PKC δ vagy β-aktin). Specifikus másodlagos HRP-ellenanyaggal jelöltük a primer ellenanyagot, majd a fehérjéket kemilumineszcens „Supersignal kit" segítségével (Pierce) tettük láthatóvá. A mennyiségi összehasonlítást denzitometriás analízissel végeztük (Alpha Imager, Flowgen). Amennyiben γP^{32}-ATP beépülését vizsgáltuk, úgy a blottokat megszárítottuk és autoradiográfiának vetettük alá (Pongrácz, Webb et al. 1999; Pongracz, Parnell et al. 2003).

4.7. Enzimaktivitás mérése

4.7.1. PKC izoenzimek, Akt/PKB, PI3K és JNK aktivitásának mérése

A megfelelő kezelést követően neutrofilekből, timocitákból vagy tímusz epiteliális sejtekből került sor enzim fehérjére specifikus ellenanyag felhasználásával az enzim immunprecipitációjára (2 óra, 4°C-on), majd a megfelelő gátló (pl. S33559) és aktiváló (pl. DAG, TPA) kontroll kezelések alkalmazása mellett mértük a γ-^{32}P-ATP beépülését az enzimspecifikus szubsztrátba (Pongrácz, Webb, et al. 1999). Más kísérletekben viszont a szubsztrátba beépült rádioaktivitás folyadék scintillációs mérésével határoztuk meg az enzim aktivitását (Griffiths, Garrone et al. 1996; Pongracz, Parnell et al. 2003).

4.7.2. PKC-k autofoszforilációja

A PKC izoenzimek autofoszforilációjának méréséhez a sejtvonalakat 4 órán keresztül 200 *m*Ci/ml [^{32}P] ortofoszfátot tartalmazó médiumban inkubáltuk, majd PKC izoenzim specifikus ellenanyag felhasználásával immunprecipitáltuk. 8% SDS-PAGE-n végzett elektroforézist követően a géleket megszárítottuk, majd autoradiografáltuk (Griffiths, Garrone et al. 1996).

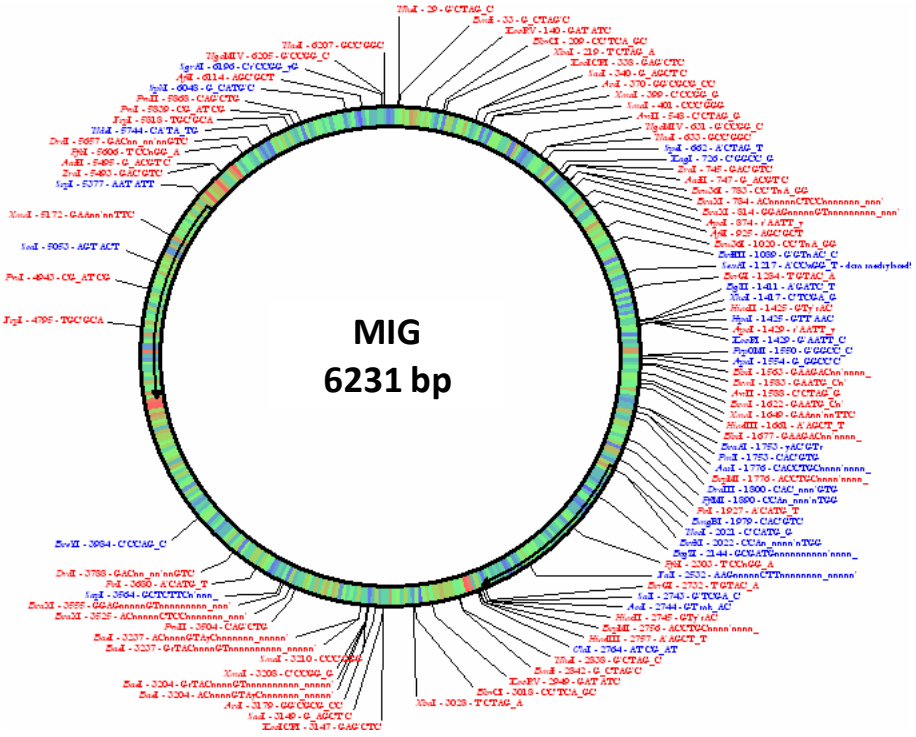
4.8. Génexpresszió módosítása

4.8.1. Rekombináns vírusok készítése

A rekombináns vírusok megkönnyítik a gének bejuttatását primer sejtekbe, mivel fiziológiás folyamatokat használnak a génbevitelre. Míg a retrovírusok osztódó sejteket, addig az adenovírusok nem osztódó, elsősorban epiteliális eredetű sejteket fertőznek meg. A retrovírusok által bejuttatott gének beépülnek a genomba, míg az adenovírusok által bejuttatott gének nem. Mind a rekombináns retro-, mind a rekombináns adenovírusok fertőzőképesek, de vad típusú sejtekben osztódni nem tudnak. A vírusok előállításához ezért speciális sejtvonalakra (293A, Phenix) van szükség, amelyek hordozzák a vírus replikációjához szükséges géneket.

4.8.1.1. Rekombináns retrovírusok

A rekombináns retrovírusok készítéséhez a MIG (**M**SCV-**I**RES-**G**FP) plazmid konstrukciót használtuk, melybe szekvenciaspecifikus primerek segítségével amplifikált célszekvenciákat klónoztunk. Így készült el a MIG-CMV-IRES-GFP, MIG-CMV-ICAT-IRES-GFP, MIG-CMV-Wnt4 -IRES-GFP és a MIG-CMV-ICNOTCH-IRES-GFP plazmid. Az elkészült vírus plazmidokat tisztítás után 293A sejtvonalba transzfektáltuk Lipofectamine 2000 felhasználásával. A sejtvonal hordozza a vírus replikációjához szükséges szekvenciákat, és a sejtvonalban termelt retrovírusokat a médiumba bocsátja ki. A médiumot három napon át gyűjtöttük, majd felhasználtuk osztódó, primer timociták megfertőzésére (Pongracz, Hare et al. 2003; Pongracz JE, Parnell SM et al. 2006).



14. ábra: Rekombináns retrovírusok előállításához génsebészeti eljárásokkal készített MIG plazmid térképe

A MIG plazmid Prof Warren Pear (Pennsilvania) ajándéka volt a kísérletek kivitelezéséhez. Az ábrán a piros és kék jelek restrikciós helyeket jelölnek, melyek megkönnyítik a restrikciós térkép elkészítését a klónozás során. A bejuttatásra váró géneket „multiple cloning site'-on valamelyik restrikciós hely kiválasztásával úgy klónoztuk, hogy a gént a restrikciós helyekre jellemző „ragadós" véggel készítettük el.

4.8.1.2. Rekombináns adenovírusok készítése

A rekombináns adenovírusok készítéséhez a kereskedelmi forgalomban lévő alapvektorokat használtunk (Hare, Pongracz J et al. 2003). Először a „shuttle" vektorba klónoztuk a módosított expressziót igénylő szekvenciát, majd a „shuttle" és „acceptor" vektort homológ rekombinációnak vetettük alá. A homológ rekombinációt követően a vektorokat baktériumokban felszaporítottuk, megtisztítottuk, linearizáltuk, majd a 293A sejtvonalba juttattuk Lipofectamine 2000 segítségével. A sejtvonalban a sikeres transzfekciót követően megfelelő mennyiségű plazmidot hordozó sejtek vírusokat kezdtek termelni. A vírustermelő sejtek elpusztultak és a kiszabaduló vírusok további sejteket fertőztek meg. Így nőtt meg a vírus koncentrációja a felülúszóban, amellyel további sejteket fertőztünk meg. A megfelelő mennyiségű fertőzött sejt elérése után a vírusokat kereskedelmi forgalomban is elérhető Ad vírus tisztító kit segítségével tisztítottuk, majd centrifugálásos módszerrel, Millipore szűrőket felhasználva koncentráltuk.

A kísérletekhez az alábbi adenovirális vektorokat készítettük: Ad-CMV-CD80-IRES-GFP, Ad-CMV-IRES-GFP.

4.8.2. siRNS kísérleti alkalmazása

Az siRNS avagy a „small interfering RNA" alkalmazása ma már bevett gyakorlat a génátíródást követő, transzlációs folyamatok megakadályozására. Kísérleteinkben kereskedelmi forgalomban elérhető siPKC δ-t használtunk a PKC δ fehérje szintjének és aktivitásának csökkentésére (Varecza, Kvell et al. 2011).

5. EREDMÉNYEK ÉS MEGBESZÉLÉS

5.1. PKC-k a mieloid típusú immunsejtek jelátvitelében

A mieloid típusú sejtek az immunológiai védelem első vonalát képviselik, melynek következtében szerepük elengedhetetlen a természetes immunválasz kialakításában. A mieloid sejtekben kialakuló betegségek ezért megbénítják az immunrendszer működését, amely súlyos, gyakran halálos patológiás folyamatok kialakulásához vezet.

Kezdeti kísérleteinkben a PKC család fiziológiás hatásainak felderítése volt a célunk, hiszen az irodalomból ismert, de ellentmondásos kísérleti eredmények következtében a terápiás szempontból ígéretes PKC-k fiziológiás szerepe felderítetlen maradt (Moore, Jenkinson et al. 1992; Rajotte, Haddad et al. 1992.). A kísérleti ellentmondások okai visszavezethetők arra, hogy a legtöbb kísérletben nem vették figyelembe a farmakológiai eredetű aktivitást módosító vegyületek, mint pl. a TPA koncentráció-függő sajátosságait és a kísérletben alkalmazott biológiai rendszereket. Gyakran még a kísérleti rendszer izoenzim összetételét sem vizsgálták meg, mégis messzemenő következtetéseket vontak le az eredményekből, amelyek éveken át hatással voltak a PKC-k fiziológiai funkciójának megítélésére. A kísérleteinkben alkalmazott aktivátorokat és inhibítorokat az 1. táblázat foglalja össze.

1. táblázat

Teljes elnevezés	Rövidítés	Aktivátor	Inhibítor	Célenzim
12-tetradecanoyl-13-phorbol acetate	TPA	+		PKC család
12-deoxyphorbol-13-phenylacetate	Dopp	+		PKC család
12-deoxyphorbol-13-phenylacetate-20-acetate	Doppa	+		PKC család (PKCβ)
thymeleatoxin	THY	+		PKC család
Bistratene A	BisA	+		PKCδ
Staurosporine	SA		+	PKC
Go6976			+	cPKC
Rottlerin			+	PKCδ
Bisindolylmaleimide I	Bis		+	cPKC
PD98059			+	MAPK-ERK1,2
LY379196			+	PKCβ

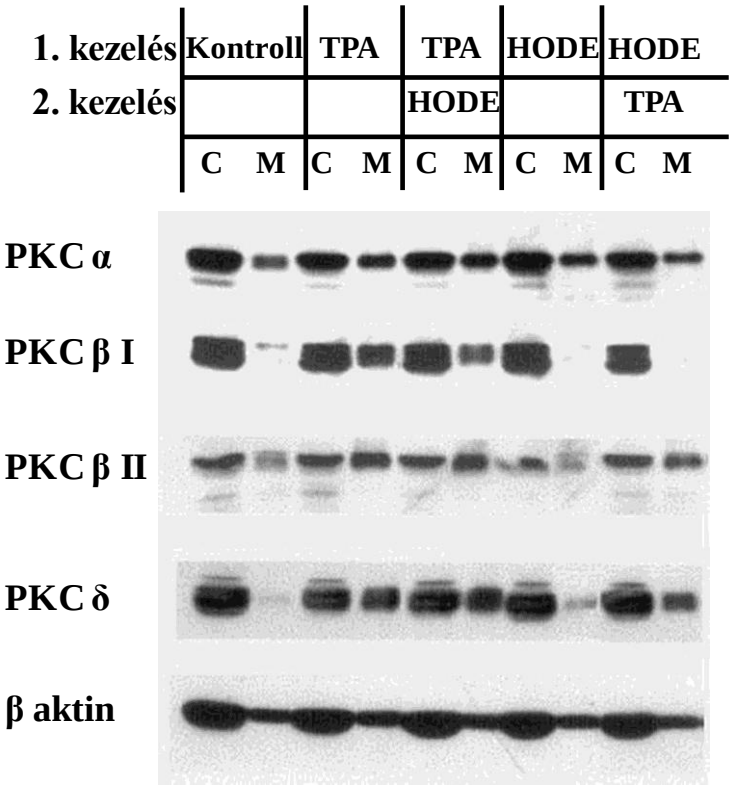
Teljes elnevezés	Rövi-dítés	Akti-vátor	Inhi-bítor	Célenzim
13-Hydroxyoctadecadienoic acid	13-HODE		+	PKC
SB202190			+	p38

5.1.1. PKC izoenzimek szerepének vizsgálata mieloid sejtekben gyulladási, proliferációs és apoptotikus folyamatok során

A mieloid sejtek előalakjaiból készített sejtvonalak alkalmasnak bizonyultak a PKC izoenzimek fiziológiás folyamatokban történő jelátviteli szerepének meghatározására, hiszen könnyű tenyészthetőségük elegendő számú sejtet biztosított a kísérletek elvégzésére. Kísérleteinkben a PKC izoenzimeket szelektíven aktiváló vagy gátló molekulákat alkalmaztunk a vizsgálatokban. Ezekben a kísérletekben azt vizsgáltuk, hogy a mind aktivációjuk módjában, mind szubsztrátspecificitásukban nagyon hasonló enzimeknek mi a fiziológiás szerepe és vajon terápiás célpontként alkalmazhatóak-e? Ezen túlmenően a kísérleti rendszereink alkalmasnak bizonyultak a specifikus PKC inhibítorokként vagy aktivátorokként ismertté vált vegyületek specificitásának és hatásmechanizmusának vizsgálatára is.

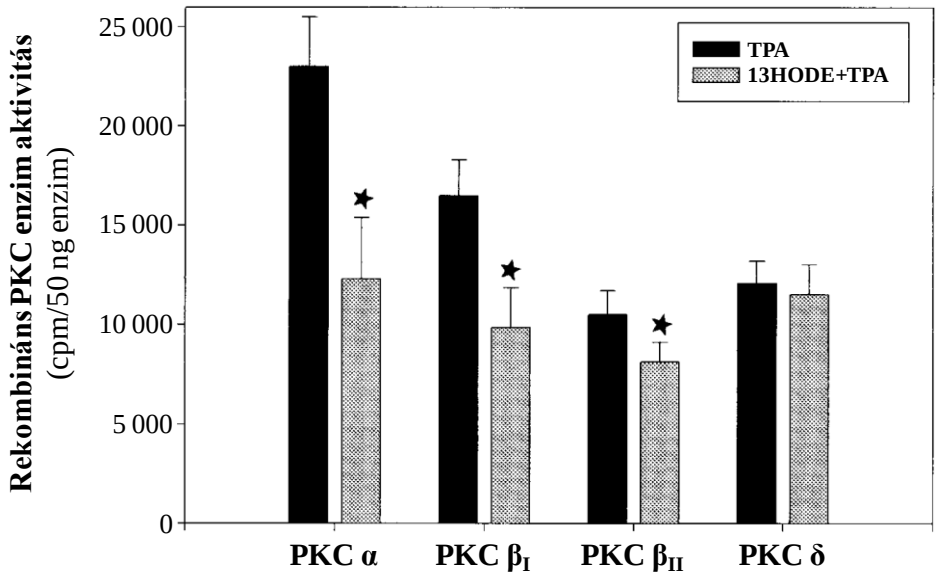
A kutatásainkban alkalmazott egyik vegyület a 13-Hydroxyoctadecadienoic sav (avagy 13-HODE). A hydroxylinoleic sav a lipoxigenáz egyik fő metabolitjaként ismert, amelyet a gyulladásos folyamatokban résztvevő mieloid típusú sejtek termelnek a gyulladásos sejtek aktivitásának módosítására. Először HUVEC-ben (human umbilical cord endothelial cell), azaz nem hematopoietikus, hanem endoteliális sejtekben bizonyították, hogy a 13-HODE beépül a DAG-ba, melynek következtében szelektíven gátolni tudja a PKC-k aktivitását. Mivel a DAG-ra mind a klasszikus (c), mind a novel (n) PKC-nak szükségük van a teljes aktiválódáshoz, feltételezhető volt, hogy e két izoenzim alcsalád együttes gátlásához vezet a 13-HODE alkalmazása. Ezért feltételeztük, hogy a 13-HODE a jelátvitel általános sejtfiziológiai tanulmányozásában is hasznosnak bizonyulhat. A természetesen is előforduló PKC inhibítor alkalmazása a mieloid típusú sejtek aktiválásának szabályozására azért is nagy jelentőségűnek tűnt, mert specifikus gátló molekulák betegségek kimenetelének módosítására nyújthatnak lehetőséget. Feltételeztük, hogy amennyiben a 13-HODE valóban képes gátolni a gyulladási folyamatokban résztvevő sejtek aktiválódását, úgy akár egy igen hatékony gyulladásgátló is kifejleszthető a kísérleteinkből nyert ismeretek alapján.

Kezdeti vizsgálataink során élő sejtekben vizsgáltuk az enzimaktiváció hatására végbemenő fehérjelokalizációs változásokat. Kísérleteinkben figyelembe vettük a jelátvitel valós időbeni sebességét. Kerültük a hosszadalmas enzimtisztítási lépéseket, mint pl DE52 és hidroxilapatit alkalmazását, amely helyett transzlokációs és immunprecipitációval kombinált enzimaktivitási vizsgálatokat végeztünk. Kísérleteinkben a promieloid, HL60-as sejtvonalat és rekombináns PKC izoformákat használva megállapítottuk, hogy a 13-HODE képes gátolni a PKC α-t, βI-t és βII-t, azaz a klasszikus PKC-kat, de nem befolyásolja a novel PKC δ aktivációját. A kísérleteket a HL60-as sejtvonalban a PKC izoformák indukcióra végbemenő transzlokációjával és immunprecipitált izoenzimek aktivitásának mérésével bizonyítottuk (15. ábra). Eredményeinket rekombináns PKC-vel végzett kísérletek is alátámasztották (16. ábra).



15. ábra: A 13-HODE hatása PKC izoenzimek transzlokációjára HL60-as sejtekben (Pongrácz, Lord et al. 1999)

Csak 10 nM TPA, 10 nM TPA és szimultán adott 3 ng/ml 13-HODE, csak 3 ng/ml 13-HODE, vagy 3 ng/ml 13-HODE 30 perccel a 10 nM TPA aktiválást megelőzően – a HL60-as sejtekből citoszol (C) és membrán (M) frakciókat izoláltunk, majd a fehérjéket SDS-PAGE gélen szeparáltuk Western blot analízishez. A blottokat PKC izoenzim specifikus ellenanyagok felhasználásával teszteltük (α, βI, βII, és δ). Anti-β-aktin ellenanyagot használtunk az egyenlő mennyiségű fehérje felvitelének bizonyítására.



16. ábra: A 13-HODE hatása rekombináns PKC isoenzimek aktivitására (Pongrácz, et al. 1999)

Rekombináns humán PKC izoenzimeket (α, βI, βII, és δ) TPA-val aktiváltuk (10 nM), amellyel szemben a 3 ng/ml 13-HODE enzimaktivitásra kifejtett hatását mértük PKC specifikus peptid szubsztrátba való rádioaktív ATP ([γ^{32}P]ATP)-ből származó P-beépülésével. A csillag jelöli a szignifikáns változásokat ($p \leq 0.05$).

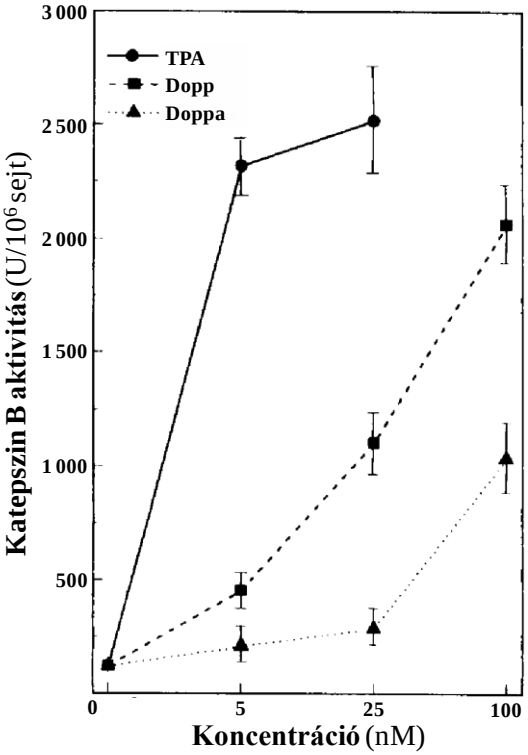
Eredményeink alapján a 13-HODE, mint természetes metabolit képes volt a klasszikus PKC izoenzimek gátlására, de a novel PKC δ-ra nem volt hatással. A kísérletek eredményeként arra a következtetésre jutottunk, hogy a PKC δ a gyulladásos folyamatok jelátvitelében feltehetően nem játszik szerepet. Mivel a PKC δ fiziológiás szerepének megértéséhez a 13-HODE-dal végzett kísérletekkel nem jutottunk közelebb, annak megállapítására, hogy a PKC δ mely fiziológiás folyamatok jeleit továbbítja, enzimspecifikus aktivitás-módosítási lehetőségeket alkalmaztunk.

PKC δ által irányított jelátviteli folyamatok vizsgálatára elsőként a Bistratene A (Bis A) elnevezésű poliétert használtuk, mely vegyületet a Heron Szigeti Korallzátony (Ausztrália) közelében lehalászott zsákállatból (*Lissoclinum bistratum)* (Degnan, Hawkins et al. 1989) izoláltak. Bis A hatását vizsgálva megállapítottuk, hogy ez a vegyület citosztatikus hatással rendelkezik, G2/M(10) fázisban állítva le a sejtosztódást (Griffiths, Garrone et al. 1996). Amennyiben a promieloid HL60 sejtvonalat kezeltük BisA-val, a sejtek megindultak a monocita/macrofág (Watters, Marshall et al. 1990) irányú differenciálódás útján, melyet differenciációs markerek, mint pl a CD11-es molekula expressziójának megjelenésével és az adhéziós képesség megnövekedésével bizonyítottunk. Kísérleteinkből arra a következtetésre

jutottunk, hogy a klasszikus PKC-kel ellentétben a novel PKC δ elsősorban a differenciálódást irányító jeltovábbításban vesz részt.

Kérdéses maradt azonban, hogy a klasszikus PKC alcsalád minden tagja proliferációt indukáló jeleket továbbít-e, vagy ezek az enzimek szerepet játszanak sejtdifferenciáció vagy apoptózis szabályozásában is? Ennek a kérdésnek megválaszolásához olyan aktivátorokra vagy inhibítorokra volt szükség, amelyek a klasszikus PKC izoenzimek differenciált aktiválására illetve gátlására képesek. Mivel a cPKC alcsalád tagjainak differenciált és specifikus gátlása az akkor rendelkezésre álló inhibítorokkal nem volt lehetséges, aktivátorok felhasználásával végeztük a kísérleteket. Pozitív kontrollként a PKC izoenzim specificitást nem mutató TPA-t használtuk, mivel a TPA potens PKC aktivátor lévén a promieloid sejtek differenciálódását és apoptózisát is indukálni tudja (Lotem, Cragoe et al. 1991; Clemens M.J., Trayner et al. 1992). Hogy a klasszikus alcsaládba tartozó PKC β izoenzim általános szerepkörét tanulmányozzuk, az U937-es sejtvonal felhasználásával végeztünk kísérleteket a TPA-nál nagyobb enzimspecificitást mutató, PKC β specifikusnak feltételezett forbolészter, Doppa (12-deoxyphorbol-13-phenylacetate-20-acetate) felhasználásával. A kísérletek elvégzését az is indokolta, hogy az eredeti Doppa forbolészter kísérletek egyebek mellett figyelmen kívül hagyták azt a tényt, hogy 6 óra inkubálást meghaladó fiziológiás hatásvizsgálatok már nem a Doppa, hanem hidrolízis terméke, a Dopp (12-deoxyphorbol-13-phenylacetate) hatását tesztelték. Dopp esetében azonban semmilyen PKC izoenzimspecificitás nem mutatható ki, ugyanazokat az izoenzimeket aktiválja, mint a TPA, csak kisebb hatékonysággal. Tehát az egyedi izoenzimek fiziológiás folyamatokban betöltött szerepére nem vonhatók le következtetések a 6 órányi inkubációt meghaladó kísérletekből.

A PKC α-t, β-t, δ-t és ζ-t expresszáló U937-es sejtvonalon végzett kísérletekben kimutattuk, hogy a PKC β gyors aktivációja az apoptotikus folyamatok megindulásához vezet, mellyel szemben a sejtek differenciációjához számos PKC izoenzim egyidejű aktiválása szükséges (17. ábra).



17. ábra: TPA, Dopp vagy Doppa indukálta differenciáció (Pongrácz, et al. 1996)

U937-es sejteket 72 órán át inkubáltuk TPA (1-25 nM), Dopp (5-100 nM) vagy Doppa (5-100 nM) jelenlétében és katepszin B aktivitásának mérésével határoztuk meg a monocita irányú differenciációt. (Az enzimaktivitást (Units/10^6 sejt) standard deviáció megjelenítésével ábrázoltuk).

A forbolészterek PKC izoenzimekre kifejtett hatását az enzimek sejten belüli lokalizációs változásaival is nyomon követtük (18. ábra), mely kísérletek alátámasztották, hogy a PKC enzimek egy órás aktivációja is messzemenő változásokat idéz elő az enzimek aktivációs szintjében és lokalizációjában.

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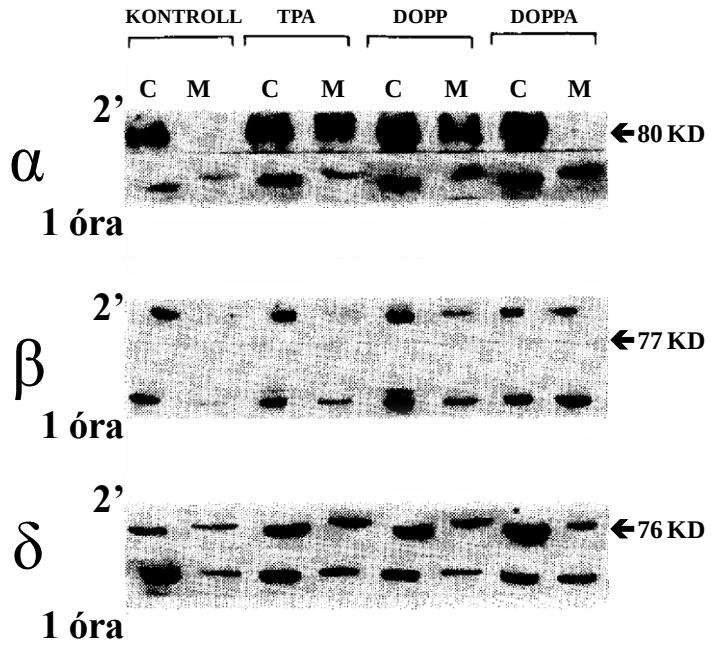


18. ábra: PKC izoenzimek aktivációja TPA, Dopp és Doppa forbolészter kezelést követően U937-es mielo-monocita sejtvonalban (Pongrácz, et al. 1996)

A kontroll sejteket csak médiummal, míg a további sejtkultúrákat 10 nM TPA-val, 25 nM Dopp vagy 25 nM Doppa-val kezeltük 2 perc és 1 óra időtartamra mielőtt a PKC izoenzimeket izoláltuk a citoszolból (C) és a membrán (M) frakcióból. A fehérjéket SDS-PAGE gélben szeparáltuk és a PKC izoenzimek jelenlétét Western-blottal határoztuk meg izoenzimspecifikus ellenanyagok felhasználásával.

A kísérleteink eredményeit alátámasztotta, hogy a PKC β mutáns HL60-as sejtvonal rezisztenssé vált a differenciálódást és az apoptózist indukáló jelekre, és ez a rezisztencia megszüntethető volt a PKC β szint helyreállításával.

Összességében kísérleteink segítségével sikerült arra rávilágítanunk, hogy a PKC izoenzimek egyedileg és enzimcsoportokként is részt vesznek specifikus jelátviteli folyamatokban. Továbbá megállapítottuk, hogy a biológiai rendszerek PKC izoenzim összetétele, azok aktivációs szintje, a bejövő jelek erőssége és az enzimek hatásirányának eredője dönti el a fiziológiás válaszképességet. Ennek tükrében azonban felmerült, hogy mennyiben relevánsak a mutáns sejtvonalakon végzett kísérletek, ha az emberi szervezetben, természetes formájukban jelen lévő immunsejtek működését kívánjuk megismerni és terápiás célzattal befolyásolni? Hogy közelebb kerülhessünk a PKC-k fiziológiás jelátvitelben betöltött szerepének megértéséhez, sejtvonalak helyett a továbbiakban primer humán neutrofilekben követtük a jelátviteli folyamatokat.

5.1.2. Gyulladásos folyamatok jelátviteli vizsgálata

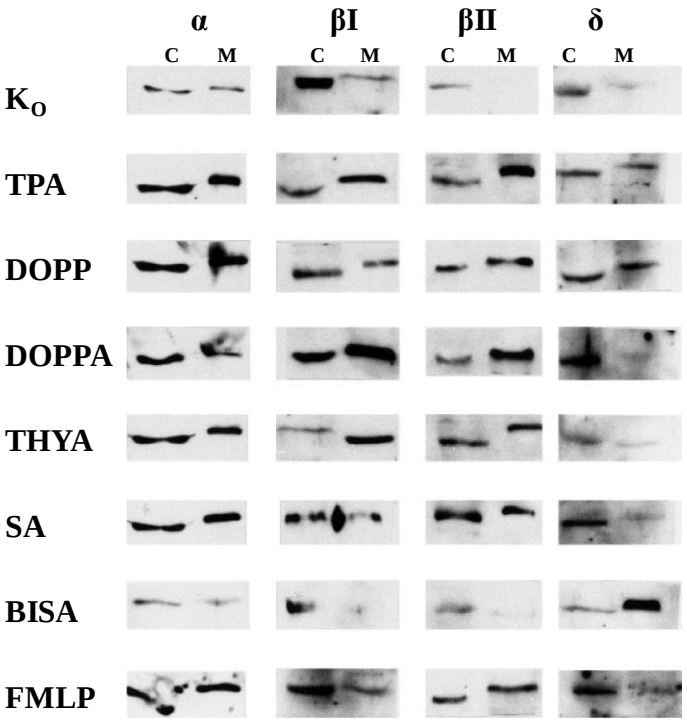
Neutrofil granulociták a legnagyobb számban jelen lévő fehérvérsejtek a keringésben. A neutrofilek a természetes immunitás igen fontos alkotói, hiszen fertőzések esetén mikróbákat fagocitálnak és pusztítanak el. A neutrofilek naponta kb $1\text{-}2\times10^{11}$ számban termelődnek, de természetes életidejük rövid, és amennyiben nincs jelen fertőzés, úgy 24-36 óra alatt apoptózissal elpusztulnak. Fertőzés esetén a neutrofilek kemoattraktánsok hatására (mikrobiális termékek pl fMLP, komplement komponensek pl C5a, és kemokinek, pl IL8) elhagyják a véráramot és a fertőzött szöveti területre migrálnak, ahol megkezdik a fagocitózist. Fagocitálást követően először lítikus enzimeket, reaktív oxigén és nitrogén gyököket termelnek, melyek segítségével elpusztítják a fertőző mikróbákat és a fertőzött sejteket egyaránt. Mindez idő alatt pro-inflammatorikus citokinek kibocsátása is megindul, mellyel még több immunsejtet vonzanak a fertőzés helyére. A fertőzés eliminálása után a neutrofilek apoptózissal elpusztulnak és maradványaikat a makrofágok fagocitálják. Érthető módon, ha a neutrofilek apoptózisa nem, vagy csak késve indul meg, úgy a fertőzésen átesett terület szöveti elemeinek pusztítása tovább folytatódik, amely krónikus szövetkárosodáshoz vezet.

Mivel az aktivált neutrofilek perzisztens jelenléte igen kiterjedt szövetkárosodást eredményezhet, a krónikus gyulladási folyamatok leállítása terápiás szempontból is igen fontos cél. A hatékony terápia megtervezéséhez azonban szükséges a neutrofilek apoptózisának molekuláris szintű megértése, és olyan molekulák megismerése, melyek terápiában való alkalmazásával szabályozhatóvá válnak a neutrofilek aktivációs és apoptotikus folyamatai.

A neutrofilek aktivációs folyamatainak vizsgálatánál figyelembe vettük, hogy a neutrofil aktiváció egyik fő lépése a NADPH oxidáz aktivációja, mely enzim NADPH felhasználásával katalizálja a reaktív oxigén gyökök termelését. A reaktív molekulagyökök termelése fontos lépés a neutrofilek sejtpusztító képességének kialakulásában. A folyamat főbb lépései a következők: az inaktív neutrofilekben a sok komponensből álló NADPH oxidáz disszociált állapotban van. Sejten kívüli stimuláló jelek hatására az aktivált neutrofilekben megindul az enzim két fő komponensének ($p47^{phox}$ és $p67^{phox}$) foszforilációja, melynek hatására igen gyorsan asszociálódik az aktív enzimkomplex. Ennek eredményeként a neutrofil képessé válik reaktív oxigén gyökök előállítására és sejtpusztító funkciójának betöltésére.

Annak eldöntésére, hogy a PKC enzimcsalád tagjai milyen szerepet játszanak a foszforilációs folyamat következtében meginduló oxigén szabadgyökök, azaz szuperoxid termelésében, számos faktort kellett figyelembe vennünk. Többek között azt, hogy noha az igen hatékony PKC aktivátor, TPA, szuperoxid termeléshez vezet, nem mindegyik neutrofil aktivációhoz vezető folyamat igényli a PKC részvételét a jelátvitelben. Ilyen pl. a C5a komplement faktor által indukált szuperoxid termelés is. Ezért kísérleteinkben már nem csak a PKC izoenzimeket direkt kapcsolódás útján aktiváló farmakológiai hatóanyagokat alkalmaztuk, hanem receptor agonistaként formyl-Methionyl-Leucyl-Phenylalanine-t (fMLP) is. Az N-formilált peptidekről bebizonyosodott, hogy a bakteriális fehérjék és a szöveti károsodás következtében kiszabaduló mitokondriumok degradációs termékei. A humán fMLP receptorhoz kötődő formilált ligandok degranulációhoz és oxigén radikálisok termeléséhez vezetnek fagocitózisra képes sejtekben. Ezért az fMLP a PKC szerepét vizsgáló kísérletekben természetes aktivációt indukáló kontrollként került alkalmazásra. A természetes neutrofil aktivációra azért is szükség volt, mivel a forbolészterek által indukált aktiváció nem áll le. A forbolészterek ugyanis a DAG kötő helyekhez eltávolíthatatlanul kapcsolódnak, ezért a PKC izoenzimek számára folyamatos aktiválási jelet jelentenek, amelynek következtében az enzimek kimerülnek és végül degradálódnak. Érthető módon, a forbolészterek által kiváltott jelek ezért hosszú távú kísérletekben nem hasonlíthatók a környezeti jelek erősségéhez és fiziológiás hatásához.

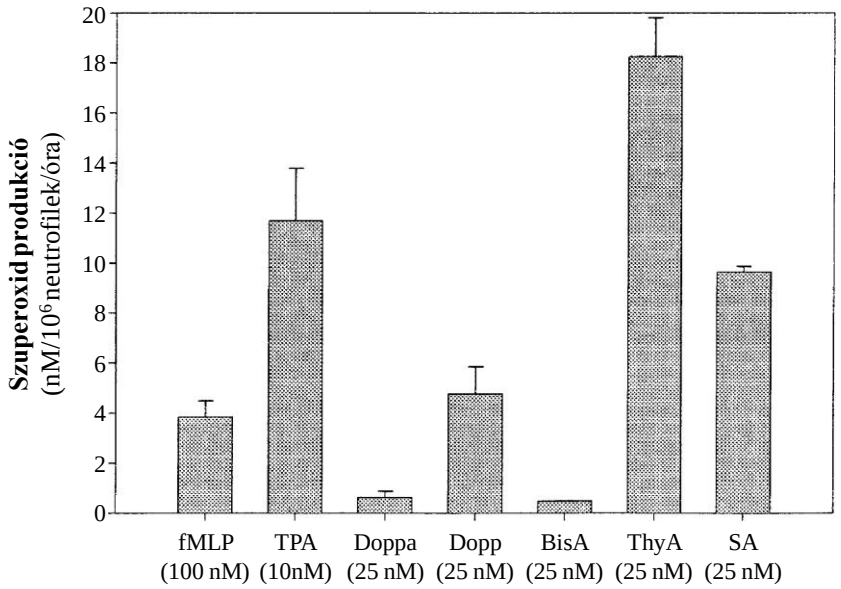
Annak eldöntésére, hogy a PKC-k szerepet játszanak-e a neutrofilek szuperoxid termelésében, forbolészterek és fMLP hatását teszteltük rövid időtartamú, PKC izoenzimeket aktiváló kísérletekben (19. ábra).



19. ábra: PKC izoenzimek aktivációja primer neutrofilekben (Pongrácz, et al. 1998)

Vénás vérből tisztított neutrofileket 10nM TPA, 25-25nM Dopp, Doppa, ThyA, SA és BisA, illetve 100 nM fMLP kezelésnek vetettük alá 2 percre, majd a kezelt és kezeletlen kontroll (Ko) sejtekből citoszol (C) és membrán (M) frakciókat izoláltunk. A fehérjék szétválasztását követően Western blottal mutattuk ki a PKC izoenzimek lokalizációját izoenzim specifikus ellenanyagok segítségével.

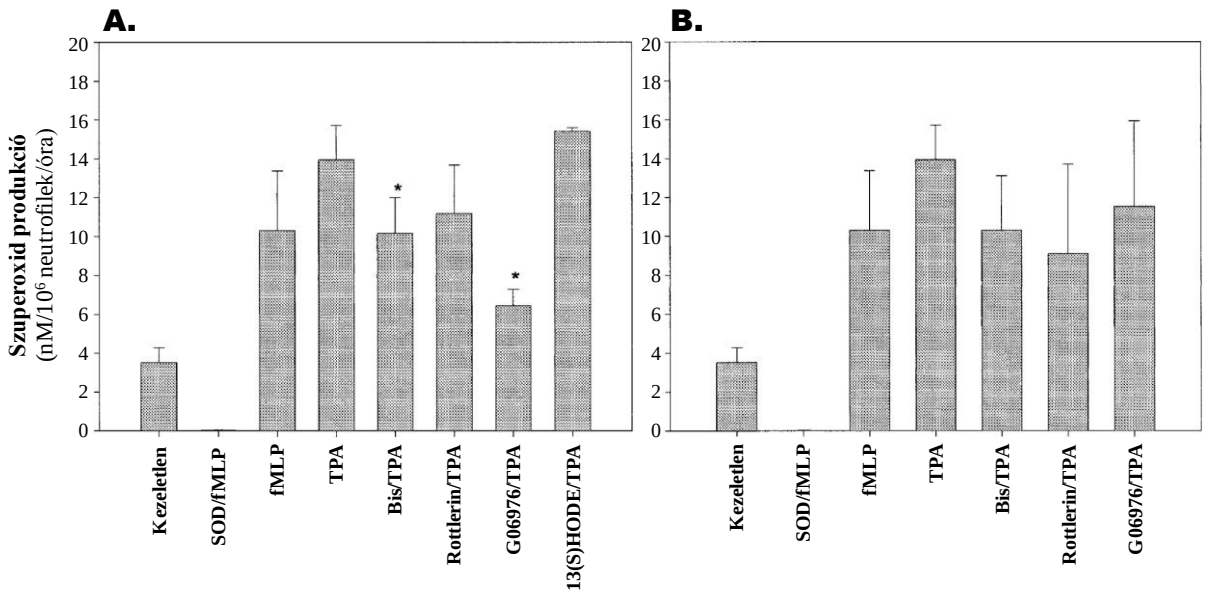
A kísérletekből megállapítható volt, hogy az fMLP kezelés leginkább PKC α és βII aktivációjához vezet, amely leginkább a TPA által indukált izoenzim transzlokációs mintázathoz hasonlított. Ez felvetette annak lehetőségét, hogy a szuperoxid termelődéséhez vezető folyamatokat klasszikus PKC-k irányítják, feltehetően PKC α. Ez annál is inkább valószínűnek tűnt, mivel a PKC β specifikus Doppa és a PKC δ specifikus BisA kezelés nem vezetett szuperoxid termelődéshez (20. ábra).



20. ábra: Szuperoxid termelés PKC aktivátorokkal és fMLP-vel kezelt humán neutrofilekben (Pongrácz, et al. 1998)

Neutrofileket 100 nM fMLP, 10 nM TPA, vagy 25 nM DOPP, SA, ThyA, BISA, vagy DOPPA jelenlétében inkubáltuk 1 órán keresztül 37^{o}C-on, majd a szuperoxid termelést mértük.

Hogy további bizonyítékot nyerjünk az izoenzimek szerepéről a neutrofilek szuperoxid termelésében, PKC aktivációt gátló farmakológiai hatóanyagokat alkalmaztunk a kísérleteinkben. Először a neutrofileket PKC inhibítorokkal előkezeltük (Bis, Rottlerin, Go6976 és 13-HODE), mielőtt a sejteket TPA-val vagy fMLP-vel aktiváltuk. Érdekes módon, míg kísérleteinkben a TPA hatását teljes mértékben alátámasztották az inhibítorokkal végzett tesztek, azaz a klasszikus alcsaládba tartozó PKC izoenzimek döntő szerephez jutottak a TPA által indukált szuperoxid termelésben, addig az fMLP receptorból induló jeleket a farmakológiai inhibítorok egyike sem gátolta (21. ábra).



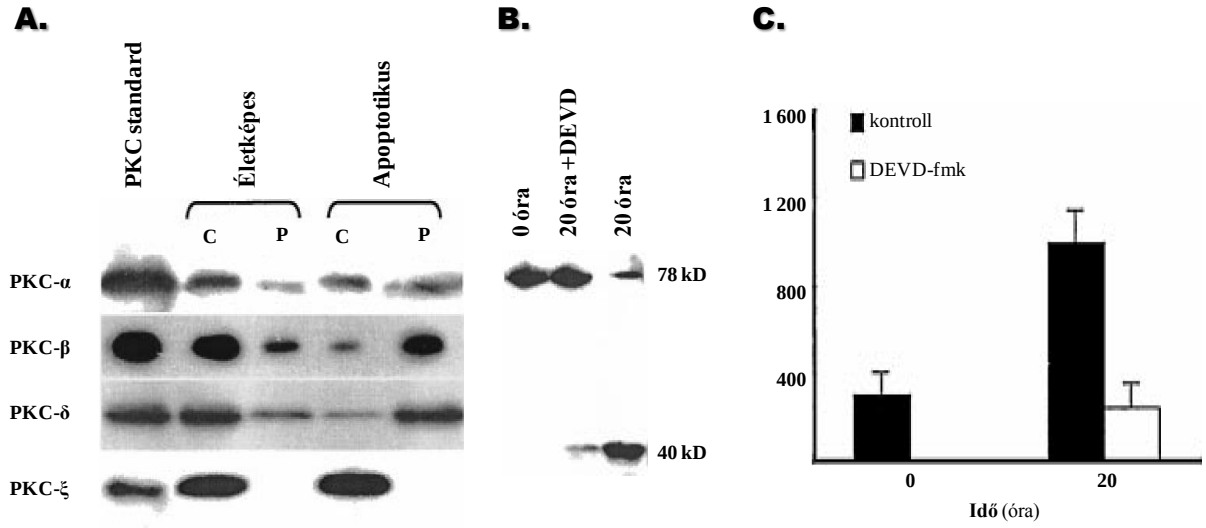
21. ábra: PKC inhibítorok fMLP vagy TPA kezelés által kiváltott szuperoxid termelésre kifejtett hatása (Pongrácz, et al. 1998)

Neutrofileket 500 nM bisindolylmaleimide 1-gyel, 20 nM Go6976-tal, 300 nM 13-HODE-dal, vagy 5 mM Rottlerinnel kezeltük 30 percig (A) 10 nM TPA vagy (B) 100 nM fMLP hozzáadását megelőzően, majd a szuperoxid termelést mértük. Statisztikailag szignifikáns különbségeket csillaggal jelöltük ($p \leq 0.05$).

A kísérleti eredményekből arra a következtetésre jutottunk, hogy számos jelátviteli út vezethet a reaktív oxigén gyökök termeléséhez. Mivel számos jelátviteli folyamat befolyásolja a neutrofilek aktivációját, félrevezető lehet a kísérleti rendszerek túlzott leegyszerűsítése és a természetes hatóanyagok eliminálása a kísérleti rendszerekből. Kísérleti eredményeink hozzájárultak annak megértéséhez is, hogy a számos neutrofil aktivációt előidéző intracelluláris mechanizmus között redundancia lehetséges, és a neutrofil aktivációs folyamatok klasszikus PKC izoenzimek aktiválásával is kiválthatók. A neutrofilek fiziológiás folyamatainak megértéséhez azonban további kísérletekre volt szükség. Kérdéses maradt ugyanis, hogy neutrofilek apoptotikus sejthalálában szerepet játszanak-e PKC függő folyamatok? Ennek a kérdésnek a megválaszolása igen fontos lehet, hiszen a gyulladásos folyamatokban aktiválódó és szövetkárosító neutrofilek eltávolítása fontos terápiás cél.

A neutrofilek apoptotikus folyamatainak vizsgálatára a véráramból frissen eltávolított neutrofileket használtunk, melyeket különböző ideig inkubáltunk. *In vitro* kísérletekben 24 óra elteltével a neutrofilek 70%-a apoptotikussá válik, ezért apoptotikus sejtek nagy számban álltak rendelkezésre a jelátviteli folyamatok vizsgálatára. Annak meghatározására, hogy az apoptotikus folyamat beindulásával mely PKC izoenzimek aktíválódnak, Western blot

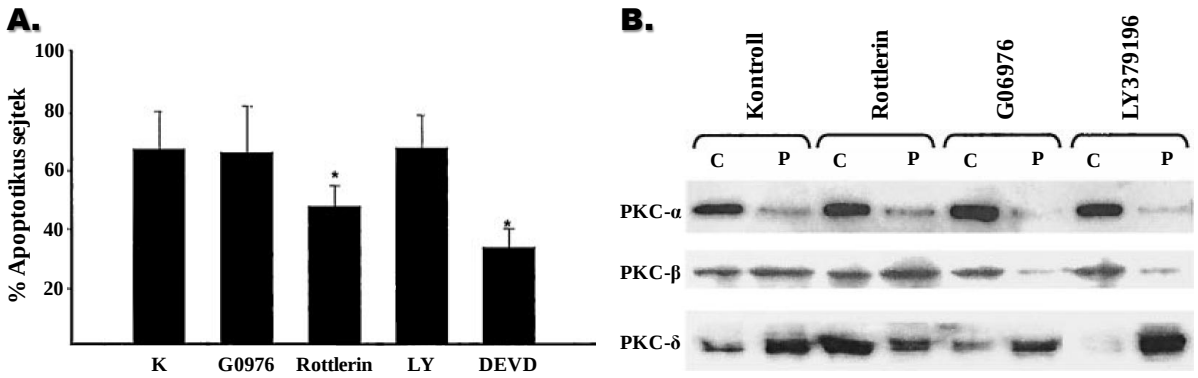
kísérleteket végeztünk PKC α, β, δ és ζ izoenzimek sejten belüli lokalizációjának vizsgálatára. Megállapítottuk, hogy az életképes sejtekkel szemben, az apoptotikus folyamatok megindulásakor PKC β és PKC δ izoenzimek találhatók a neutrofilek partikuláris frakcióiban (22/A. ábra), amelyből arra következtettünk, hogy ez a két PKC izoenzim fontos szerepet tölt be a neutrofilek apoptotikus folyamatainak szabályozásában.



22. ábra: PKC izoenzimek aktivációja neutrofil apoptózis során (Pongrácz, Webb et al. 1999)

A) PKC izoenzimek aktivációjának vizsgálatát a citoszolból (C) a partikuláris (P) frakcióba történő transzlokációjuk detektálásával végeztük frissen izolált és 20 órán át inkubált neutrofilokból, Western blot technikával, anti-PKC izoenzim ellenanyag alkalmazásával. 10 ng rekombináns human PKC-α, -β, -δ, és -ζ képviselte a standardot. B) teljes sejt extraktumokat készítettünk frissen izolált és 20 órán keresztül 20mM DEVD-fmk jelenlétében vagy hiányában inkubált neutrofilekből, majd Western blottal detektáltuk a teljes méretű (78 kDa) és kaszpáz által hasított (40 kDa) PKC δ fragmentumot. C) DEVD-fmk kaszpáz 3-ra kifejtett gátló hatásának bizonyítása frissen izolált és 20 órán át inhibítor jelenlétében vagy hiányában inkubált neutrofilekben. Az enzimaktivitást fluoreszcensen jelölt kaszpáz 3 szubsztrát alkalmazásával végeztük (fluoreszcens egység/1×10^6 sejt).

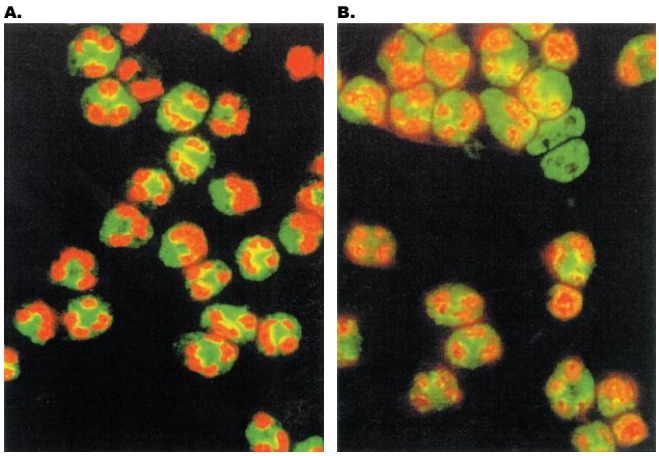
Annak meghatározására, hogy PKC β és δ izoenzimek valóban szabályozzák-e az apoptotikus folyamatok lezajlását, további kísérleteket végeztünk PKC izoenzimek gátlását előidéző farmakológiai inhibítorokkal. Kísérleti rendszerünkben Go6976, mint a klasszikus (PKC-α, -β, és -γ), LY379196 mint a PKC β specifikus (Jirousek, Gillig et al. 1996), és Rottlerin, mint PKC δ specifikus (Gschwendt, Müller et al. 1994) inhibítorokat teszteltük. Mivel a PKC δ izoenzim kaszpáz-3 hasítás útján is aktiválható, ezért kaszpáz-3 inhibítort (DEVD-t) is alkalmaztunk a kísérletekben. Míg a klasszikus PKC-kat általánosan gátló, és a PKC β specifikus inhibítorok nem voltak hatással az apoptotikus folyamat megindulására, addig mind a DEVD (22/B. és C. ábra), mind a Rottlerin gátolta a programozott sejthalált (23. ábra).



23. ábra: PKC δ és kaszpáz 3 inhibítorok neutrofil apoptózisra kifejtett hatása (Pongrácz, Webb et al. 1999)

Neutrofileket 20 órán keresztül inkubáltuk PKC inhibítorok Go6976 (20 nM), Rottlerin (10nM) vagy LY379196 (10 nM) vagy kaszpáz 3 inhibitor DEVD-fmk (20 mM) jelenlétében, illetve inhibítorok hiányában. *A,* az apoptotikus morfológia jelenlétének vizsgálatát May-Grünwald Giemsa festéssel láthatóvá tett neutrofileken végeztük. A statisztikai szignifikanciát csillaggal jelöltük $p \leq 0.05$. *B,* a kísérletben használt inhibítorok hatását bizonyítja az izoenzimek citoszolból partikuláris frakcióba történő transzlokációjának gátlása.

Azt, hogy a PKC δ fontos szerepet tölt-e be a neutrofilek apoptotikus sejthalálának jelátvitelében, enzimdepléciós acelluláris kísérletekkel is alátámasztottuk. Ezekkel a kísérletekkel tovább bizonyítottuk, hogy a neutrofil apoptózis PKC δ függő folyamat. További kísérleteink arra is rávilágítottak, hogy apoptózis során a PKC δ a sejtmagban lokalizálódik (24. ábra), ahol feltételezhetően az apoptózist irányító enzimatikus folyamatokban résztvevő enzimeket és transzkripciós faktorokat aktiválja.



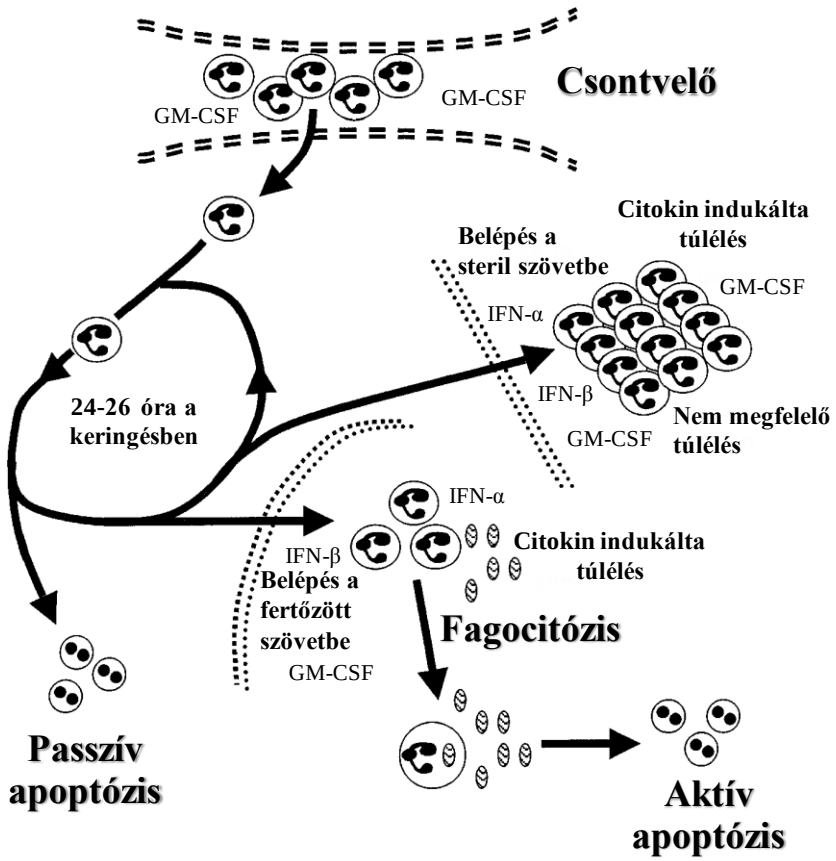
24. ábra: PKC δ szubcelluláris lokalizációja egészséges és apoptotikus neutrofilekben (Pongrácz, Webb et al. 1999)

Az ábrán frissen izolált neutrofilek (*A*) és 8 órán át sejtkultúrában inkubált neutrofilek (*B*) láthatóak. A PKC δ izoenzimet anti-PKC δ ellenanyaggal tettük láthatóvá (FITC, zöld), míg a sejtmagot propidium

jodiddal (vörös) jelöltük. Ahol a vörös (sejtmag) és a zöld (PKC δ) festés azonos helyen koncentrálódik, ott a kialakuló sárga szín a PKC δ jelenlétét jelzi a sejtmag anyagában.

Kísérleteink rámutattak, hogy PKC δ noha fontos, nem lehet egyetlen eleme az apoptotikus kaszkádnak. Többek között azért, mert a PKC δ–t aktiváló kaszpáz-3-nak is számos egyéb szubsztrátja ismeretes (pl. az apoptózis-specifikus endonukleáz inhibítora, citoszkeletális fehérjék, stb.), tehát kizárólag a PKC δ-ra koncentráló potenciális terápiás eljárások nem lehetnek hatékonyak. Kísérleteinkkel azt is alátámasztottuk, hogy a p38 MAP kináz szerepet játszik a stressz által indukált, de nem a spontán úton kialakuló neutrofil apoptózisban. Ez alátámasztotta korábbi eredményeinket, melyek szerint egyetlen sejtben párhuzamosan jelen lehetnek olyan jelátviteli kaszkádok, melyek ugyanahhoz a fiziológiás válaszreakcióhoz vezetnek, de mindezek nem szükségképpen aktiválódnak egyidejűleg.

Noha kutatásaink során sikerült azonosítanunk a PKC család fontos szerepét mind az apoptotikus sejthalál, mind a sejtek túlélése szempontjából, a kísérletekből az is nyilvánvalóvá vált, hogy a szöveti környezetből származó extracelluláris jeleket szorosabb kapcsolatba kell állítani a sejten belüli jelátviteli folyamatokkal ahhoz, hogy terápiás szempontból is alkalmazható ismereteket nyerjünk a jelátvitel minőségi és mennyiségi viszonyairól. Ennek tükrében a primer neutrofilek által nyújtott lehetőségek limitáltnak tűntek, hiszen az érett neutrofilek „fiziológiás programjában” a sejthalál kitüntetett helyet foglal el. Mivel a PKC-k párhuzamosan több jelátviteli rendszerben is szerepet játszanak, neutrofilekben nehezen azonosítható, hogy mely bejövő jelek eredőjeként kerül sor a fiziológiás változásokra. További nehézséget jelentett, hogy a teminálisan differenciálódott és rövid életidejű neutrofilek génexpressziós módosítása majdnem lehetetlen feladat, hiszen igen érzékenyen reagálnak külső behatásokra (pl. elektroporáció vagy kémia membrán-permabilitás növelők, stb), amelyek a sejtek pusztulásához vezetnek. Ezért a PKC-k aktivitásának módosítására a farmakológiai hatóanyagokra voltunk kénytelenek szorítkozni, melyek specificitása egyre inkább megkérdőjelezhetővé vált.



25. ábra: A neutrofilek citokin-depriváció következtében apoptózissal elpusztulnak (Webb, Wang et al. 2000)

A csontvelőben történő fejlődésük és differenciálódásuk során a fejlődő sejtek túlélési jeleket kapnak, pl GM-CSF formájában. A keringésbe való kikerülésük során a túlélést szabályozó citokin szint lecsökkenésének következtében beindul az apoptózis gépezete. A fertőzések helyén azonban, ahol a citokin szintek magasak, a neutrofilek élettartama megnövekszik.

Összességében kísérleteink eredményeként nyilvánvalóvá vált, hogy a szöveti környezet komplex hatása befolyásolja az egyes sejtek túlélését, aktiválódását, illetve halálát kiváltó jelátviteli folyamatokat (25. ábra). Hogy a jelátviteli folyamatok szabályozását megérthessük, elengedhetetlenné vált olyan kísérleti rendszerek alkalmazása, amelyekben a komplex szöveti környezetben párhuzamosan érnek ingerek fiziológiás válaszreakciók széles skálájára képes sejteket.

5.2. Jelátvitel a tímusz organogenezise és a timociták fejlődése során

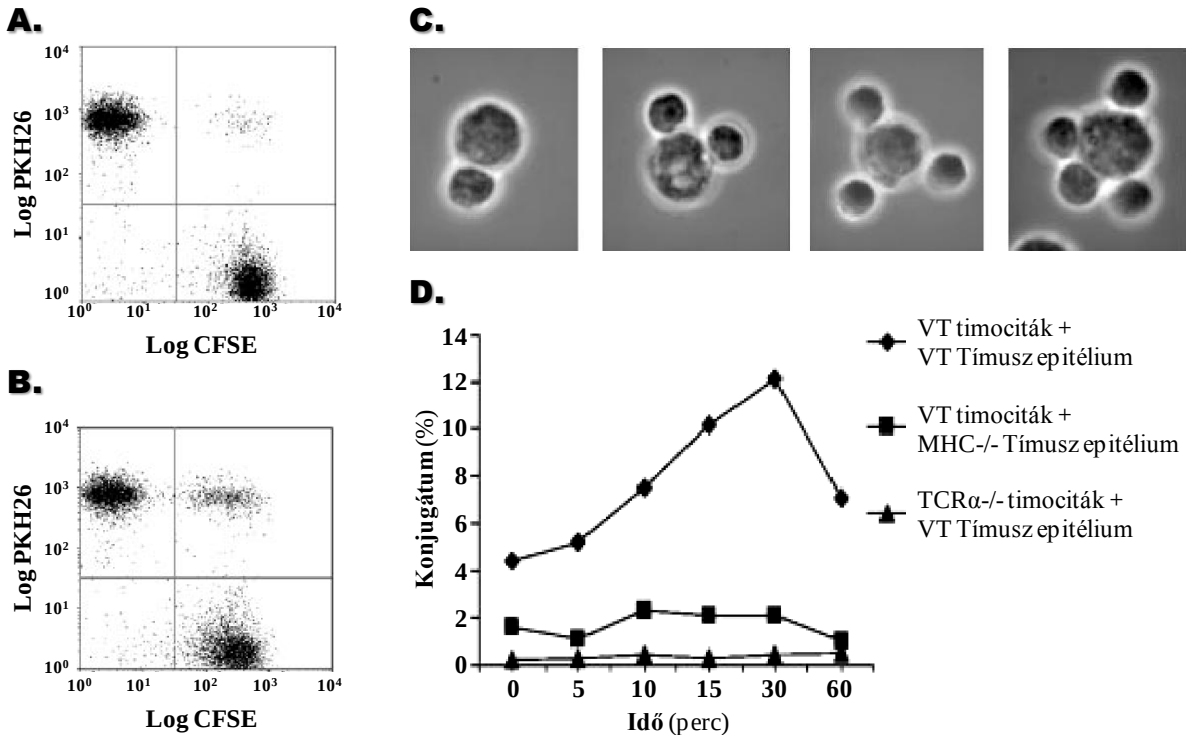
A csecsemőmirigyben, avagy a tímuszban végbemenő sejtes és szöveti változások, mind a szerv kialakulása, mind a benne fejlődő T-sejtek differenciálódása és szelekciója során ideális környezetet biztosítanak a jelátviteli rendszerek vizsgálatára. Különös tekintettel arra, hogy a

sejtek túlélését, illetve a halálát kiváltó szelekciós faktorok folyamatos jelenléte lehetőséget biztosít mennyiségi változások mérésére és az intra- és extracelluláris jelátviteli molekulák szerepének pontosabb meghatározására. A tímusz különlegessége az is, hogy a fejlődés, osztódás, differenciálódás és sejthalál ugyanabban a szövetben, párhuzamusan megy végbe úgy, hogy a fenti folyamatokat kiváltó sejtes interakciók illetve szekretált faktorok hatásának eredője szabja meg a szövet minden sejtjének válaszreakcióit. Az egyik olyan sejttípus a tímuszban, amelyre számos jel hat egyidejűleg, az a fejlődő timociták csoportja.

A tímuszban fejlődő T-sejtek számos fontos osztódási, differenciációs és szelekciós lépésen esnek át. Ezek közül a T sejt receptorhoz (TCR-hoz) kapcsolódik mind a sejtek pozitív, mind negatív szelekciójának folyamata, melynek eredményeként eldől, hogy a periférián milyen TCR repertoárral rendelkeznek majd a T-sejtek. A TCR specificitásának biztosításához viszont elengedhetetlen, hogy csak MHC-I-hez vagy MHC-II-höz kötött antigént ismerhessenek fel az érett, perifériális T-sejtek. Ezért a T-sejtek MHC (emberben HLA), illetve antigén-MHC-t felismerő képességének tesztelése igen fontos lépés a timociták fejlődésében. Sem az MHC-t, sem az antigén-MHC-t fel nem ismerő T-sejtekre nincs szüksége az immunrendszernek, hiszen ezek haszontalanok az immunválasz kialakulása szempontjából. Továbbá azokra a sejtekre sincs szükség, amelyek igen nagy affinitással és aviditással kötődnek az antigén-MHC komplexhez, hiszen így igen erős, szabályozhatatlan immunválasz alakul ki, gyakran saját antigénekkel szemben. Ennek elkerülése érdekében számos szelekciós folyamat megy végbe a tímuszban. Noha a TCR-ból származó jelek erőssége közti különbségnek tulajdonítják a $CD4^+CD8^+$, azaz DP timociták túléléséhez (pozitív szelekció) vagy apoptózisához (negatív szelekcióhoz) vezető folyamatok megindulását, valójában a „jel erősségének” pontos mibenlétét intracelluláris jelátviteli szinten nehéz meghatározni.

Kísérleteinkben abból a megfigyelésből indultunk ki, hogy az antigén-MHC komplexet felismerő T-sejtek kapcsolódási erősségének tesztelése elsősorban a tímusz epiteliális sejtjeire háruló feladat. A tímusz epiteliális sejtek ugyanis képesek mind MHCI, mind MHCII expressziójára. A fejlődő timociták a kortikális és medulláris epitéliumon tesztelik először a TCR génátrendeződés eredményeként létrejövő receptor fehérjék antigén és MHC felismerő képességét. Az antigén-MHC-komplexet felismerő TCR-ből származó jeleken túlmenően számos ko-receptor-ligand kapcsolat szabja meg a jel erősségét, amely vagy a timociták túléléséhez vagy apoptotikus sejthalálához vezet.

A TCR-ből származó jelátviteli folyamatok vizsgálatára és annak megállapítására, hogy mekkora erősséggel kötődik a timocita az epitéliumhoz, először azt vizsgáltuk, hogy mely molekulákra van szükség ahhoz, hogy a timociták komplexet képezzenek az epitélium membránján található peptid-MHC molekulakomplexszel. *In vitro* kísérletekben TCRα-/-, azaz funkcióképtelen TCR-ral rendelkező timocitákat, és MHC-val nem rendelkező, azaz DK (MHCI/II-/-) epitéliumot használtunk a vad típusú, Balb/c egerekből származó szövetek és sejtek kontrolljaként (26. ábra).

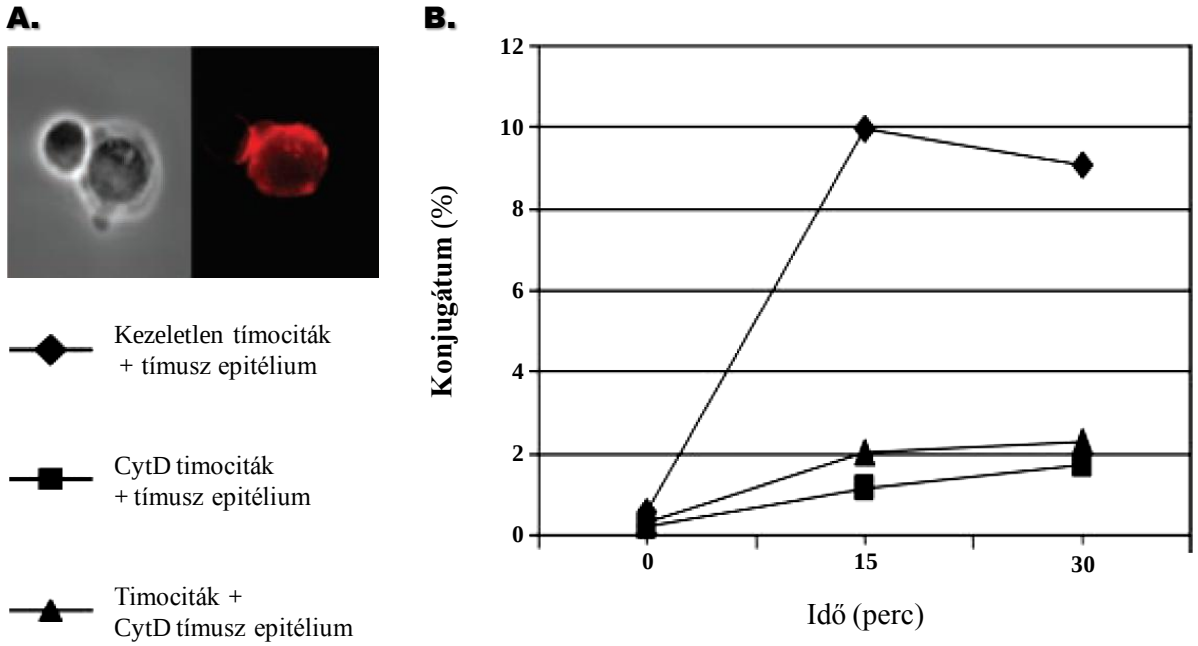


26. ábra: $CD4^+8^+$ timociták TCR és MHC függő konjugátumot képeznek a tímusz epitéliummal (Hare, Pongracz J et al. 2003).

A $CD4^+8^+$ timocitákat PKH26, míg a tímusz epiteliális sejteket CFSE fiziológiás festéssel jeleztük. A sejteket 1:1 arányban kevertük össze, majd vagy azonnal (*A*) vagy 30 percig, 37°C-on történt inkubációt követően áramlási citometriával (*B*) vagy mikroszkóppal analizáltuk (*C*). A konjugátumok formálódásának alakulását 0 és 60 perc között a PKH26 és a CFSE események gyakoriságát mérve határoztuk meg (*D*, rombusz). Kontrollként MHC-/- tímusz epitéliumot kevertünk vad típusú timocitákkal (*D*, négyszög), vagy TCRα-/- timocitákat vad típusú (VT) tímusz epiteliális sejtekkel (*D*, háromszögek).

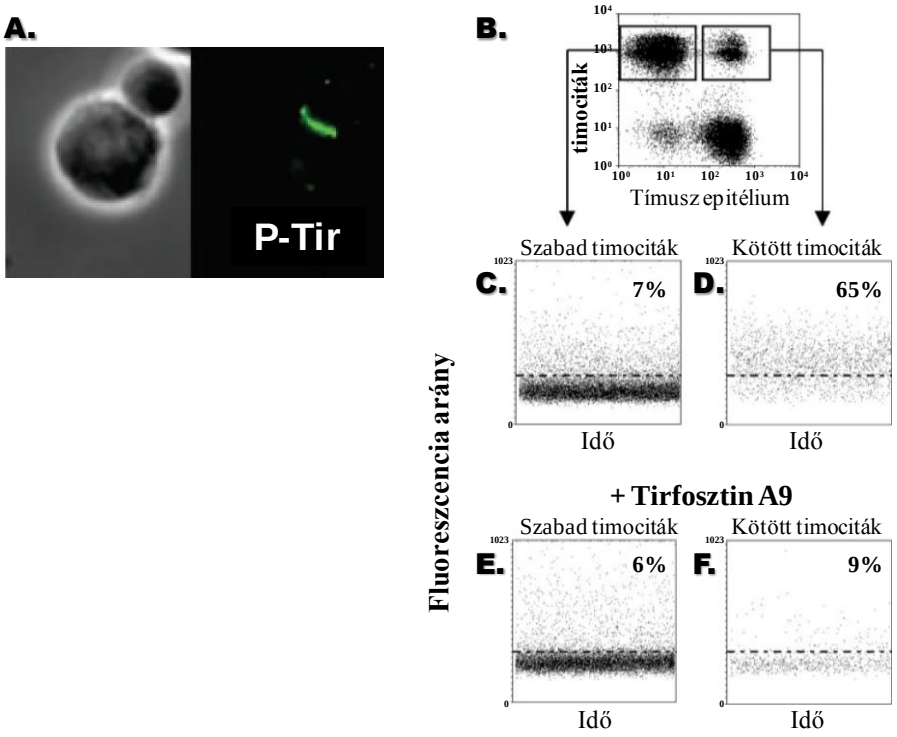
Kísérleteinkben megállapítottuk, hogy a timocitáknak rendelkezniük kell funkcióképes TCR-rel, míg az epiteliális sejteknek MHC-val ahhoz, hogy a szelekciós folyamatok megindulhassanak. A fenti kísérletben olyan kísérleti rendszer összeállítására került sor, amelyben a timociták válaszreakciói kiválóan nyomonkövethetővé váltak differenciációs

markerek segítségével, míg a sejten belüli jelátviteli változások is tesztelhetők maradtak. Megállapítottuk, hogy a timocita-epitélium komplexben az aktív receptor-ligand kapcsolat az aktin citoszkeleton koncentrált polimerizációjával jár mind a timocitákban, mind az epiteliális sejtekben (27. ábra). Továbbá ez a kapcsolat a timocitákban tirozin foszforilációtól függő Ca ion szint növekedéséhez vezetett, azaz aktív jelátvitel megindulását észleltük (28. ábra).



27. ábra: A timocita-epitél sejt interakció az aktin citoszkeleton polimerizációját eredményezi (Hare, Pongracz J et al. 2003)

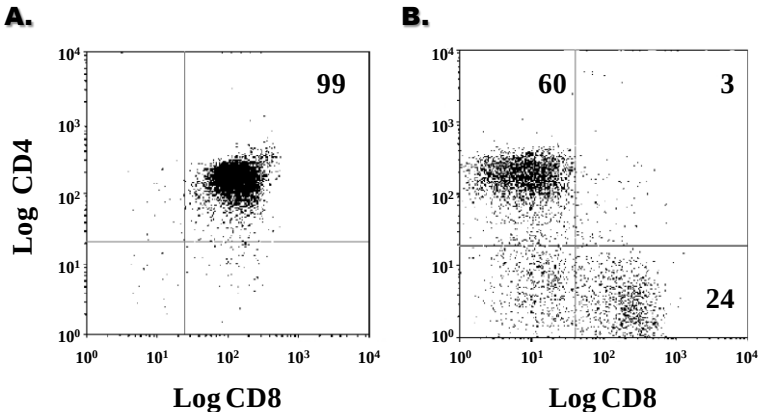
MHC-/- egerekből származó $CD4^+8^+$ timocitákat tímusz epitéliummal inkubáltuk 30 percen keresztül, majd a kitapadt sejteket permeabilizálva falloidint használtunk az aktin citoszkeleton polimerizációjának vizsgálatára (*A*). A konjugátumok képződésének analíziséhez áramlási citomeriával végeztünk vizsgálatokat citokalazin (cyt) D-kezelt timociták és kezeletlen tímusz epiteliális sejtek (négyszögek), vagy kezeletlen timociták és citokalazin D-kezelt epitélium (háromszögek), illetve kontrollként kezeletlen timocita és epitélium (rombuszok) (*B*) felhasználásával.



28. ábra: A timocita-epiteliális sejt interakció növeli a timocitákban az intracelluláris kálcium szintet és foszfotirozin akkumulációt idéz elő (Hare, Pongracz J et al. 2003)

$CD4^+8^+$ timocitákat tímusz epitéliummal inkubáltuk 30 percen keresztül, majd a kitapadt sejteket permeabilizálva foszforilált tirozint felismerő ellenanyaggal inkubáltuk a konjugátumokat (A). Az MHC-/- egerekből származó $CD4^+8^+$ timocitákat Indo-1-gyel kezeltük, majd PKH26-tal jelöltük, míg a tímusz epiteliális sejteket CFSE fiziológiás festéssel jelöltük. A sejteket 1:1 arányú keverést követően 5 percig inkubáltuk, majd a konjugátum-képződést áramlási citometriával analizáltuk (*B*). A citoszolban levő kálcium koncentrációt a nem konjugálódott szabad (C) és az epiteliumhoz kapcsolódó timocitákban (D) áramlási citometriával analizáltuk. Annak megállapítására, hogy a kálcium-beáramlás a tirozin kináz aktivációjának következménye-e, az Indo-1/PKH26-jelzett timocitákat 50 μM tirfosztin A9-cel inkubáltuk a konjugátum képződést megelőzően, majd a kálcium beáramlását mértük szabad (E) és epiteliumhoz kötött timocitákban (*F*).

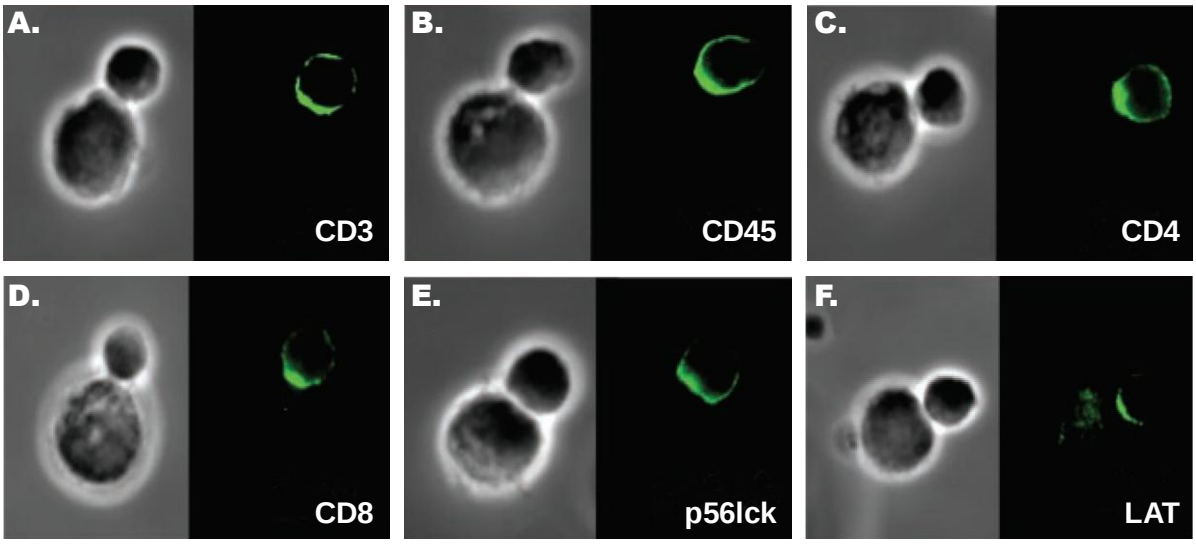
A fenti kísérleti rendszerben aktivált DP timocitákat ezután tímusz reaggregátum kultúrákban inkubáltuk tovább hét napon át. A háromdimenziós (3D) tímusz epiteliális szövet megfelelő mikrokörnyezetet biztosít a T-sejtek teljes kifejlődéséhez. Ebben a mikrokörnyezetben a hét napos inkubációs idő lehetőséget biztosít a T-sejtek kifejlődésére és a pozitív szelekció folyamatának nyomon követésére. A fenti kísérleti rendszer alkalmazása egyértelművé tette, hogy az *in vitro* sejtszuszpenzióban epiteliális sejtekkel konjugáltatott timociták olyan túlélési jelet kapnak, ami lehetővé teszi a $CD4^+$ helper és a $CD8^+$ citotoxikus T-sejtekké történő differenciálódásukat, azaz a DP timociták pozitív szelekcióját. Ez a folyamat a sejtfelszíni markerek karakterisztikus változásával nyomon követhető volt (29. ábra).



29. ábra: A timocita-epitéliális sejtek közötti konjugátum képződés a pozitív szelekció indukálását jelenti (Hare, Pongracz J et al. 2003)

A tisztított, vad típusú Balb/c timociták és epitéliális sejtek konjugátumait reaggregátum szervkultúrába helyeztük (A), majd 7 napos inkubálást követően a timocitákat TCR, CD4 és CD8 expresszió szempontjából analizáltuk áramlási citometria segítségével (B).

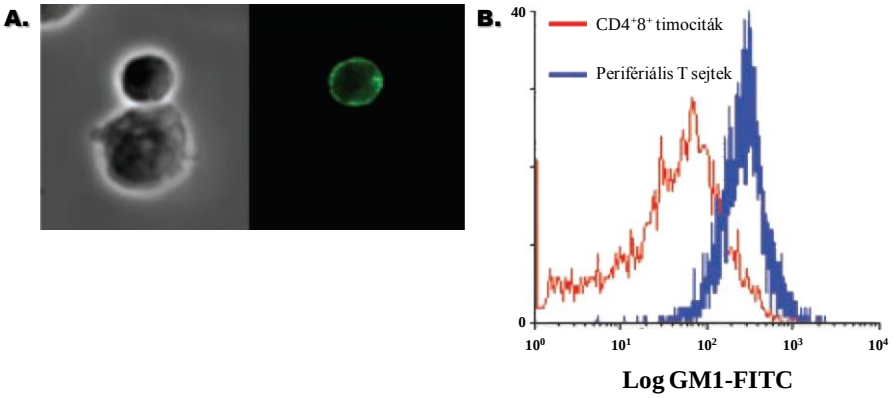
A sejtek egymásra hatásának következtében meginduló pozitív szelekcióhoz vezető folyamatok során számos intracelluláris jelátviteli molekula aktiválódása is megfigyelhető volt. Hogy a molekuláris aktivációt láthatóvá tegyük, a timocita-epitéliális sejt konjugátumban a két sejt közötti kapcsolat helyén sejtfelszíni, és sejten belüli jelátviteli molekulákat jelöltünk meg. A sejtek közötti interakció helyére a CD3, CD45, CD4, CD8 sejtfelszíni és, a p56lck tirozin kináz, LAT adapter protein és PKCΘ belső jelátviteli molekulák koncentrálódtak (30. ábra).



30. ábra: A tímusz epitéliummal való érintkezés a timocitákban molekuláris átrendeződéshez vezet (Hare, Pongracz J et al. 2003)

MHC-/- egerekből származó $CD4^+8^+$ timocitákat vad típusú tímusz epitéliális sejtekkel inkubáltuk 30 percen keresztül, majd tárgylemezhez tapadásuk után permeabilizáltuk a sejteket és jelátviteli molekulákra specifikus ellenanyagokkal megjelöltük: CD3 (*A*), CD45 (*B*), CD4 (*C*), CD8 (*D*), p56*lck* (*E*), és LAT (*F*). Amíg a polarizáció szembetűnő volt minden olyan timocitán, amely tímusz epitéliummal konjugátumot képezett, addig a nem konjugált sejtek nem polarizálódtak.

Noha számos jelátviteli molekula koncentrálódott az epitélium-timocita kapcsolat pontjára, ez a folyamat mégis függetlennek bizonyult a „lipid raft”-ok avagy „lipid tutajok” jelenlététől (31. ábra), melyeket korábban elengedhetetlennek feltételeztek az aktív jelátvitel kialakulásához. Az a felfedezés, mely szerint „lipid tutajok” nem alakulnak ki a tímuszon belül fejlődő T-sejtek jelátvitelekor, különösen fontosnak bizonyult, hiszen rámutatott a tímuszban zajló TCR-függő szelekciós és a periférián végbemenő TCR-függő aktivációs jelek jeltovábbítási különbségeire.

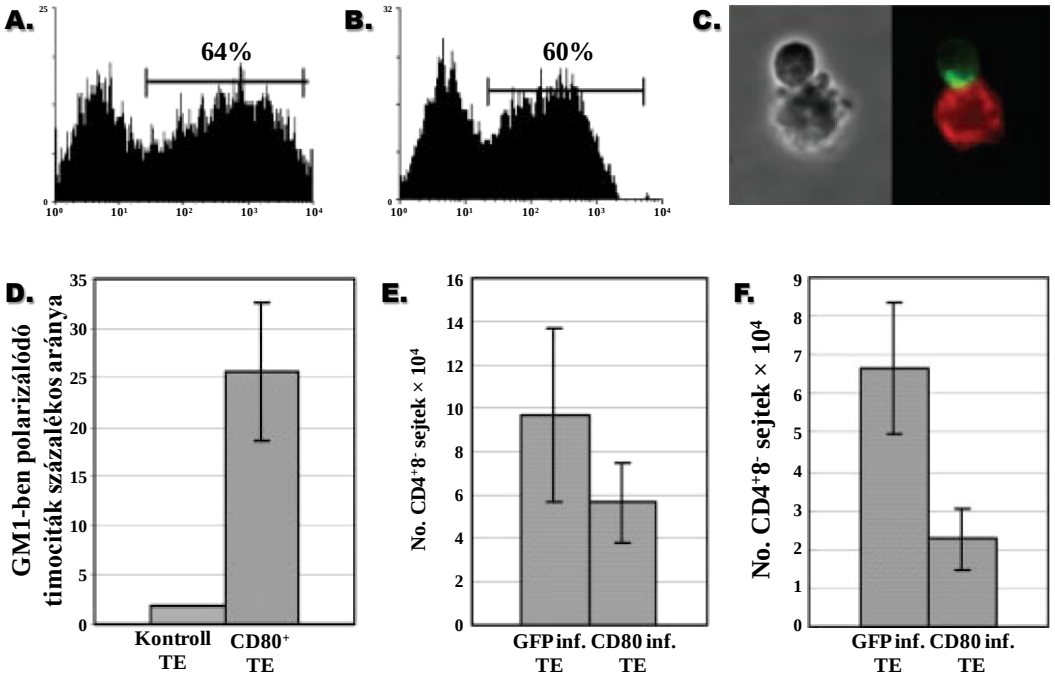


31. ábra: A timociták pozitív szelekciójának megkezdődése nem jár együtt a GM1 lipid-tutajok akkumulálódásával (Hare, Pongracz J et al. 2003)

Az MHC-/- egér törzsből származó $CD4^+8^+$ timocitákat kolera-toxin-FITC-cel jelöltük, majd vad típusú tímusz epitéliummal inkubáltuk 30 percen át. A sejteket ezután tárgylemezre tapasztottuk ki, hogy a GM1 polarizáció vizsgálható legyen (*A*). Mind $CD4^+8^+$ timociták, mind perifériás T-sejtek kolera-toxin-FITC-cel kerültek megjelölésre, majd áramlási citometriával analizáltuk a változásokat (*B*).

A jelátviteli különbségeket analizálva feltételeztük, hogy a CD28-B7 (CD80 és CD86) kölcsönhatás, mint ko-stimulációs jel lehet az egyik különbség a perifériás T-sejtek és a fejlődő, DP T-sejtek TCR-függő jelátvitele között. Különösen fontosnak tűnt ennek a feltételezésnek a vizsgálata, hiszen a tímuszban a kortikális epiteliális sejtek az érett, perifériás antigén prezentáló sejtekkel (APC) ellentétben nem expresszálnak CD80 vagy CD86 ko-stimulációs ligandokat. Az elmélet ellenőrzése kezdetben technikai problémákba ütközött, ugyanis a tímusz epiteliális sejteken szükség volt a fehérjeexpresszió módosítására. További nehézségként merült fel, hogy a sejtfelszínen expresszáltatott fehérjéknek funkcióképesnek kellett lenniük, miközben a tímusz epiteliális sejtek életképességét meg kellett őrizzük a fehérjeexpresszió módosítását előidéző vektorok bejuttatását követően is. A korábban használt génexpressziót módosító módszerek nem vezettek volna eredményre, mivel a fenti módszerek alkalmazásához a tímusz epiteliumot kétdimenziós kultúrákban kellett volna tenyészteni. Kétdimenziós szövettenyészetekben a tímusz epiteliális sejtek azonban elveszítik azon képességüket, hogy a T-sejtek fejlődését és differenciálódását támogassák. Éppen ezért, végül rekombináns adenovirális (rAd) génbevitelre esett a választásunk, amely igen hatékonynak bizonyult az epiteliális sejtek fehérjeexpressziójának módosításában akár intakt tímusz lebenyek esetében is. Kísérleteink rámutattak arra, hogy a rAd-sal CD80 expresszióra kényszerített, majd fejlődő timocitákkal reaggregáltatott tímusz epiteliális sejtek „lipid tutajok" kialakulását indukálták a timocitákban, amelynek következményeként

megnövekedett az apoptózis és csökkent a pozitív szelekció (). Megállapítottuk tehát, hogy a ko-stimulációs molekulák jelenléte módosítja a TCR-függő szelekciós jelek erősségét.



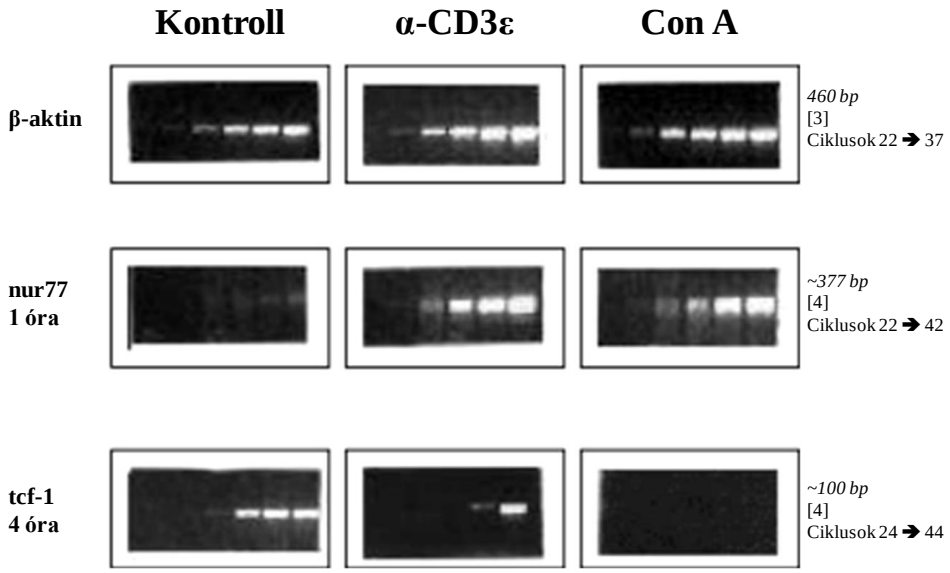
32. ábra: CD80 expresszióra kényszerített tímusz epitélium megindítja a lipid tutajok kialakulását és csökkenti a pozitív szelekciót (Hare, Pongracz J et al. 2003)

Deoxiguanozinnal kezelt embrionális tímusz lebenyekből származó tímusz epitéliumba rAd-CD80-GFP-t és kontroll, rAd-GFP-t juttattuk be. A fertőzött sejteket áramlási citometriával analizáltuk GFP és CD80 expresszióra (*A* és *B*). MHC-/- egér törzsből származó $CD4^+8^+$ timocitákat kolera toxin FITC-cel jelöltük, majd CD80-at expresszáló tímusz epitéliummal inkubáltuk 30 percen keresztül. A sejtek kitapasztása után, a sejteket anti-CD80 ellenanyaggal, majd anti-egér rhodaminnal jelöltük. A GM1 polarizációt mutató (C) CD80-hoz kötött timociták kvantitálását a *D* ábra mutatja., A DP timocitákból és a rAd-sal előkezelt epitéliumból reaggregátumokat készítettünk, majd öt napi inkubálás után a timociták CD4 és CD8 expresszióját analizáltuk áramlási citometria segítségével (*E*) és (*F*).

Noha a ko-receptorok jelenléte, illetve hiánya támpontot nyújtott a szelekciós jelek erősségét meghatározó folyamatok eredetére, a koncentráltan megjelenő jelátviteli molekulák a timocita-epitélium érintkezési felületen nem adtak intracelluláris magyarázatot a szelekciós jelek erőssége között fellépő különbségekre.

Hogy a szöveti környezetből származó túlélést, illetve sejthalált indukáló sejten belüli jelek közötti különbségeket azonosítani tudjuk, két, a perifférián „high avidity” jelet utánzó és ezért T-sejtaktivációt előidéző faktort, anti-CD3ε ellenanyagot és concanavalin A (ConA)-t alkalmaztunk. Azért esett a választásunk az ellenanyagra és ConA-ra, mert fejlődő timocitákra más hatást gyakorolnak, mint perifériális T-sejtekre. Míg az anti-CD3ε ellenanyag a DP

timociták apoptotikus halálát idézi elő, addig ConA kezelésük pozitív szelekciót és következésképpen túlélést indukál. A kísérleteket intakt embrionális (E15) tímusz lebenyekben végeztük annak bizonyítását követően, hogy mind az ellenanyag, mind a ConA bediffundálnak a lebenyekbe. Így a két anyag hatását az amúgy érintetlen szöveti mikrokörnyezetben vizsgálhattuk. Az anti-CD3ε ellenanyag és a ConA hatása közötti különbség molekuláris szinten már koránt sem bizonyult annyira egyértelműnek, mint a fejlődő T-sejtekre kifejtett szelekciós hatásuk. Korábbi kísérletek bizonyították, hogy a timociták TCR által indukált apoptotikus folyamatai során a nur77 (Cheng 1997; Winoto 1997) transzkripciós faktor expressziója megnő. Meglepő módon azonban mind az anti-CD3ε ellenanyag, mind a ConA növelte a nur77 expresszióját megközelítően azonos szinten. Továbbá, a szelekciós folyamatok megindulását jelző, mind a negatív, mind a pozitív szelekció során expressziós csökkenést mutató tcf1 génre, gyakorlatilag azonos hatással voltak, csökkentve annak transzkripcióját (33. ábra).

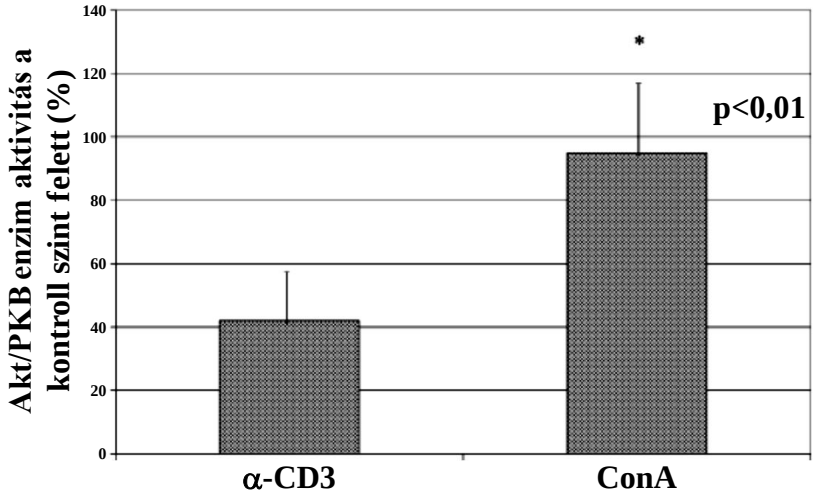


33. ábra: Anti-CD3 ellenanyag és ConA hatása nur77 és tcf1 gének expressziójára (Pongracz, Parnell et al. 2003)

Az anti-CD3ε (5 μg/ml) és Con A (5 μg/ml) fiziológiás hatásai MHC DK timociták génexpressziójára szervkultúrában történt inkubációt követően. *nur77 és tcf1* gének szemikvantitatív RT-PCR analízise TCR ligációt 1 és 4 órát követően. (PCR reakcióban β-aktin-t alkalmaztunk belső kontrollnak). A reakciótermékek méretét az ábra jobb oldalán tüntettük fel.

Korábbi kísérleteinkben a TCR-epitélium konjugációjának helyére akkumulálódott számos kináz jelenlétéből arra következtettünk, hogy esetleg a lipid „second-messenger”-ek felszabadulása lehet más a két hatóanyag jelenlétében, amely aktívan módosíthatja a túlélési

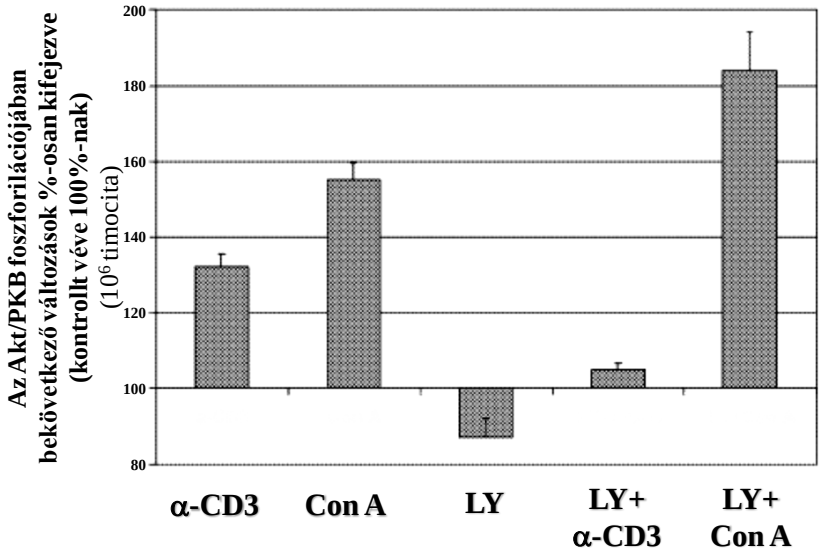
és apoptotikus jeleket. A külső receptor-ligand interakciók differenciált lipid „second-messenger" indukciója módosult kinázaktivitást eredményezhet, mely a túlélési folyamatokban döntő szerepet játszó Akt/PKB enzim differenciált aktiválását idézheti elő, megváltoztatva ezzel a túlélés, illetve a sejthalál kiváltását indukáló jelek arányát. A feltételezés kísérletes bizonyításához a timociták anti-CD3ε és ConA kezelését követően a sejt lizátumból immunprecipitáltuk az Akt/PKB enzimet, majd aktivitásméréssel bizonyítottuk, hogy a ConA kezelés következtében az enzim aktivitása megközelítően kétszeresére nő az anti-CD3ε-nal kezelt timocitákból izolált Akt/PKB aktivációs értékeivel összehasonlítva (34. ábra).



34. ábra: Akt/PKB enzim aktivitása anti-CD3ε és ConA kezelést követően (Pongracz, Parnell et al. 2003)

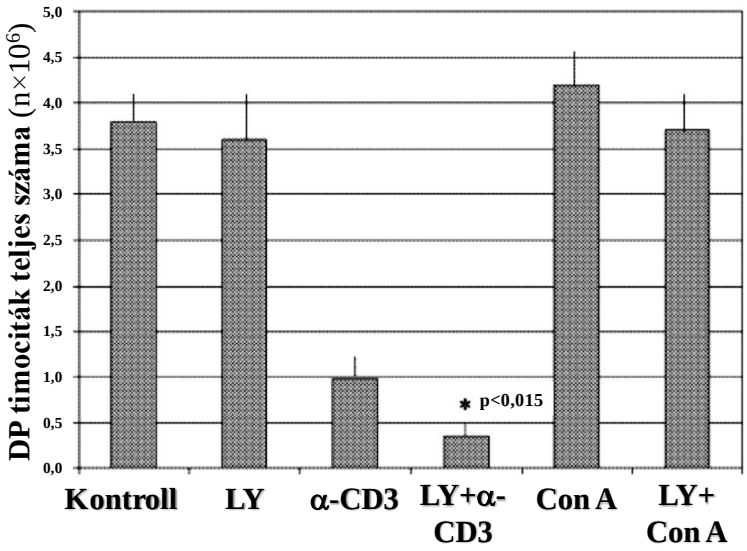
MHC DK tímusz lebenyeket anti-CD3ε (5 μg/ml) és Con A-val (5 μg/ml) kezeltük 4 órán keresztül. Egymillió timocitát lizáltunk a mérésekhez, melyekből az Akt/PKB enzimet immunprecipitáltuk, majd a γ^{32}P-ATP beépülésével szubsztrátba történő beépülésével mértük az enzim aktivációs szintjét.

Ebből az eredményből arra következtettünk, hogy az Akt/PKB fontos szerepet tölthet be a túlélési folyamatok megindításában, viszont arra nem derült fény, hogy milyen folyamatok vezethetnek az Akt/PKB differenciált aktiválásához. Mivel számos megfigyelés azt bizonyította, hogy az Akt/PKB aktivációját a PI3K irányítja (Bondeva 1999), a TCR-ből kiinduló és a PI3K-t aktiváló jelek módosítására PI3K specifikus inhibítorokat alkalmaztunk. Az inhibítorokkal történt előinkubálást követően az intakt tímusz lebenyekben lévő timocitákat anti-CD3ε és ConA hatásának tettük ki, majd mértük az Akt/PKB enzim aktivitását és a DP timociták számát és életképességét (35. és 36. ábra).



35. ábra: Akt/PKB foszforilációja PI3K inhibítor, majd anti-CD3ε és ConA kezelést követően (Pongracz, Parnell et al. 2003)

MHC-/- egér embriókból származó tímusz lebenyeket PI3K specifikus inhibítorral LY294002 (1.5 μM, 37°C), anti-CD3ε (5 μg/ml) illetve Con A (5 μ g/ml) jelenlétében inkubáltuk, majd a timociták lizálását követően az immunprecipitált Akt/PKB foszforilációs szintjét mértük.



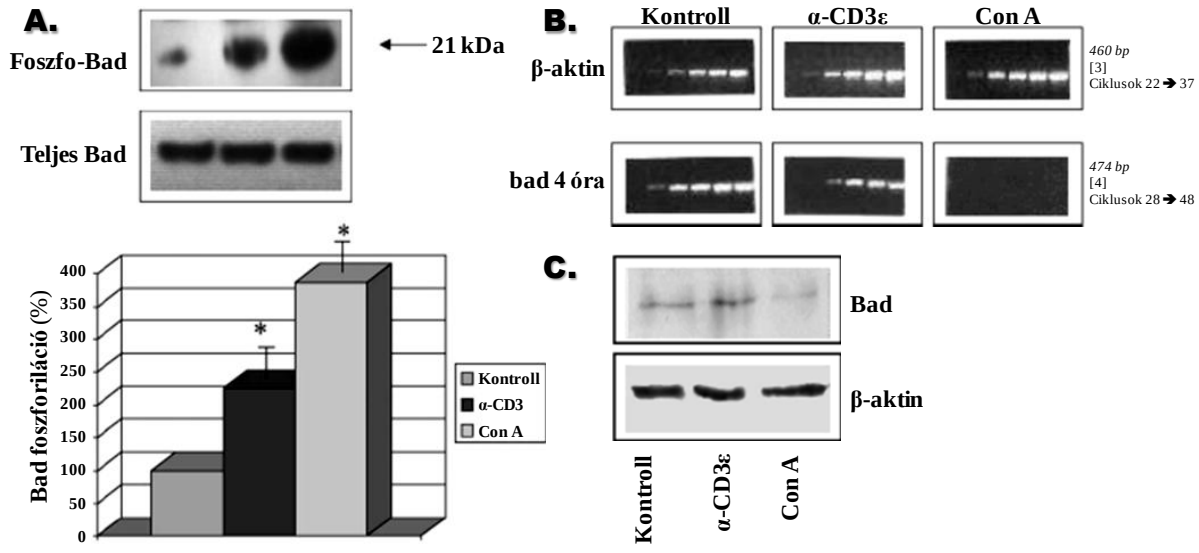
36. ábra: A PI3K gátlásának fiziológiás hatása (Pongracz, Parnell et al. 2003)

MHC-/- egér embriókból származó tímusz lebenyeket PI3K specifikus inhibítorral LY294002 (1.5 μM, 37°C, 1 óra) előinkubáltuk, azután anti-CD3ε (5 μg/ml), illetve Con A-val (5 μ g/ml) kezeltük 16 órán keresztül, majd a timociták $CD4^+$ és $CD8^+$ expresszióját analizáltuk áramlási citométerrel. A szignifikáns különbséget csillaggal jelöltük.

Mivel a PI3K csökkent aktivitása csak az anti-CD3ε-ellenanyaggal keresztkötött TCR esetében csökkentette a timociták túlélését, felmerült annak a lehetősége, hogy a túlélési folyamatok szabályozásában résztvevő Akt/PKB a PI3K-tól teljesen függetlenül is

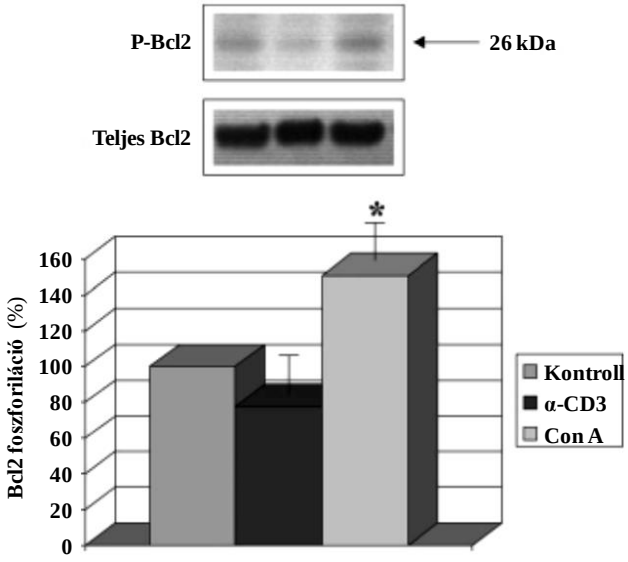
aktiválható, amely magyarázatot szolgáltatott volna a timociták túlélésében mutatkozó különbségekre. Ennek tisztázására, mind az Akt/PKB-ból, mind az Akt/PKB-hoz vezető jelátviteli útvonalakat behatóbb vizsgálatnak vetettük alá.

Az apoptózist és életképességet szabályozó Bcl molekulacsaládra esett a választásunk a „down-stream" elemek vizsgálatában. Különösen, mivel a Bcl család számos tagja szubsztrátja az Akt/PKB, illetve a túlélést és apoptózist irányító PKC enzimeknek. Vizsgálataink kimutatták, hogy mennyiségi különbségek is döntő hatást gyakorolnak a jelátvitelre. A pro-apoptotikus Bad foszforilációjának következménye az anti-apoptotikus BclXL felszabadulása, amely gátolja a mitokondriumokból a citokróm C felszabadulását és ezzel a kaszpáz-aktiválta apoptózis megindulását. Kísérleteink rávilágítottak arra, hogy a ConA sokkal nagyobb mértékben indukálja a Bad molekula foszforilációját, mint az anti-CD3ε kezelés (37. ábra), amelynek lehet a következménye az anti-apoptotikus BclXL fokozott felszabadulása. Ezen túlmenően a ConA, ellentétben az anti-CD3ε ellenanyag hatásával, idővel a bad-gén átíródásának teljes gátlását eredményezte, miközben az anti-apoptotikus molekula, a Bcl2 foszforilációját és ezzel aktivitását növelte (38. ábra).



37. ábra: Bad foszforiláció és mRNS szintek (Pongracz, Parnell et al. 2003)

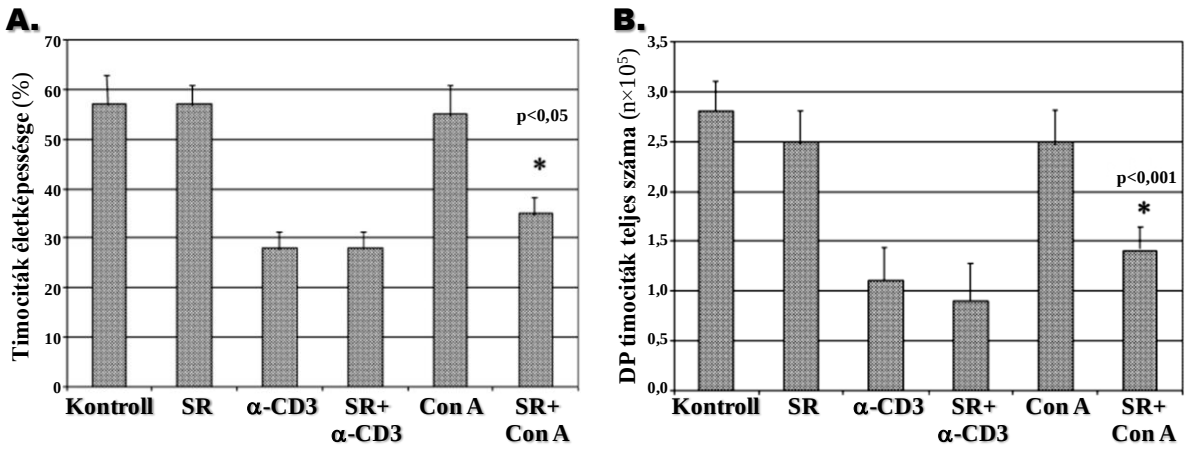
(A) MHC-/- egér embriókból származó tímusz lebenyeket anti-CD3ε (5 μg/ml) illetve Con A (5 μ g/ml) jelenlétében inkubáltuk 4 órán át, majd timocitákból ($1x10^6$/kezelés) a Bad molekulákat immunprecipitáltuk. A fehérjék gélen történő szeparálását követően (15% SDS-PAGE) blottoltuk, majd anti-foszfo-Bad ellenanyag felhasználásával vizsgáltuk a fehérjék foszforilációs szintjét. (B, C) 12 órás anti-CD3ε (5 μg/ml), illetve Con A (5 μ g/ml) kezelést követő Bad mRNS és protein expressziója.



38. ábra: Bcl2 foszforiláció (Pongracz, Parnell et al. 2003)

MHC-/- egér embriókból származó tímusz lebenyeket anti-CD3ε (5 μg/ml), illetve Con A (5 μg/ml) kezelés közben γ^{32}P-ATP jelenlétében inkubáltuk. A timociták feltárása után a Bcl2-t immunprecipitáltuk és foszforilációs szintjét mértük.

Mivel a Bcl2 molekula foszforilációja gyakorlatilag azonnali (másodperceken belüli), foszfatázok (PP2A) által katalizált de-foszforilációhoz vezet, megvizsgáltuk, hogy a de-foszforiláció miért nem következik be ConA kezelést követően? Mivel a PP2A foszfatázt ceramidok aktiválják, kémiai inhibítorokkal gátoltuk mind a savas sfingomielináz (SR33557), mind a ceramid szintáz (fumosin) aktivitását, majd megvizsgáltuk a DP timociták túlélését anti-CD3ε ellenanyag, illetve ConA kezelést követően (39. ábra).



39. ábra: A savas sfingomielináz inhibítor (SR33557) hatása a DP timociták viabilitására (Pongracz, Parnell et al. 2003)

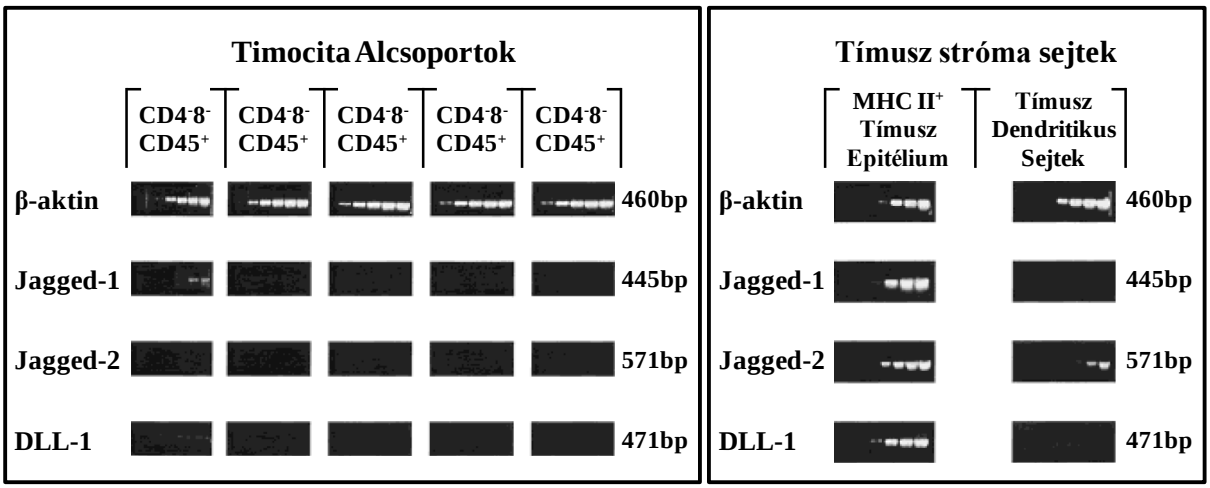
MHC-/- egér embriókból származó tímusz lebenyeket 30 μM SR33557-ral, majd anti-CD3ε (5 μg/ml) illetve Con A-val (5 μ g/ml) kezeltük. A viabilitást propidium jodiddal (A), míg a DP timociták deplécióját (B) anti-CD4-PE és CD8-FITC ellenanyagok felhasználásával, áramlási citometriával mértük.

Várakozásainkkal ellentétben, a ceramid termelésének gátlása nem akadályozta meg az ellenanyag által kiváltott timocita depléciót. A ceramid termelés gátlása viszont gátolta a ConA által kiváltott túlélési jelátvitelt, ami arra utal, hogy a ConA által indukált túlélési folyamat nagymértékben függ a savas sfingomielináz aktivitásától. Ennek következtében, és a korábbi elméletünkkel ellentétben nem a PP2A foszfatáz, hanem a proliferációs folyamatokat is szabályozó (Kolesnik 1995) PKC ζ aktiválásához elengedhetetlen (Lozano 1994) ceramid-termelés játszik fontos szerepet.

Kísérleteink bizonyították, hogy a TCR-ból származó jelek módosíthatók és egyéb receptor-ligand kapcsolatokból párhuzamosan indított jelátviteli kaszkádok mennyiségi viszonyai döntő hatással bírnak a fejlődő timociták túlélésére, szelekciójára. A TCR-függő szelekciós jeleken túlmenően azonban, a túlélést és a timociták differenciálódását egyéb, a szöveti fejlődést szabályozó fő jelátviteli rendszerek is befolyásolják. Ezek közül kísérleteink során kettővel, a Notch és a Wnt jelátviteli rendszerek hatásaival foglalkoztunk behatóbban, míg a BMP hatásaival érintőlegesen.

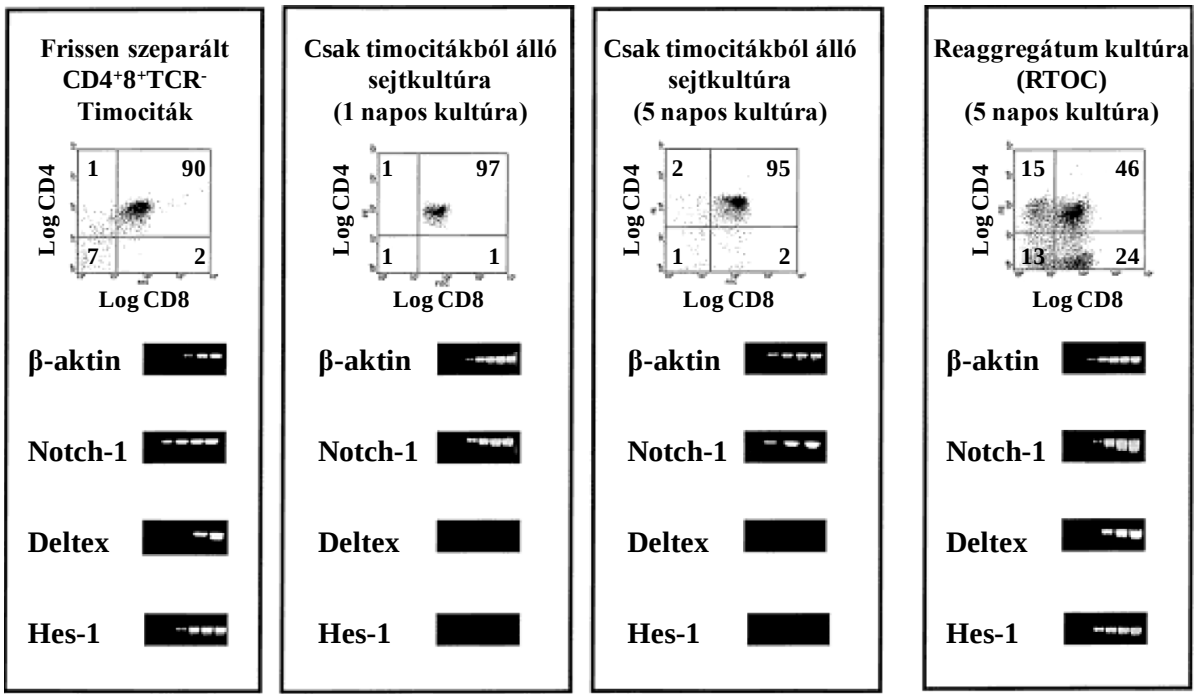
A Notch jelátvitel hatásának vizsgálatával további betekintést nyertünk a negatív és pozitív szelekció komplex jeltáviteli szabályozásába. Kísérletinkkel bizonyítottuk, hogy a Notch ligandok expresszió ja nem a timociták, hanem a tímusz epitéliális sejtek feladata (40. ábra), és

hogy a tímusz epitéliummal való érintkezés hiányában a timociták fejlődése elakad, melyet a Notch jelátvitel célgénjeinek (Hes1 és Deltex) megszűnő transzkripciója is jelez (41. ábra).



40. ábra: A tímuszban a Notch ligand expresszió az MHC class II^+ epithelium-ra korlátozódik (Anderson, Pongracz et al. 2001)

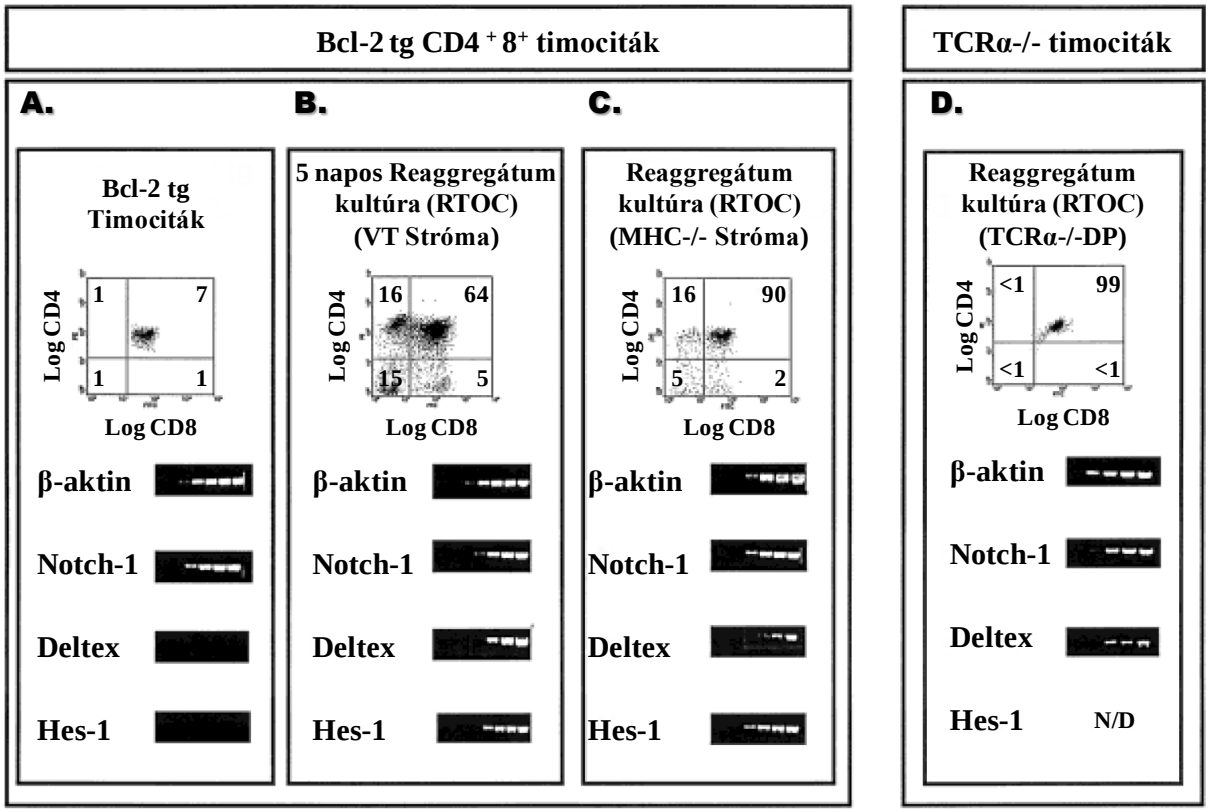
Jagged-1, Jagged-2 és Delta-like-1 expressziót timocitákból és tímusz stróma sejtekből előállított, cDNS-ben mértünk. Belső kontrollként β-aktin-t használtunk. A PCR termékek méreteit az ábra jobb oldalán tűntettük fel (bp).



41. ábra: Tímusz epiteliális sejtek szükségesek és szabályozzák a Notch jelátvitelt DP ($CD4^+8^+$) timocitákban (Anderson, Pongracz et al. 2001)

Bcl-2 transzgén $CD4^+8^+TCR^-$ timocitákat (A) vagy epitélium jelenléte nélkül inkubáltuk egy éjszakán át (B), vagy még öt napon keresztül tímusz stróma hiányában (C) vagy jelenlétében (D) reaggregált kultúrában. A timocitákban CD4, CD8 illetve Notch-1, Deltex és Hes-1mRNA szinteket analizáltunk.

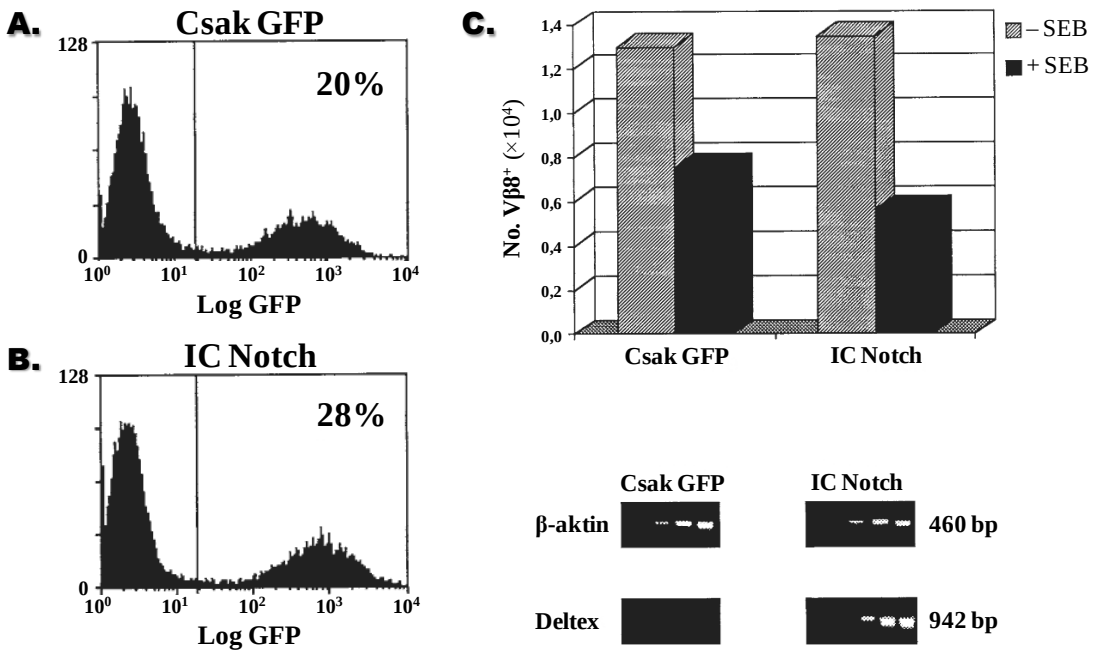
Kísérleteink azt is igazolták, hogy a Notch jelátvitel célgénjeinek expressziójára kizárólag az aktivált Notch receptor jelenléte hat, de ez nem elegendő a timociták differenciációjának megindulásához. Azt is bizonyítottuk, hogy a TCR-MHC interakcióból származó jelek nincsenek befolyással a Notch célgénekre (42. ábra), de a tímusz epitélium kapcsolatból származó TCR-MHC interakciók jelei szükségesek a pozitív szelekció megindulásához.



42. ábra: Tímusz epiteliális sejtek aktiválják a Notch jelátvitelt CD4^{+}8^{+} timocitákban a TCR-MHC interakció hiányában is (Anderson, Pongracz et al. 2001)

CD4^{+}8^{+}TCR^{-} timociták bcl-2 transzgén újszülöttekből egy éjszakán át tartó inkubálást követően (A), majd reaggregáltatva vad típusú (VT) (B) vagy MHC–/– tímusz strómával (C) öt napon keresztül. Hasonlóképpen, TCRα-/- timocitákat vad típusú tímusz strómával inkubáltuk együtt (D), majd a timocitákban CD4, CD8 fehérje illetve Notch-1, Deltex és Hes-1mRNS szinteket analizáltunk.

Hogy kideritsük, hogy a tímusz epitéliumból származó Notch jelátvitel aktiválása védelmet nyújt-e negatív szelekciós szignálokkal szemben, rekombináns retrovírussal permanensen aktív (IC-Notch) Notch-ot juttattunk a timocitákba, majd megvizsgáltuk, hogy a dendritikus sejtek által prezentált staphylococcus enterotoxin szuperantigén (SEB) által indukált sejthalállal szemben ellenállókká válnak-e (43. ábra).



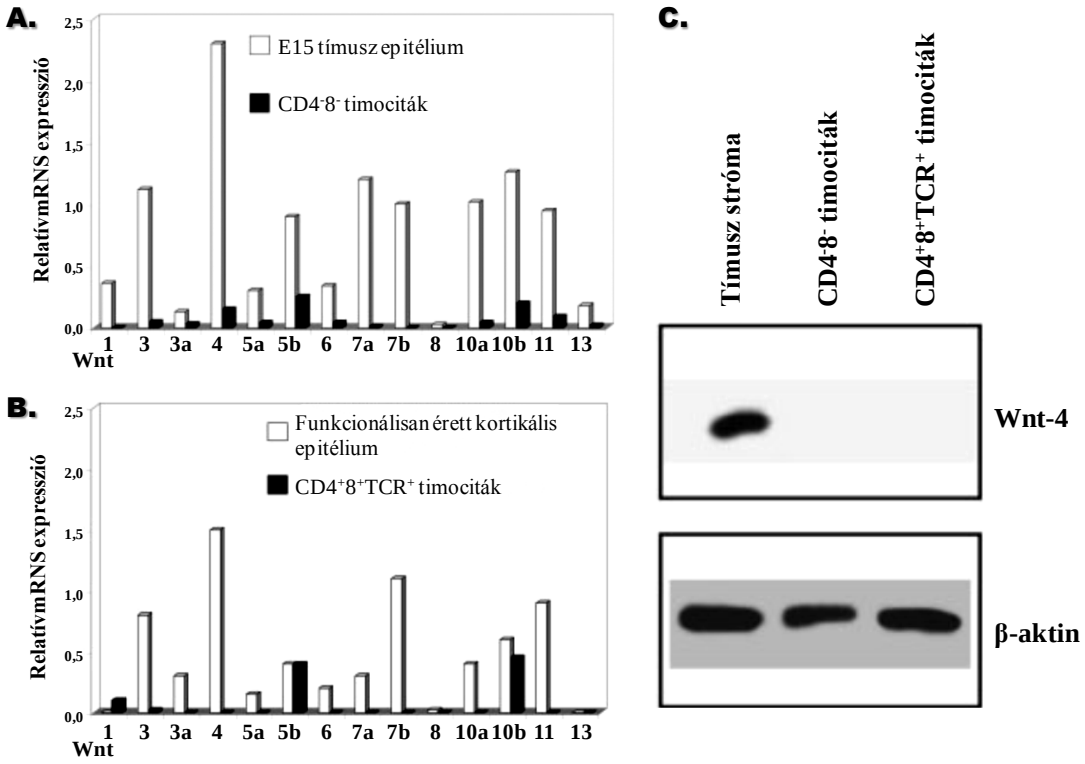
43. ábra: Konstitutívan aktív Notch1 expresszió nem védi meg a DP timocitákat a szuperantigén mediálta negatív szelekciótól (Hare, Pongrácz et al. 2002)

A kísérletekben Bcl-2 transzgén újszülött egerekből származó timocitákba IC-Notch-GFP-t, illetve a kontroll csoportba GFP-t juttattunk rRetrovírus felhasználásával. A timocitákat egy napos inkubálás után 10:1 arányban SEB-t (10 μg/ml) prezentáló illetve nem prezentáló dendritikus sejtek jelenlétében 48 órán tovább inkubáltuk. A deltex expressziót mRNA szinten PCR-rel vizsgáltuk, míg GFP és T-sejt receptor expressziós analízisét áramlási citometriával végeztük.

Kísérleti eredményeink bizonyították, hogy a timocitákat nem védi meg az aktív Notch jelátvitel a szuperantigén által indukált sejthaláltól. Továbbá ezzel a kísérlettel azt is sikerült bizonyítanunk, hogy a pozitív szelekciót követően még egy szelekciós lépés vár a fejlődő T-sejtekre a tímusz dendritikus sejtekben dús, medulláris epiteliális mikrokörnyezetében, mely tovább finomítja a periférián megjelenő T-sejtek TCR repertoárját.

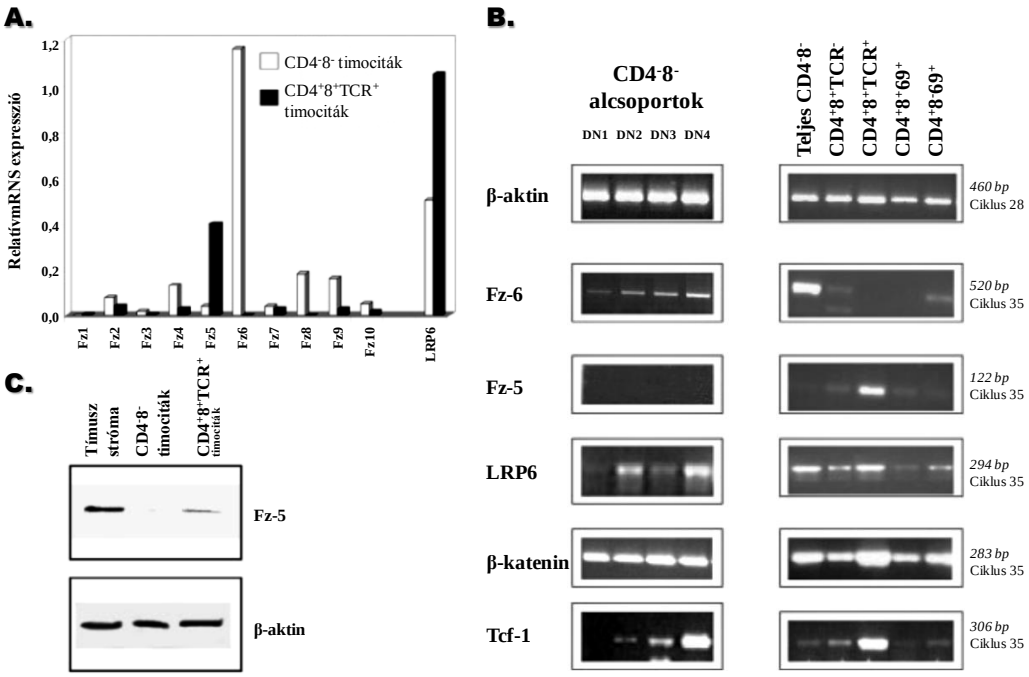
A sejtek túlélését, differenciációját, öregedését azonban nem kizárólag a Notch jelátvitel szabályozza. A Wnt jelátvitel komplex és komplikált jelátviteli rendszereiről is feltételezhető volt, hogy befolyásolják a T-sejtek érési és differenciálódási folyamatait. Első vizsgálatainkkal arra kívántunk fényt deríteni, hogy honnan származnak a Wnt ligandok a tímuszban, és hogy a T-sejtek rendelkeznek-e Wnt receptorokkal? A tisztított timocita és tímusz epiteliális sejtpopulációkon végzett génexpressziós vizsgálatok rávilágítottak arra, hogy a Wnt rendszer ligandjait elsősorban a tímusz epiteliális sejtek termelik (44. ábra). Ellentétben azonban a Notch jelátvitellel, ezek a ligandok szekretáltak és a T-sejteken a tíz receptoruk közül csak limitált számú Fz található. A timociták által különböző fejlődési lépésekben más-más Fz receptor jelenlétét azonosítottuk, amiből arra következtettünk, hogy a

Wnt jelátvitel jól körülhatárolható folyamatokat szabályoz a timociták fejlődése és differenciálódás során (45. ábra).



44. ábra: Wnt család tagjait szelektíven expresszálja a tímusz epitélium (Pongracz, Hare et al. 2003)

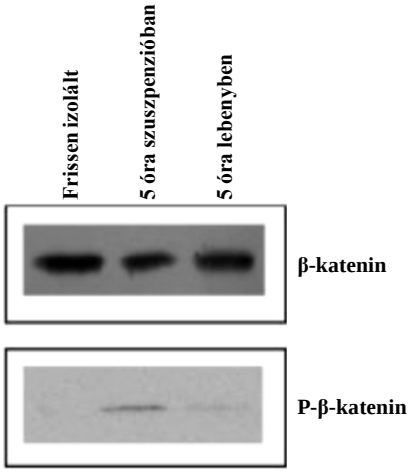
(A) Wnt-ok relatív expresszióját E15 tímusz epitéliumban és DN timocitákban vagy (B) funkcionálisan érett kortikális epitéliumban és DP timocitákban vizsgáltuk RT-PCR reakcióval. Belső kontrollként β-aktin-t használtunk. (C) Wnt-4 fehérje expressziójának vizsgálata CD45 depletált (limfocita és makrofág mentes) tímusz strómában, DN és DP timocitákban Western-blot analízissel. Az egységes fehérje felvitelt anti-β-aktin ellenanyaggal mutattuk ki.



45. ábra: A Fz receptor család tagjait szelektíven expresszálják a timociták és a tímusz epitélium (Pongracz, Hare et al. 2003)

(A) Frizzled receptorok és ko-receptor (LRP6) expressziója a fejlődő DN és TCR^+DP T-sejtken. (B) A DN timociták négy alpopulációjában (DN1-DN4), TCR^- és TCR^+ DP timocitákban, illetve a szelekciós folyamatok megindulását jelző $CD69^+$DP és a pozitív szelekción sikeresen átjutott „single" pozitív (SP, $CD4^+8^-69^+$) timocitákon vizsgáltuk a Wnt jelátvitelhez szükséges receptorok Fz5 és Fz6 expresszióját, illetve a Wnt kanonikus jelátviteli út fő jelátviteli molekulájának, a β-kateninnek és célgénjének a Tcf-1 transzkripciós faktornak a jelenlétét RT-PCR reakcióval. (C) Fz5 fehérjeexpressziót Western-blottal vizsgáltuk DN és DP timocitákban, illetve CD45 depletált tímusz stróma állományban.

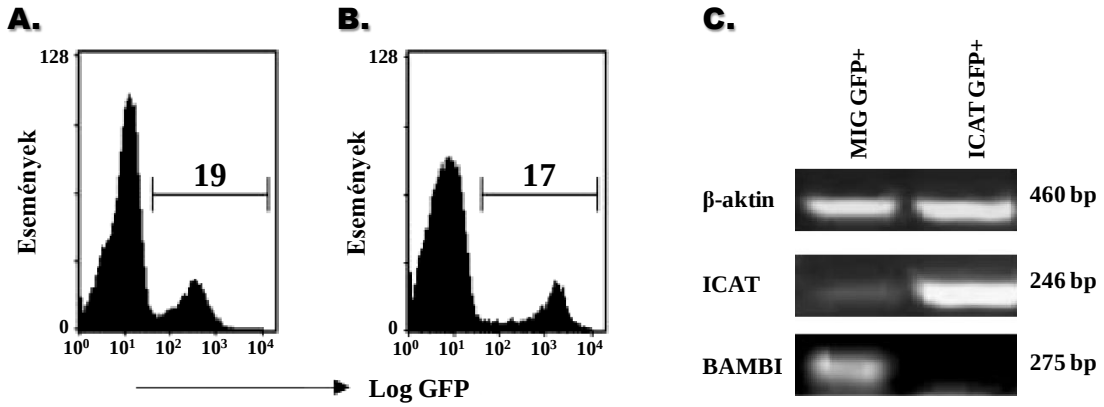
Kísérleti eredményeink arra engedtek következtetni, hogy a Wnt jelátvitelre a tímuszban fejlődő timocitáknak elsősorban a DN-DP fejlődési átmenetnél van szükségük, hiszen mind a receptorexpresszió, mind a jelátviteli molekulák jelenléte ebben a fejlődési stádiumban a legmarkánsabb. Annak vizsgálatára, hogy a Wnt jeleket valóban a tímusz epitéliumból kapják-e a fejlődő T-sejtek, timocitákat tímusz strómamentes környezetben inkubáltuk öt órán át, majd megvizsgáltuk a β-katenin molekula expressziós és foszforilációs szintjét. A foszforiláció csak a stróma nélkül inkubált timocitákban emelkedett, amiből arra következtettünk, hogy a timociták által termelt kis mennyiségű Wnt ligand nem elegendő a kanonikus Wnt jelátvitel fenntartására a tímusz epitélium hiányában. Mivel a β-katenin foszforilációja a fehérje proteolitikus degradációjához szükséges, a fehérjeszint is csökkenő tendenciát mutatott az inkubációs periódus végére (46. ábra).



46. ábra: β-katenin stabilitásának vizsgálata Western blottal (Pongracz, Hare et al. 2003)

DN ($CD4^-CD8^-$) timocitákat vagy frissen nyertük ki E15 embrionális tímuszból, vagy 5 órás inkubálás után tímusz stróma hiányában, illetve jelenlétében inkubáltuk. A timocita fehérjék szeparálása után anti-β-katenin és anti-foszfo-β-katenin ellenanyaggal mutattuk ki a fehérje szintű változásokat.

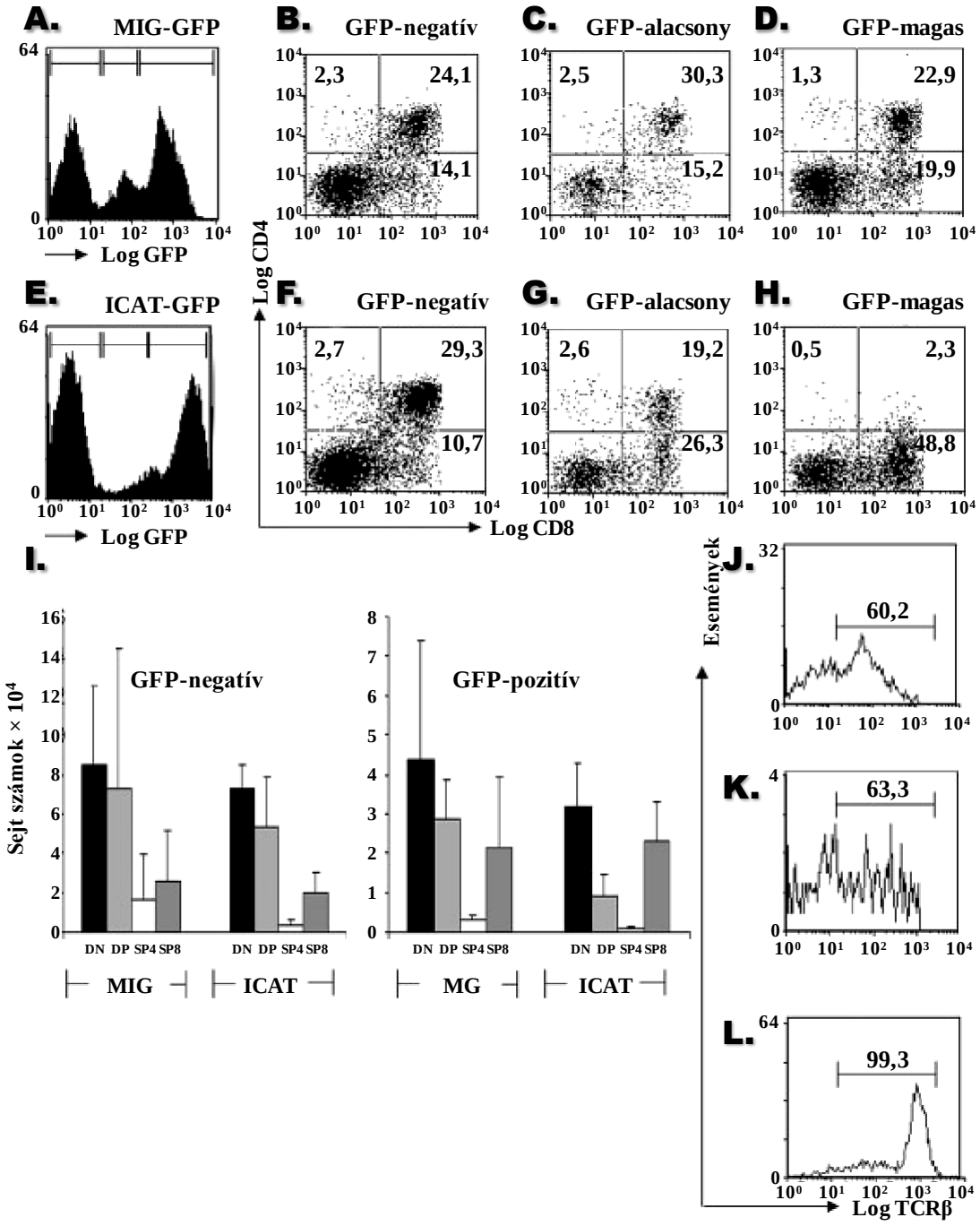
Annak megállapítására, hogy csak a sejtek túléléséhez szükséges vagy a timociták fejlődési lépéseihez is elengedhetetlen-e a kanonikus Wnt jelátvitel, a β-katenin-függő jeltovábbítás módosítására volt szükség. A sejtekben fiziológiásan is jelenlévő β-katenin inhibítor, ICAT, génjének számos példányát rekombináns retrovírussal juttattuk a fejlődő timocitákba, ezzel elérve azt, hogy a fejlődő timociták nagy százalékában igen magas szinten expresszálódjon a β-katenin jelátvitelt gátló molekula. Az ICAT molekula expressziós szintjét és aktivitását a kísérletek megkezdése előtt határoztuk meg. A Wnt jelátvitel célgénjeinek egyike a „bone morphogenic protein" (BMP) és „activin membrane-bound inhibitor", azaz BAMBI, amely a TGFβ/BMP jelátvitelt szabályozza. A BAMBI expressziós szintjét RT-PCR-ral mértük, amely az ICAT-et túlexpresszáló sejtekben detektálhatatlan szintre csökkent (47. ábra). Ebből arra következtettünk, hogy az ICAT molekula aktívan gátolja a β-katenin függő Wnt jelátvitelt. Ebben a kísérleti rendszerben már vizsgálhattuk a β-katenin jelátvitel fiziológiás hatását a fejlődő timocitákban.



47. ábra: ICAT túlexpresszáltatása T-sejt prekurzorokban
(Pongracz JE, Parnell SM et al. 2006)

Retrovirális konstrukciókkal (MIG-GFP és MIG-ICAT) rekombináns retrovírusokat állítottunk elő, melyekkel megfertőztük a DN azaz $CD4^-8^-$ timocitákat (A, B). A fertőzött, azaz GFP-t expresszáló sejteket MoFlo sejt szorter segítségével elválasztottuk a GFP negatív populációtól, majd az ICAT szintjét illetve az ICAT aktivitásának nyomkövetésére szolgáló BAMBI szintjét vizsgáltuk RT-PCR reakcióval.

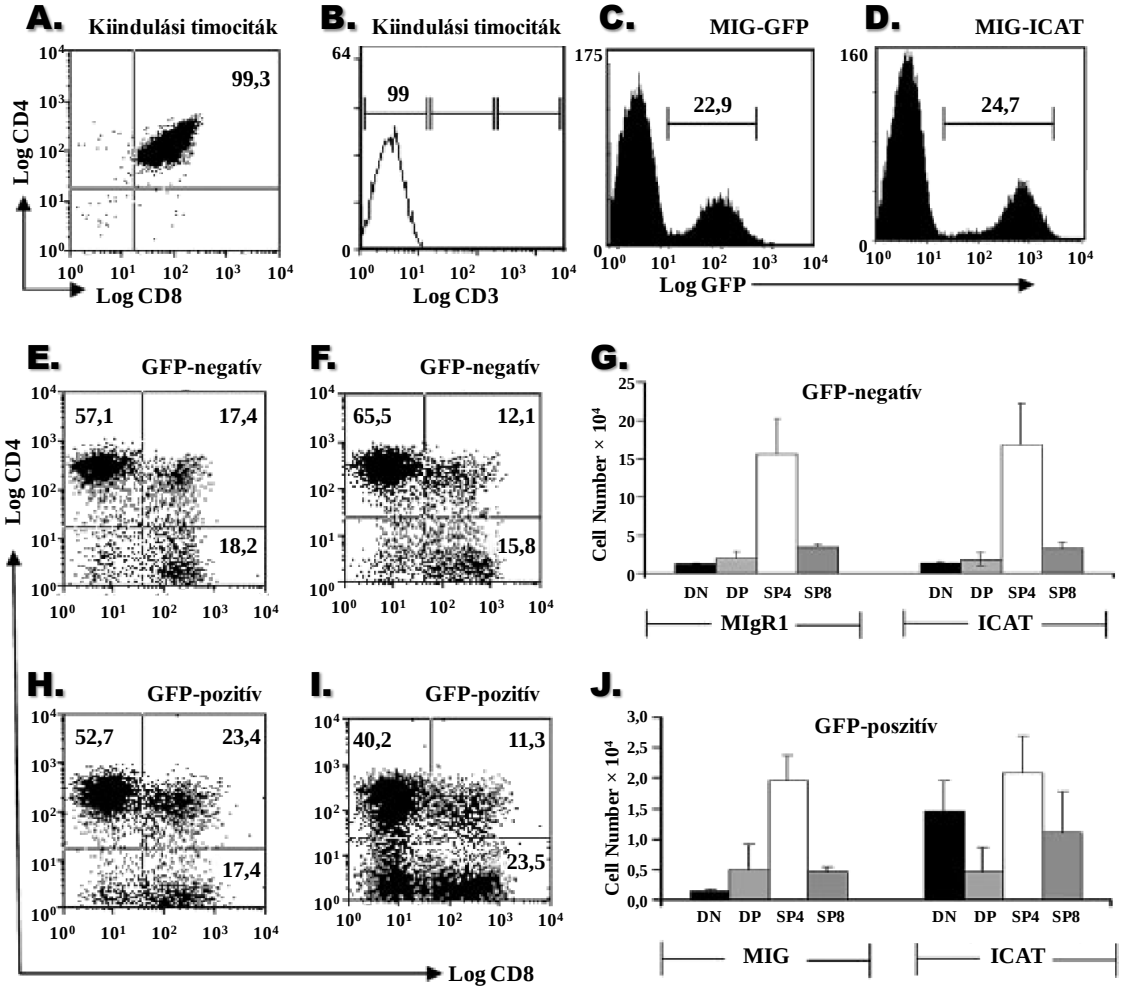
Miután bizonyítottuk, hogy az ICAT aktív, a rekombináns retrovírussal módosított tisztított DN timocitákat (48. ábra) illetve tisztított DP timocitákat (49. ábra) külön-külön reaggregált tímusz kultúrákban tenyésztettük tovább. Ezekben a kultúrákban minden jel, a TCR-MHC jelátviteltől, a Notch szignálokon keresztül, a Wnt ligandokon és receptorokon át rendelkezésre állt a timociták fejlődéséhez és differenciálódásához. Az egyetlen kivételt, a kanonikus Wnt jelátvitel jelentette a timocitákon belül, amelyet hatékonyan blokkolt az inhibítor jelenléte. A DN timocitákkal végzett kísérletek bizonyították, hogy a Wnt jelátvitel a DN-ból a DP fejlődési állapotba való átmenethez szükséges. Erre már a Fz receptorok expressziós mintázatának vizsgálatából is következtetni lehetett, hiszen a timociták Wnt receptorokat elsősorban a DN3, DN4 és DP fejlődési állapotban expresszálnak. Az inhibítor jelenlétében végzett kísérletek rámutattak arra, hogy az ICAT-et expresszáló sejtek a DN, illetve a $DN^-TCR\beta^+$, a DP fejlődési állapotot közvetlenül megelőző differenciálódási szinten rekedtek meg a fejlődésben.



48. ábra: ICAT túlexpresszáltatása a DP átmenet előtt blokkolja a T sejtek fejlődését (Pongracz JE, Parnell SM et al. 2006)

Embrionális májból származó $CD45^+$ T-sejt prekurzor sejteket MIG-GFP vagy MIG-ICAT retrovirussal fertőztünk, majd limfocita-mentes tímusz lebenyeket kolonizáltattunk a módosított timocitákkal. 11 nap inkubálást követően a sejteket áramlási citométerrel analizáltuk. (A, E) GFP expresszió MIG-GFP és MIG-ICAT kultúrákban, ahol GFP^-, GFPalacsony és GFPmagas sejt populációkat külön-külön további analízisnek vetettünk alá (B–D, F–H). Minden alcsoportban megvizsgáltuk a CD4 és CD8 [(B–D) expresszióját a MIG-GFP, (F–H) és MIG-ICAT kultúrákban. A T sejt receptor génátrendeződésének és a DP átmenetet közvetlenül megelőző TCRβ lánc expresszióját is vizsgáltuk $CD4^-8^+$ sejtekben mind MIG-GFP (J), mind MIG-ICAT (K) kultúrákban és felnőtt tímuszból származó $CD4^-8^+$ sejtek expressziós szintjével hasonlítottuk össze (L). A kultúrák abszolút sejtszámát is megjelenítettük (I) három független kísérlet átlagaként.

A DP timocitákkal végzett kísérletek rámutattak arra is, hogy a további differenciálódási szakasz nem igényli a kanonikus Wnt jelátvitelt, hiszen a timociták SP ($CD4^+8^-$ helper és $CD4^-8^+$ citotoxikus) sejtekké értek a kanonikus Wnt inhibítor jelenlétében.



49. ábra: ICAT hatása a DP $CD4^+8^+$ timociták fejlődésére
(Pongracz JE, Parnell SM et al. 2006)

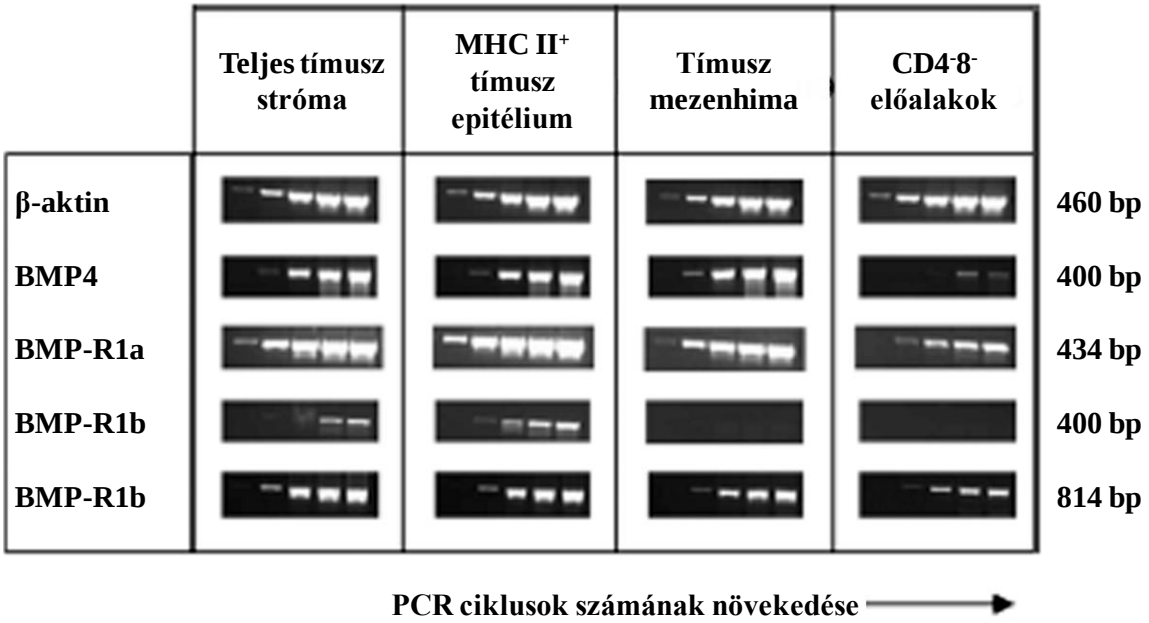
Frissen izolált $CD4^+8^+CD3^-$ sejteket (A,B) MIG-GFP vagy MIG-ICAT retrovirussal fertőztük, majd reaggregált tímusz szervkultúrákban tenyésztettük 5 napon át. Az inkubálást követően a sejteket áramlási citométerrel analizáltuk GFP expresszióra MIG-GFP (C) és MIG-ICAT (D) kultúrákban, illetve CD4 és CD8 (E–I) expressziós mintázatra. Érett $CD4^+8^-$ és $CD4^-8^+$ T-sejtek minden kezelést követően megtalálhatók, azt mutatva, hogy a kanonikus jelátvitel gátlása nem akadályozza a DP-SP fejlődési átmenetet (E-I). A kísérletekből visszanyert sejtszámokat a G és J grafikonokon összesítettük három független kísérlet alapján.

A fő jelátviteli rendszerek és fiziológiás hatásaik komplex és egymás hatását szabályozó kapcsolata kísérleteink alapján nyilvánvalóvá vált. A kutatásaink azonban arra is fényt derítettek, hogy az általunk vizsgált jelátviteli rendszerek nem csak a T-sejtek fejlődésére hathatnak. Rávilágítottunk arra is, hogy a T-sejtek fejlődéséhez és differenciálódásához

olyannyira nélkülözhetetlen tímusz epitéliális sejtek is hordozzák azokat a receptorokat és intracelluláris jelátviteli molekulákat, amelyek képessé teszik a mikrokörnyezet e fontos elemeit a jelátviteli rendszerek által közölt környezeti információ megválaszolására.

A BMP jelátviteli rendszerben a receptor-ligand jelenlétét vizsgálva hasonló expressziós mintázatokra bukkantunk, mint a Wnt és Notch jelátviteli rendszerek esetében. Kísérleteink rámutattak arra, hogy a BMP4-et, amelyre a timocitáknak is szükségük van a fejlődésükhöz (Tsai, Lee et al. 2003) a tímusz stróma sejtek és nem a timociták termelik (50. ábra), míg a receptorok –kivéve a BMPR1b receptort az MHCII+ tímusz epitéliumon- mind a timocitákon, mind a tímusz stróma sejteken megtalálhatók. A fő jelátviteli rendszerek receptor és ligand expressziójának mintázatából arra a következtetésre jutottunk, hogy ugyanazok a jelátviteli rendszerek szabályozzák a tímusz epitélium fejlődését is, mint a timocitákét.

A sejten belüli jelátviteli molekulák alaposabb vizsgálata is előrevetítette a jelátvitel megértésének további nehézségeit. Csak példaként, a kanonikus Wnt jelátvitel célgénje, a már korábbi kísérletekben „read-out" génként alkalmazott BAMBI, nem kizárólag a TGFβ és a BMP jelátvitelt szabályozza. A BAMBI molekula kapcsolatot tud létesíteni a Wnt jelátvitel számos elemével, így a Wnt receptor Fz5-tel, a Fz ko-receptor LRP6-tal és a Wnt jelátvitelben fontos Dvl2-vel, amely interakció útján erősíti a Fz5 receptorból származó jeleket (Lin, Gao et al. 2008). Ezáltal a BAMBI párhuzamosan három jelátviteli útat is befolyásol.



50. ábra: A tímusz epiteliális sejtek rendelkeznek receptorral a BMP-kre (Anderson, Jenkinson et al. 2006)

Szemikvantitatív RT-PCR reakcióval vizsgálva E14 embriókból származó DN timociták, illetve deoxiguanozinnal kezelt tímusz lebenyekből izolált különféle stróma sejt populációk BMP és BMP receptor mRNS szintjét analizáltuk.

A timociták fejlődését vizsgálva megállapítottuk, hogy a timociták proliferációjában és differenciációjában fontos szerepet betöltő jelátviteli folyamatok aktiválási jelei a tímusz epiteliumból származnak. Ezek nélkül, azaz Notch avagy Wnt jelek nélkül, a timociták nem érik el azt a fejlődési szintet, amely elengedhetetlen a receptorláncok génjeinek átrendeződéséhez. Amennyiben nincsenek működőképes receptorok a fejlődő timociták felszínén, úgy a funkcióképtelen sejtek apoptotikus sejthalállal elpusztulnak. A funkcióképes TCR kialakulása után azonban minden egyéb jelet felülírnak a TCR függő szelekciós szignálok.

Az immunológiai válaszreakciókban igen fontos szerepet játszó T-sejtek fejlődése igen komplex folyamat. A T-sejtek fejlődésére, differenciálódására és szelekciójára a tímusz strómaállományától függő sejtes kölcsönhatások és jelátviteli hálózatok szövevénye, azaz a speciális mikrokörnyezet igen nagy befolyással bír. Ezek a jelek azonban nem csak a T-sejtek fejlődését szabályozzák, hanem visszahatnak az epiteliális sejthálózat fenntartására is.

5.3. Jelátvitel a tímusz atrófiája során

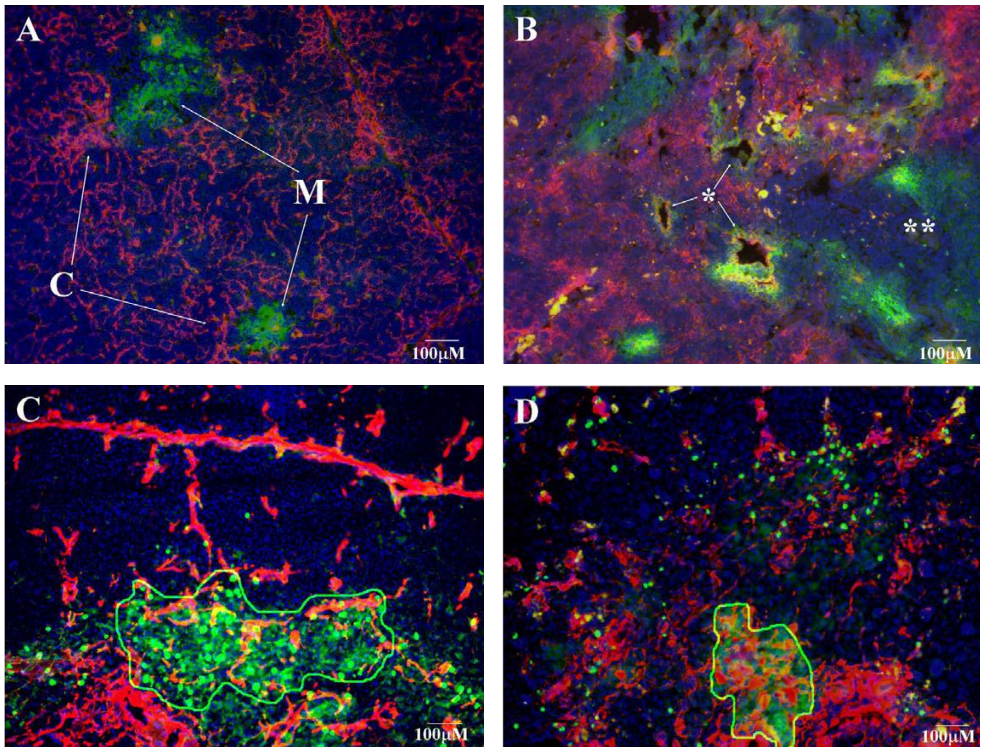
Annak megértése érdekében, hogy a T-sejtek fejlődését támogató tímusz epitélium hogyan alakul ki, a Wnt jelátvitel alaposabb megértése tűnt a legcélravezetőbb kutatási iránynak. A Wnt jelátvitel különösen fontosnak ígérkezett, mivel a Wnt4 jelátviteli molekula hiányában a tímusz epitélium karakterét kialakító transzkripciós faktor, a FoxN1 transzkripciója nem indul meg, azaz a tímusz epitélium nem alakul ki. A tímusz epitéliumban zajló jelátvitel megértéséhez azonban olyan kísérleti rendszer kialakítására volt szükség, amelyben a célgének azonosításával a Wnt jelátvitel jól nyomon követhető. Ehhez olyan komplex „microarray" kísérleteket végeztünk, amelyekben a szelektív Wnt célgének azonosítását kívántuk elvégezni. A kísérletekhez a Fz receptorból származó jelek továbbításában fontos szerepet betöltő PKC (PKC δ) molekula módosulatait alkalmaztuk, mely kutatások végül teljesen új irányt adtak a jelátviteli rendszerek és a tímuszban zajló folyamatok megismerésének.

5.3.1. Fiziológiás tímusz atrófia

A tímusz epitéliális sejtekben végzett Wnt jelátviteli vizsgálatok, melyekhez módosított aktivitással rendelkező PKC δ molekulákat is használtunk, a korábbi évtizedekben „thymopoietin"-nek nevezett molekula génexpressziós szintjének Wnt függő változásait azonosították. A gén mai neve lamin asszociált protein 2α, azaz LAP2α, mely fontos szerepet tölt be sejtek, különösen a mezenhimális eredetű fibroblasztok adipoid irányú transzdifferenciációjának szabályozásában. Ezzel az eredménnyel felmerült annak a lehetősége, hogy egy olyan jelátviteli rendszer néhány elemét sikerült azonosítanunk, amely fontos szerepet játszik az öregedés folyamán lezajló tímusz atrófia szabályozásában.

A fenti feltételezés alátámasztására megkezdtük az öregedő vad típusú Balb/c egerek tímuszának vizsgálatát (51. ábra), mely rávilágított arra, hogy az öregedés egy bizonyos fázisában az epitéliális és mezenhimális markerek ko-lokalizálnak. Ez arra engedett következtetni, hogy a korábbi elméletekkel ellentétben az öregedés során a tímusz epitéliális sejtek nem valószínű, hogy apoptózissal elpusztulnak és az így támadó szövethiányt a környezetből beáramló zsírsejtek töltenék meg. Sokkal inkább úgy tűnik, hogy az epitéliális sejtek először epitéliális-mezenhimális tranzíción, azaz EMT-n esnek át, amelynek

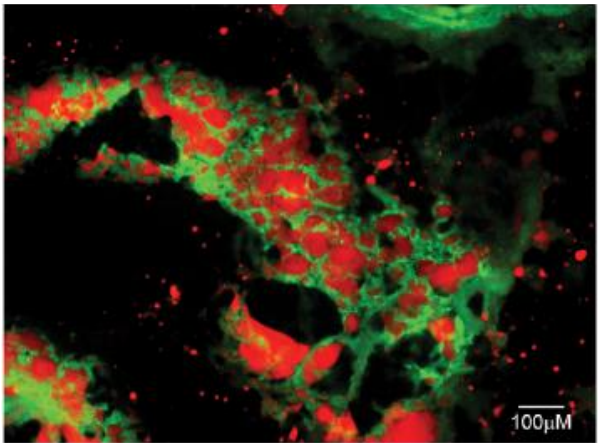
következményeként a már jól ismert fibroblaszt-adipocita transzdifferenciációra is képesek lesznek.



51. ábra: Az epiteliális hálózat dezintegrációja (Kvell, Varecza et al. 2010)

(A) 1 hónapos Balb/c egér tímuszának metszete, (B) 1 éves Balb/c egér tímusz metszete, ahol a zöld festés, anti-EpCAM1-FITC, a tímusz epiteliumot azonosítja, míg az anti-Ly51-PE (vörös) festés a kortikális és medulláris epitélium között tesz különbséget. A sejtmagot DAPI-val festettük (kék). 'M' =medulláris epitélium (EpCAM1^{++}, Ly51^{-}), 'C'= kortikális epitélium (EpCAM1$^{+/-+}$, Ly51^{+}). A B ábrán a csillag (*) degeneratív vakuólumokat jelöl, míg a két csillag (**) az epiteliális festődés teljes hiányát jelzi. (C) 2 hónapos, míg (D) 9 hónapos Balb/c egér tímusz metszetében ábrázolja az epiteliális anti-EpCAM1-FITC (zöld), és a mezenhimális marker ER-TR7-PE (piros) expresszióját. A sejtmagot itt is DAPI (kék) jelzi. A medullát folyamatos zöld vonallal emeltük ki.

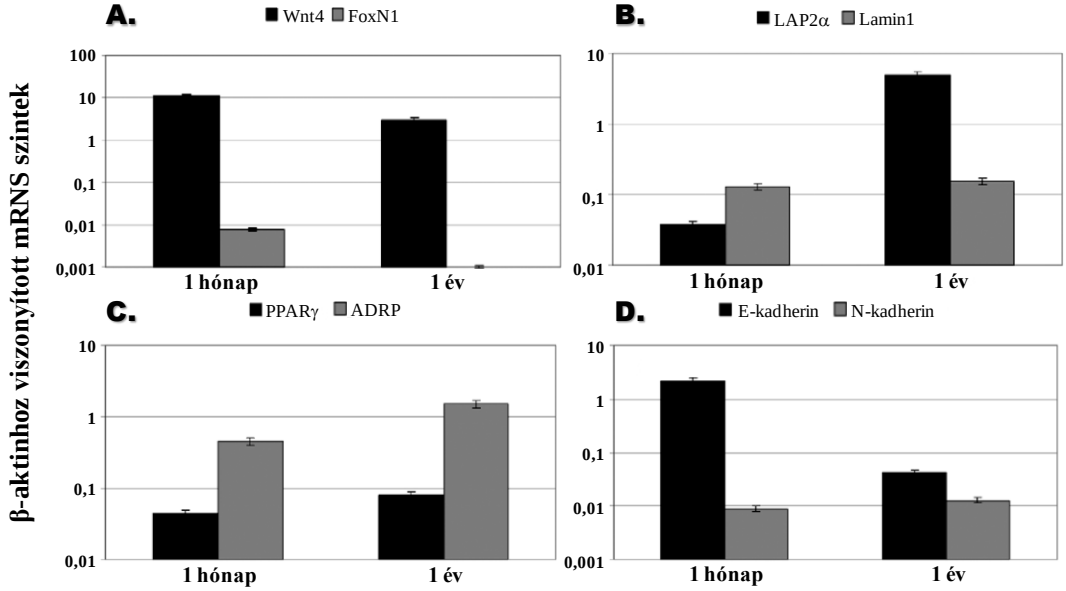
A felhalmozódott lipidek jelenlétét az általunk készített GFP transzgén Balb/c egér (Kvell, Czömpöly et al. 2010) tímuszának vizsgálatával mutattuk ki (52. ábra). Az egér minden sejtje expresszálja a zöld fluoreszcens fehérjét, melynek következtében jól látszik a tímusz epiteliális sejtekben a sejtmembránhoz szoruló zöld citoplazma a vörösen festődő zsír vakuólumokkal szemben.



52. ábra: Adipoid involúció (Kvell, Varecza et al. 2010)

Másfél éves GFP transzgén Balb/c egér tímusz metszete. GFP (zöld), LipidTox Red (vörös) a lipidekkel töltött vakuólumokat jelzi a sejteken belül.

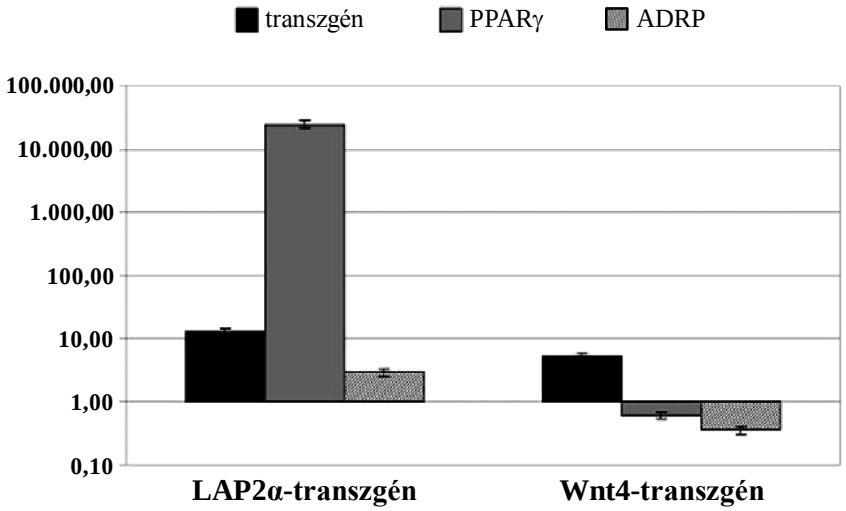
A tímusz epitéliumban zajló öregedési folyamatok molekuláris vizsgálatának megkezdésekor a Wnt4, illetve célgénje, a tímusz epitélium karakterét meghatározó FoxN1 transzkripciós faktor expressziós vizsgálatára koncentráltunk. Öregedés során mind a Wnt4, de különösen a FoxN1 expressziója és az epitéliális sejtek differenciációs szintjét jelző E-kadherin csökkenést mutatott, míg ezzel párhuzamosan megnövekedett a LAP2α szintje, és a zsírosodást szabályozó PPARγ és ADRP transzkripciója (53. ábra).



53. ábra: Molekuláris változások adipoid involúció során (Kvell, Varecza et al. 2010).

Molekuláris változások MACS tisztított (Ep-CAM1^{+}) tímusz epitéliumban. A tímusz epitéliáls sejtek mRNS-éből készített cDNS-ből quantitatív RT-PCR-rel mértük a génexpressziós változásokat. Az Y tengely logaritmikus skálát mutat.

A kezdeti kísérletekben azonosított molekulák a tímusz epitélium elzsírosodási folyamataiban betöltött szerepét transzgén sejtvonalakban végzett kísérletekkel támasztottuk alá. Míg a LAP2α gén tranziens expressziója megnövekedett PPARγ és ADRP expresszióhoz vezetett, addig a Wnt4 expressziós szintjének növekedése mind a PPARγ-t, mind az ADRP-t a kontroll szint alá csökkentette (54. ábra).



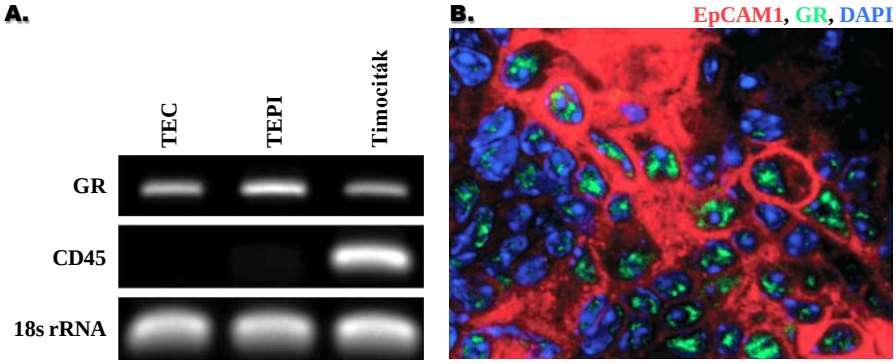
54. ábra: LAP2α-t és Wnt4-et túlexpresszáló tímusz epiteliális (TEP1) sejtvonal molekuláris változásai (Kvell, Varecza et al. 2010)

LAP2α-t és Wnt4-et túlexpresszáló tímusz epiteliális (TEP1) sejtvonal, melyben a génexpressziót kvantitív RT-PCR-rel mértük. Amíg a LAP2α expressziója tranziens volt, úgy a Wnt4-et rekombináns retrovírussal juttattuk be a sejtekbe, majd GFP alapján választottuk ki a Wnt4-et túlexpresszáló sejtpopulációt.

Ezzel bizonyítottá vált, hogy a kor előrehaladtával lecsökkenő Wnt4 szint valószínűleg igen nagy fontossággal bír, hiszen lehetővé teszi a tímusz epitélium epiteliális karakterének elvesztését és ezzel az EMT kialakulását. Ez, párhuzamosan az adipoid transzdifferenciációt szabályozó LAP2α szintjének növekedésével, az öregedő tímusz elzsírosodásához vezet. A kérdés, hogy milyen fiziológiás változások állhatnak az öregedési tímusz elzsírosodás hátterében, megválaszolatlan maradt. Korábbi feltételezések szerint a tímusz öregedése a szex hormonok termelődésének megindulásával köthető össze. Ezt látszott alátámasztani, hogy akár sebészeti, akár kémiai kasztrálás a tímusz atrófiáját megállítani látszott. További bizonyítékot a terápiás glükokortikoidok által indukált átmeneti tímusz atrófia szolgáltatott (Fletcher, Lowen et al. 2009). Felmerült a kérdés, hogy a glükokortikoidok által indukált tímusz involúció ugyanazokat a molekuláris folyamatokat indítja-e meg, mint amelyeket a tímusz fiziológiás öregedése során sikerült azonosítanunk?

5.3.2. Glükokortikoidok (GC) által indukált tímusz atrófia

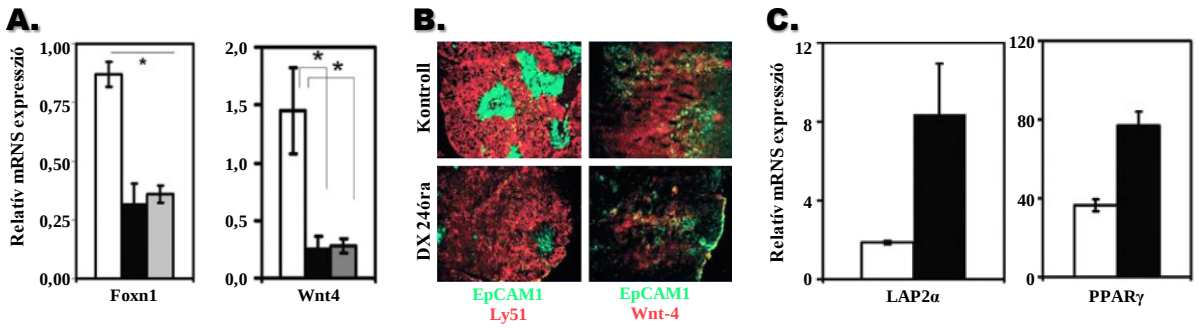
Hogy biztosak lehessünk abban, hogy a tímusz epitéliumot nem másodlagos hatásaként a timociták GC-ok indukálta pusztulása készteti atrófiára, hanem ez a folyamat a GC-ra adott direkt válasz, első lépésként bizonyítottuk, hogy a timociták mellett a tímusz epiteliális sejtek és a kísérletekben alkalmazható tímusz epiteliális sejtvonal, a TEP1 rendelkezik GC receptorral (55. ábra). Ezzel alátámasztottuk azt a feltételezést, hogy a tímusz epiteliális sejtek érzékenyek lehetnek a szteroid szintek változására.



55. ábra: Glükokortikoid receptor (GR) expressziója tímusz epiteliális sejtekben (TEC) és tímusz epiteliális (TEP1) sejtvonalon (Talaber, Kvell et al. 2011)

Tímusz epitéliumból, TEP1 sejtvonalból tisztított mRNS-ből készült cDNS-en RT-PCR-ral teszteltük a GR expresszióját. Timocitából izolált mRNS-ből készült cDNS- használtunk pozitív kontrollként. A tímusz epiteliális sejtek tisztaságát CD45 specifikus primerekkel teszteltük. Belső kontroll-nak 18S rRNS-t használtunk (A). GR fehérjét konfokális mikroszkópiával teszteltük anti-GR-FITC-jelzett ellenanyaggal (zöld) kezeletlen, felnőtt BALB/c tímuszban. Tímusz epiteliális sejteket anti-EpCAM1 ellenanyaggal azonosítottuk anti-patkány-Northern Light 637 másodlagos ellenanyag segítségével (vörös). A sejtmagot DAPI-val tettük láthatóvá (kék) (B).

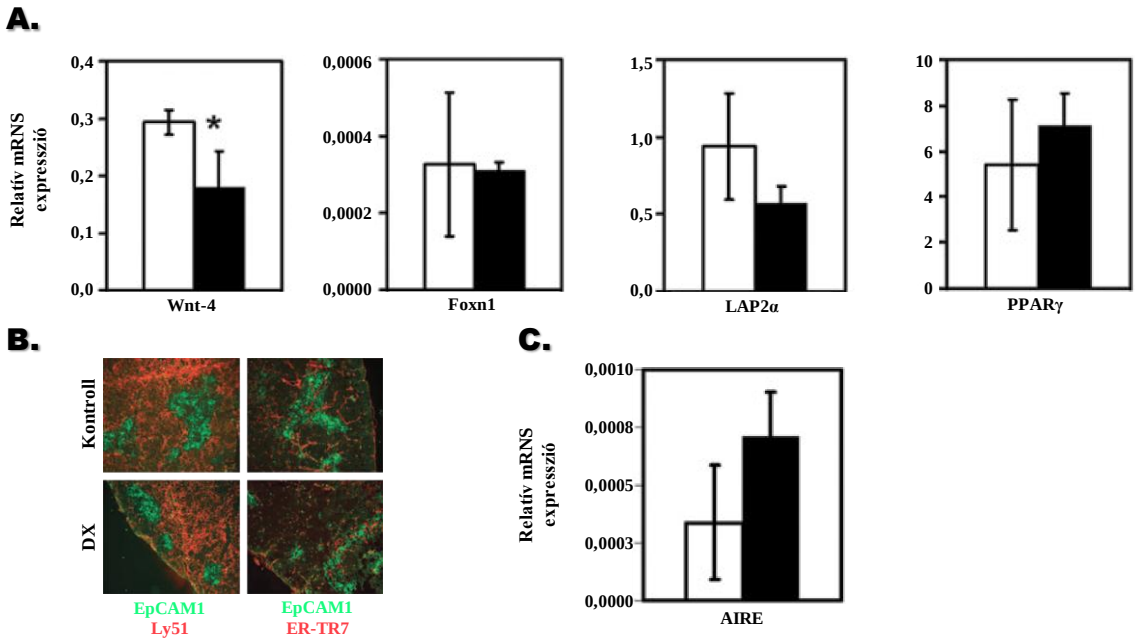
Balb/c egerek dexametazon kezelését követően mértük mind a Wnt4, mind a FoxN1 gének expressziós szintjét. Mindkét gén transzkripciója drasztikus csökkenést mutatott, míg az adipoid differenciációt szabályozó gének emelkedett expresszióját detektáltuk. A kezelés molekuláris szinten felgyorsított fiziológiás öregedés jeleit mutatta. Ezzel párhuzamosan a tímusz morfológiájában is involúciós változások váltak kimutathatóvá (56. ábra).



56. ábra: Dexametazon indukálta génexpressziós és morfológiai változások az egér tímuszban (Talaber, Kvell et al. 2011)

Foxn1 és Wnt4 gének expressziós mintázata (A) kontroll és DX-kezelt, tisztított tímusz epitéliumban 24 óra elteltével. A szürke oszlopok reprezentálják a 168 órával később mért génexpressziót. A csillaggal jelölt értékek szignifikáns különbségeket jelölnek (*$p<0.05$). (B) Tímusz metszetek PBS- és DX-kezelt egerek tímuszából 24 órával a stimulust követően. Anti-EpCAM1-FITC (zöld) és anti-Ly51-PE (vörös) ellenanyagok segítségével mutattuk ki a kortikális és medulláris epitéliumot. A festés elsősorban a medulla depléciójáta mutatta. Ezzel párhuzamosan a Wnt4 fehérje expressziós szintje is csökkent DX kezelés hatására (B, jobb). Wnt4-Northern Lights557 (vörös) és EpCAM1-FITC (zöld) (24 h). (C) LAP2α, PPARγ és ADRP expressziója tímusz epitéliumban 168 órával a DX injekciót követően. A gén expressziót 18S rRNS-re normalizáltuk.

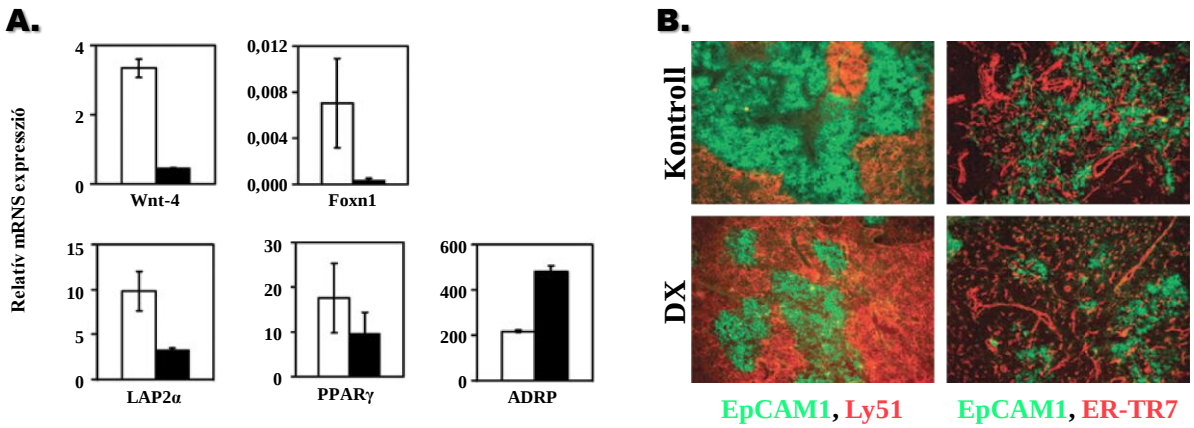
A folyamat azonban megfordíthatónak bizonyult, amennyiben csak egyszeri alkalommal kezeltük az állatokat dexametozonnal, de három hónapnak így is el kellett telnie ahhoz, hogy a tímusz epitélium molekuláris alapszintje helyreálljon (57. ábra).



57. ábra: A tímusz szövet regenerációja három hónapot igényel egyszeri dexametazon injekciót követően (Talaber, Kvell et al. 2011)

(A) Az oszlopdiagrammok a génexpresszió változását mutatják DX kezelést követően. A Wnt4 szint még 3 hónap elteltével is szignifikánsan alacsonyabb maradt (*$p<0.05$), LAP2α és PPARγ szintje nem változott, míg az ADRP expressziós szintje csökkent. Kontroll és DX kezelt tímusz metszetek morfológiai analízise három hónappal a DX kezelést követően EpCAM-Ly51 (B, bal) és EpCAM-ER-TR7 (tímusz epitélium és fibroblaszt marker) (B, jobb). A (C) panel a medulláris marker, autoimmun regulátor (AIRE) expresszióját mutatja, a kontrollhoz képest szignifikáns különbség nem mutatkozik.

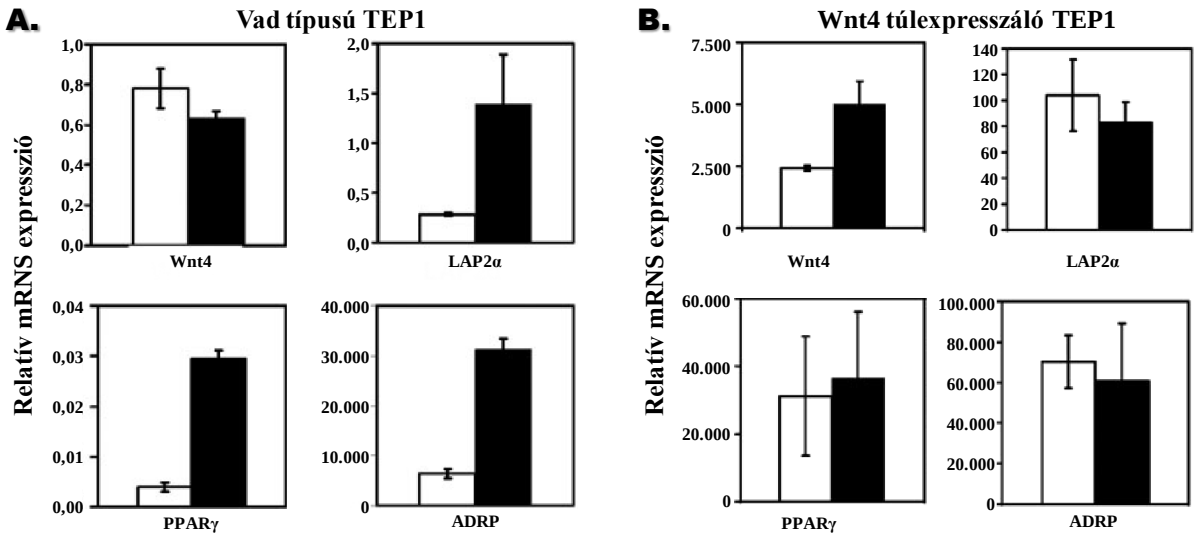
A gyulladáscsökkentő terápiában gyakran alkalmazott, ismételt glükokortikoid kezelés viszont más molekuláris eredménnyel járt. Sem a Wnt4, sem a FoxN1 szint nem tért vissza a kontroll szintre, ami azt jelzi, hogy huzamos szteroid terápia felgyorsult és visszafordíthatatlan tímusz epitéliális öregedéshez vezet. Kísérleteink arra is rámutattak, hogy az elzsírosodási folyamatokban a LAP2α feltehetően átmeneti szerepet tölt be, és mire az elzsírosodást szabályozó ADRP szint jelentősen megnövekszik, addigra a LAP2α-ra már nincs szükség az elzsírosodási folyamat befejezéséhez. Eredményeink rámutattak arra is, hogy a tímusz medulláris állománya sokkal érzékenyebb az öregedési jelekre, mint a kortex. Ennek következményeként a timociták negatív szelekciójának mikrokörnyezete károsodik, amelynek végeredményeként a felszaporodott autoreaktív T-sejtek növelik az autoimmun betegségek kialakulásának kockázatát.



58. ábra: Ismételt dexametazon kezelés hatása a tímusz epitélium génexpressziójára és a tímusz szövet morfológiájára (Talaber, Kvell et al. 2011)

(A) Wnt4, Foxn1, LAP2α, PPARγ, és ADRP expressziós szintje kontroll szöveti szinthez hasonlítva. EpCAM1-Ly51 festés tímusz metszeteken kimutatható a medulla kontroll-hoz viszonyított csökkent mennyisége (B). ER-TR7 festés nem mutatott szignifikáns különbséget, de inkább pöttyözött mintázatot mutatott.

Mivel kezdeti kísérleteinkben a Wnt4 túlexpresszáltatása hatékonynak bizonyult az elzsírosodást szabályozó gének expressziós szintjének csökkentésében, megvizsgáltuk annak lehetőségét, hogy a Wnt4 képes-e megakadályozni a szteroid indukálta génexpressziós változásokat? Kísérleteinkből kitűnik, hogy a Wnt4 molekula folyamatos jelenléte a kísérleti rendszerben képes a dexametazon indukálta LAP2α, PPARγ és ADRP expresszióját azonos szinten tartani a kísérleti kontroll sejtekével (59. ábra), azaz az öregedési elzsírosodási folyamatokat megakadályozni.

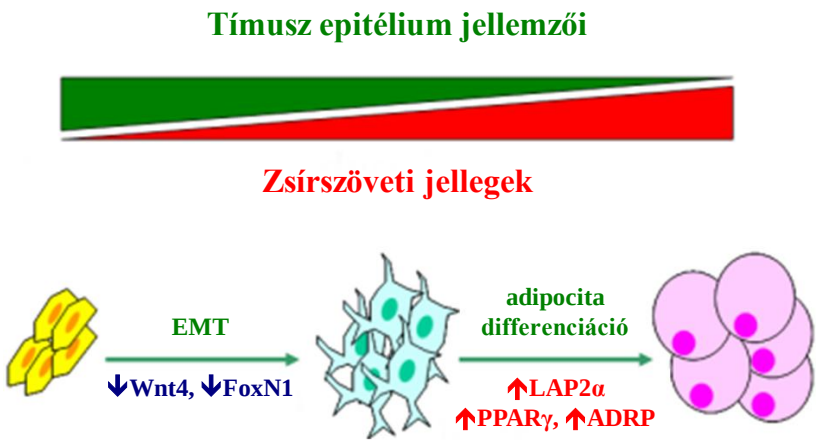


59. ábra: Wnt4 túlexpressziója a TEP1 sejtvonalban képes megvédeni a sejteket a dexametazon (DX)-indukálta adipoid transzdifferenciációtól (Talaber, Kvell et al. 2011)

Génexpressziós változások a TEP1 sejtvonalban 168 órányi DX-kezelés után. A Wnt4, LAP2α, PPARγ, és ADRP mRNA szintjeit teszteltük oldószer és DX-kezelt normál (A) és Wnt4-túlexpresszáló TEP1 (B) sejtvonalakon. A fehér oszlopok az oldószerrel kezelt, míg a fekete oszlopok a DX kezelt sejtek eredményeit mutatják. A géneexpresszió szintje markánsan különbözik.

A Wnt4 szerepének jelentőségét látszanak alátámasztani Aspinall és munkatársainak kísérletei (Henson, Pido-Lopez et al. 2004; Henson, Snelgrove et al. 2005), akik kimutatták, hogy IL7 kezelés a timocita állomány fenntartásához vezet, melynek következtében a tímusz epitélium által alkotott szöveti hálózat kortikális és medulláris epitéliumra osztható szöveti morfológiája is fenntartható. Mivel korábbi kísérleteinkben kimutattuk, hogy azon kevés Wnt-ok közül, amit a timociták termelnek, a Wnt4 az egyik, feltételezhetjük, hogy a nagy mennyiségben jelen lévő timociták hasonló hatást gyakorolnak a tímusz epitéliumra, mint a Wnt4 molekula túlexpresszáltatása.

Kísérleteink összegzéseként felállítottunk egy modellt, melyben összefoglaltuk a fiziológiás, és glükokortikoidok által indukált, felgyorsított öregedés molekuláris mechanizmusának közös elemeit. Azaz a csökkent Wnt4 és FoxN1 szint lehetőséget biztosít az EMT kialakulására, amely során az így keletkezett fibroblaszt-szerű sejtek képesek a megnövekedett LAP2α, PPARγ és ADRP hatására az elzsírosodási folyamatot befejezni (60. ábra). Eredményeink szignifikáns előrelépést jelentettek a tímusz öregedésének megismerésében, mivel a számos morfológiai és molekuláris megfigyelés leíró jellegű interpretációja helyett a mi kísérleteink vezettek az eddigi eredményeket is újraértelmező, általános tímusz öregedési elmélet felállításához.

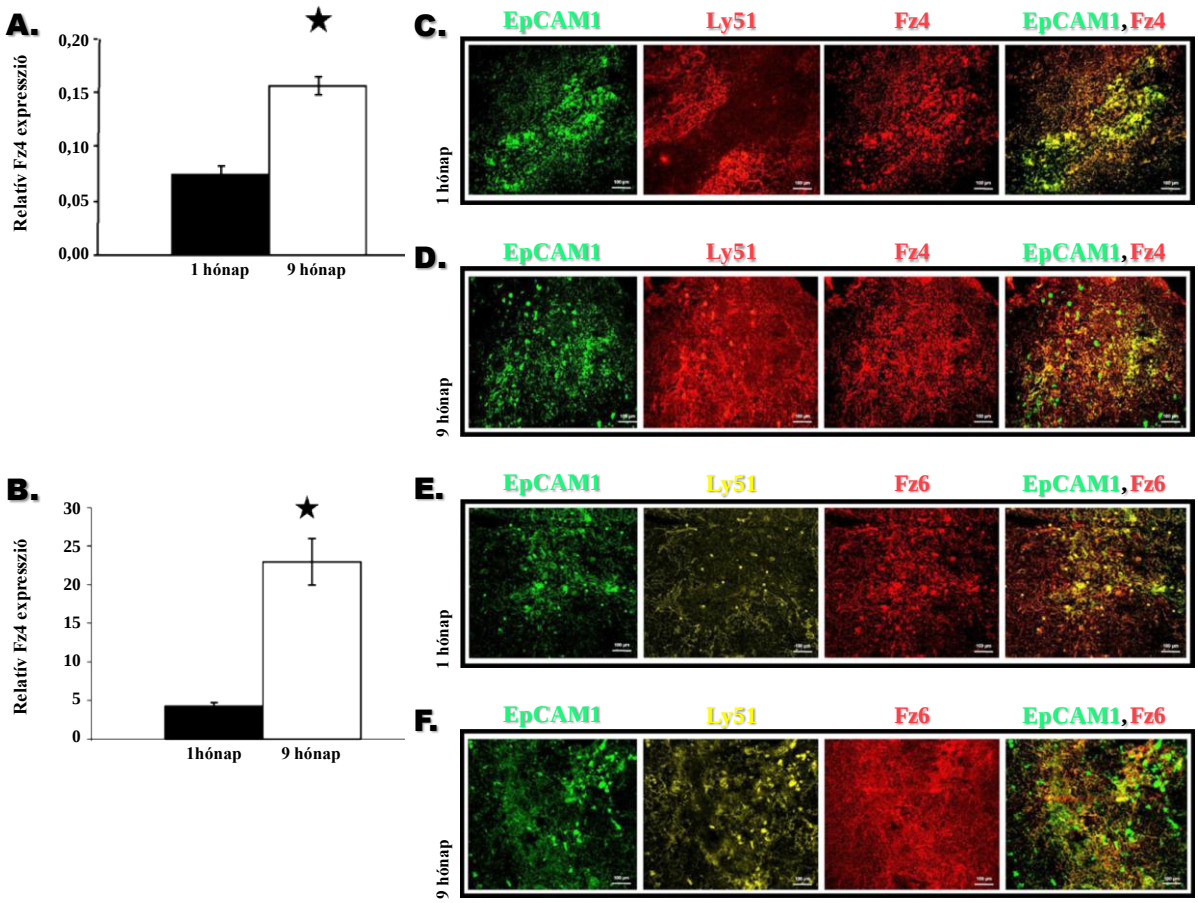


60. ábra: Fiziológiás és GC indukálta öregedési modell (Kvell, Varecza et al. 2010)

A tímusz epitélium öregedési modelljében feltételezzük, hogy az epiteliális sejtekben lezajló változások az epitéliumot érzékennyé teszik az EMT-re, majd ezt követően a kialakuló fibroblaszt-jellegű sejtek már átalakulhatnak adipocitákká.

Mivel a LAP2α által szabályozott elzsírosodási folyamat felismerése a módosított PKC δ aktivitás eredményeként jött létre, a jelátviteli folyamatok vizsgálatára külön figyelmet fordítottunk. Irodalmi adatok alátámasztották, hogy a Wnt4 jelátviteléhez Fz4 és Fz6 receptorok szükségesek (Lyons, Muller et al. 2004). Megállapítottuk, hogy ezekkel a Wnt recoptorokkal, a tímusz epitéliumnak mind a kortex, mind a medulla állománya rendelkezik (Pongracz, Hare et al. 2003). Azt sem hagyhattuk figyelmen kívül, hogy a Fz receptorokból induló jelek átviteléhez PKC δ szükséges, ugyanis PKC δ foszforilálja a Dvl molekulákat a jelátviteli kaszkád megindításához. Kérdéses volt azonban, hogy változik-e ez a folyamat az öregedés során? Illetve, ismerve a PKC δ jelátviteli útvonalakban betöltött változatos szerepét, nem változik-e a PKC δ feladatköre az öregedési folyamatokban.

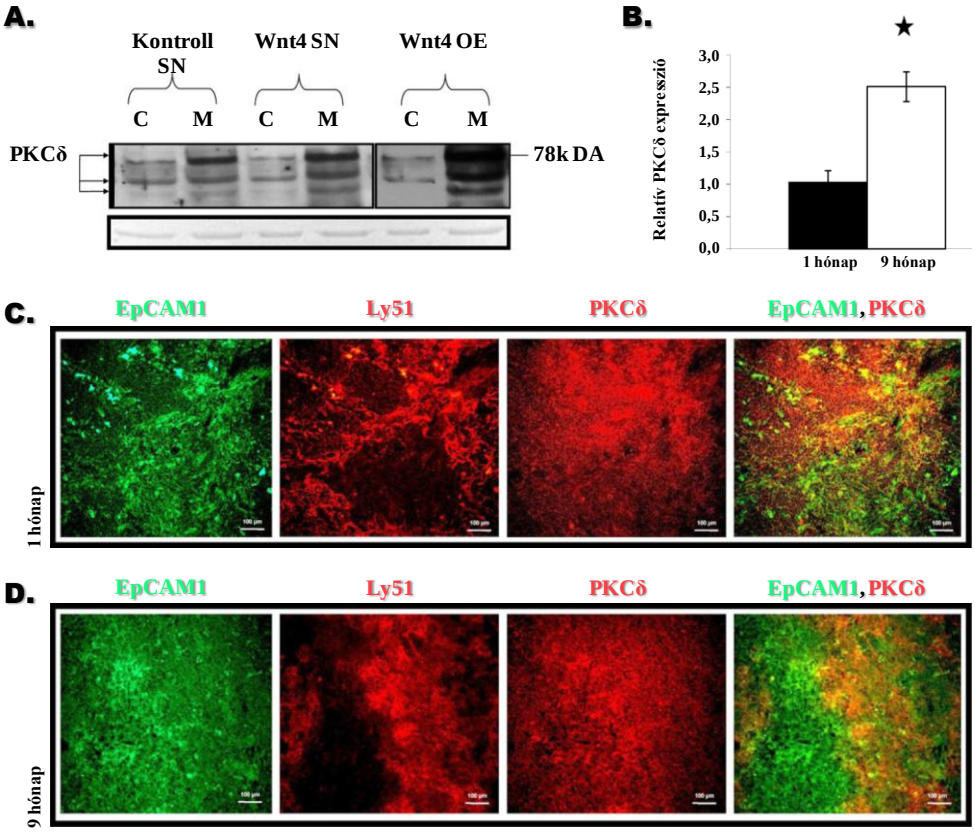
A felmerülő kérdéskör vizsgálatát a Fz4, Fz6 és a PKC δ gének expressziós szintjének vizsgálatával kezdtük korosodó, de még nem kifejezetten öreg egerekben. Azért választottunk fiatalabb, azaz 18 és 12 hónapos egerek helyett 9 hónapos egér tímuszokat, mert a korábbi kísérleteink arra engedtek következtetni, hogy a karakterisztikus molekuláris jellemzők időlegesek és kísérleteinkben nem a végpontot, hanem az ehhez vezető változásokat akartuk behatárolni. Meglepő módon mindhárom gén (Fz4, Fz6 és a PKC δ) emelkedett expressziót mutatott mind mRNS, mind protein szinten az öregedés előrehaladtával (61. és 62. ábra). Azonban, amíg a fiatal tímuszban a Fz4 és Fz6 receptorok elsősorban medulláris lokalizációt mutattak, addig a korosodó állatok tímuszában a kortexben is megnövekedett a receptorok expressziója (61. ábra).



61. ábra: Fz4 és Fz6 receptorok expressziós szintjének módosulása öregedés során (Varecza, Kvell et al. 2011)

(A) és (B) Fz-4 és Fz-6 Q-RT-PCR analízise fiatal (1 hónapos) és korosodó (9 hónapos) tisztított tímusz epiteliumban. Belső kontrollnak β-aktin-t használtunk. A statisztikailag szignifikáns különbségeket csillag jelöli. (C)–(F) Fz-4 és Fz-6 fehérje expresszió és mintázat bemutatása anti-Fz-4-NL663 és anti-Fz-6-NL663 ellenanyagok felhasználásával. A tímusz morfológiáját anti-EpCAM1-FITC és anti-Ly51-PE markerek festésével tettük láthatóvá.

Ellentétben a receptorfehérjék tímusz epiteliumban történő megoszlásának változásával, a PKC δ fehérje a fiatal egerekben elsősorban a kortikális epiteliumban található, míg a kor előrahaladtával az expresszós szint növekszik a medulláris régióban is (62. ábra).

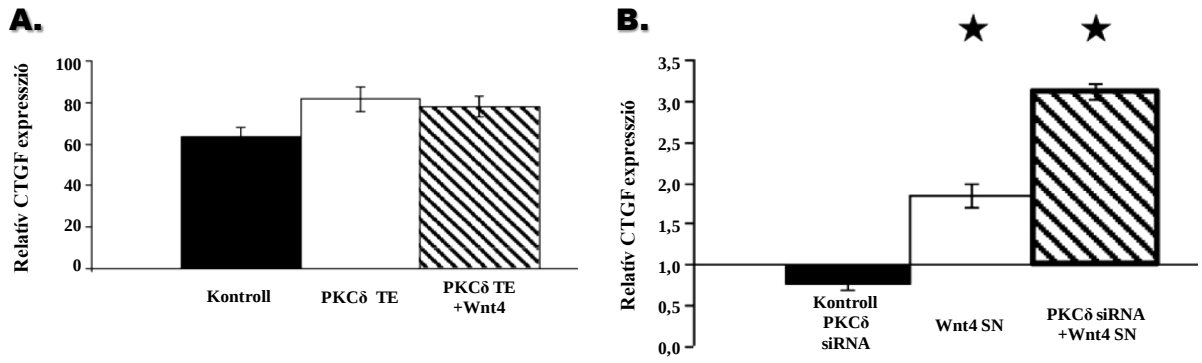


62. ábra: PKC δ Wnt4 jelátvitelben és az öregedés során (Varecza, Kvell et al. 2011)

(A) Wnt4 kezelés hatására a PKC δ citoszolból a membrán frakcióba transzlokálódik, melyet Western blot analízissel mutattunk ki. A kísérletben kontroll, Wnt4 kezelt és Wnt4 túlexpresszáló TEP1 sejtvonalat használtunk. „Ponceau red" festéssel győződtünk meg az felvitt fehérjék egyenlő szintjéről. (B) PKC δ expresszió változásának mérése öregedés során Q-RT-PCR-val. Tímusz epiteliális sejteket fiatal (1 hónapos) és korosodó (9 hónapos) egerekből tisztítottunk, melyekből RNS-t, majd cDNS-t készítettünk. Belső kontrollnak β-aktin-t alkalmaztunk. A statisztikailag szignifikáns különbségeket csillag jelöli. (C) és (D) PKC δ fehérje expressziós változásai a kor előrahaladtával. Tímusz metszeteket fiatal (1 hónapos) és korosodó (9 hónapos) egerekből anti-PKC δ-NL663, anti-EpCAM1-FITC és anti-Ly51-PE ellenanyagokkal festettük.

A kísérleti eredményeink arra engedtek következtetni, hogy a PKC δ fontos szerepet játszik az öregedési jelek transzdukciójában, de arra a kezdeti elemzések nem adtak magyarázatot, hogy milyen szinten kapcsolódik be a PKC δ az öregedési folyamatok szabályozásába. Hogy a Wnt4 és PKC δ közötti kapcsolatot megérthessük, elengedhetetlenné vált a Wnt4 specifikus célgének azonosítása. Kísérleteinkben számos gént azonosítottunk a TEP1 sejtvonalban,

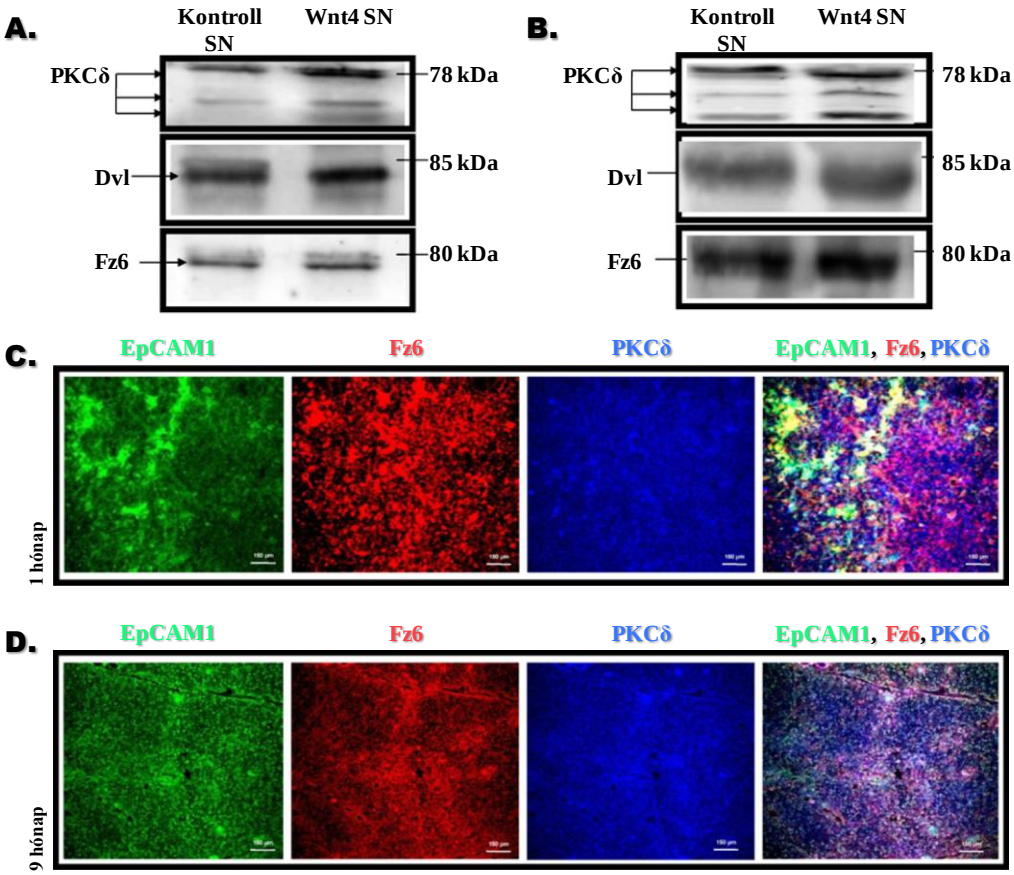
amelyek transzkripciója szignifikánsan nőtt vagy csökkent Wnt4 illetve Wnt7b kezelés hatására. Noha elméletileg a FoxN1 transzkripciós faktor megfelelő lett volna specifikus célgénként, a TEP1 sejtvonal nem expresszál FoxN1-et, míg a primer tímusz epitéliumban a célgének azonosítása ütközött nehézségbe. A célgének azonosítását ezért a TEP1 sejtvonalon végeztük el és az adatok összehasonlító elemzése után végül a „connective tissue growth factor”-t (CTGF) választottuk, mint megbízható célgént. Ez a gén ugyanis Wnt4 kezelés hatására emelkedést mutatott mind a sejtvonalakban, mind a primer tímusz epitéliális sejtekben. Ahhoz, hogy a PKC δ szerepére fényt deríthessünk, a sejtekben a PKC δ enzim aktivitását módosítottuk. Az aktivitás növelése érdekében PKC δ-t túlexpresszáltattuk a TEP1 sejtvonalban. A vad típusú PKC δ gént retrovirális úton juttattuk a sejtekbe. PKC δ expresszió csökkentéséhez viszont kereskedelmi forgalomban is beszerezhető siPKC δ-t használtuk. A megszokott, lineáris jelátviteli gondolatmenetet követve, azt vártuk, hogy mivel Wnt4 hatására aktiválódik a PKC δ, ezért a PKC δ túlexpresszáló sejtekben a Wnt4 célgén szintje emelkedni fog, míg az siPKC δ jelenlétében csökkenni. Ezzel szemben a feltételezett kimenettel ellentétes eredményeket kaptunk (63. ábra). Azaz, a PKC δ túlexpresszáltatása Wnt4 hatására nem növelte a CTGF expresszióját, míg a PKC δ enzim szintjének csökkenése szignifikáns CTGF expresszió növekedéshez vezetett. Az eredmények alapján arra következtettünk, hogy a PKC δ feltehetően egy negatív visszacsatolási rendszer része és a kanonikus Wnt jelátvitel célgénjeinek átíródásához vezető jelátviteli folyamatokban csak sokkal kisebb mértékben vagy egyáltalán nem vesz részt. Ennek azonban ellentmondani látszott az a tény, hogy a receptorból történő jelátvitel megindulásához Dvl foszforilációra van szükség, amely viszont PKC δ–függő folyamat. Hogy ezt a látszólagos ellentmondást feloldjuk, alaposabb vizsgálatnak vetettük alá a Wnt4 receptorok, Fz4 és Fz6, eddig ismert funkcióit és megvizsgáltuk ugyanebből a szempontból az általunk választott Wnt4 specifikus célgén, a CTGF eddig ismert funkcióit is.



63. ábra: CTGF expresszió módosított PKC δ aktivitás jelenlétében

A) CTGF gén expressziójának vizsgálata Q-RT-PCR analízissel kontroll és Wnt4 kezelt PKC δ túlexpresszáló TEP1 sejtvonalban. (B) CTGF gén expressziójának vizsgálata Q-RT-PCR analízissel kontroll és Wnt4 kezelt siPKC δ előkezelt TEP1 sejtvonalban. Belső kontrollnak β-aktin-t alkalmaztunk. A statisztikailag szignifikáns különbségeket csillag jelöli.

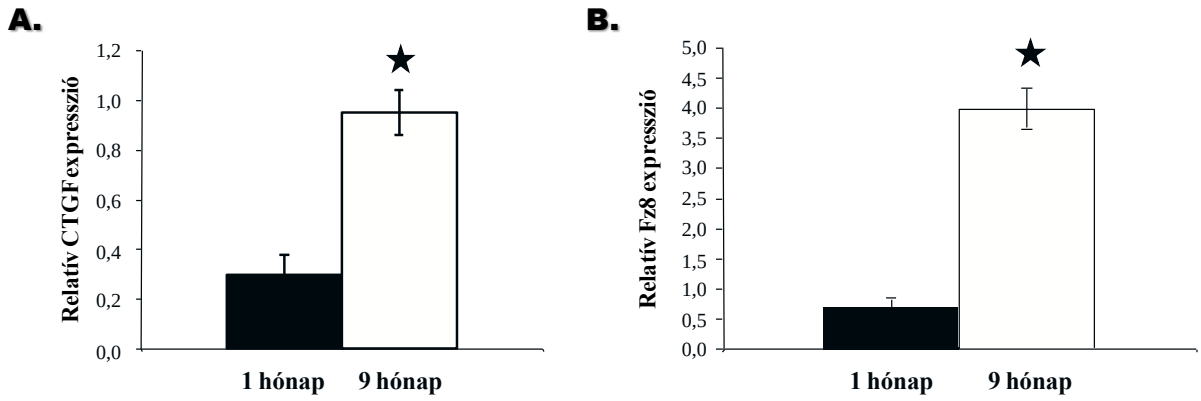
A receptorok immunprecipitációjával kontroll és Wnt4 kezelt sejtekben megvizsgáltuk, hogy a Wnt receptorokhoz kapcsolható-e a jelátvitel szempontjából fontos két molekula, a PKC δ és a Dvl (64. ábra). Míg mindkét receptor esetében kimutatható volt a jelátvitelhez szükséges molekulák jelenléte, addig arra is fény derült, hogy Wnt4 kezelést követően a Fz6 molekulához emelkedett szinten kapcsolódik mind a PKC δ, mind a Dvl. Ebből arra következtettünk, hogy a PKC δ preferenciálisan a Fz6 receptorból származó Wnt4 jelek továbbításához szükséges. Kísérleteink nem zárták ki a Fz4 receptorból történő jelátvitelt, hiszen csak fehérje szinteket mértünk és a molekulák aktivációs szintjét nem analizáltuk. A molekulák jelenléte azonban arra enged következtetni, hogy Wnt4 jelátviteléhez minden molekuláris feltétel adott volt mindkét receptor esetében. A receptorok fiziológiás hatásairól eddig ismert adatokat összehasonlítva, arra is fény derült azonban, hogy a Fz6 receptort gyakran asszociálják (Golan, Yaniv et al. 2004) negatív, kanonikus jelátvitelt gátló jelek transzdukciójával. A Fz6 receptor TAK1, NLK kinázok aktiválásával a TCF1 transzkripciós faktor foszforilációján (Ishitani, Kishida et al. 2003) és ebből eredő gátlásán keresztül akadályozza meg a kanonikus Wnt célgének átíródását. Amennyiben a Fz6 receptor valóban gátló receptorként funkcionál a tímusz epitélium Wnt4 jelátvitele tekintetében, akkor a célgén átíródása feltehetően a Fz4-ből származó jelátviteli kaszkád aktiválásának köszönhető. Ezzel is alátámasztva azt a feltételezést, hogy az immunprecipitációval kimutatott Fz-Dvl-PKC δ komplex jó indikátora a Fz4 és a Fz6 receptor aktivitásának.



64. ábra: Fz4, Fz6, Dvl és PKC δ ko-lokalizációjának analízise (Varecza, Kvell et al. 2011)

(A) és (B) Western blot analízis anti-Fz6 (A) és anti-Fz4-ellenanyaggal (B) történő immunoprecipitációt követően. Wnt4 kezelést követően a PKC δ fokozottan asszociálódik a Fz-6 receptorhoz kapcsolódó Dvl molekulával. (C) és (D) 1 és 9 hónapos egerek tímuszából készült metszeteken anti-PKCδ–NL663, anti-Fz-6-NL557 és anti-EpCAM1-FITC ellenanyagokkal készült festés látható. A három festés egymásra rétegzését az utolsó kép ábrázolja.

Az irodalmi adatok alapján arra is fény derült, hogy a megszokott funkcióin és ligand-receptor kapcsolatán túlmenően, a CTGF a Fz8 receptorhoz is képes kapcsolódni (Luo, Kang et al. 2004). A Fz8 receptorból származó jelek viszont a GSK3β enzim aktiválásához és ezzel a β-katenin proteoszomális degradációjához vezetnek. Ennek következménye a kanonikus Wnt jelátviteli rendszer gátlása. Az eddigi eredményeink tükrében megvizsgáltuk a CTGF és Fz8 gének expressziós szintjének változását is az öregedés során (65. ábra).



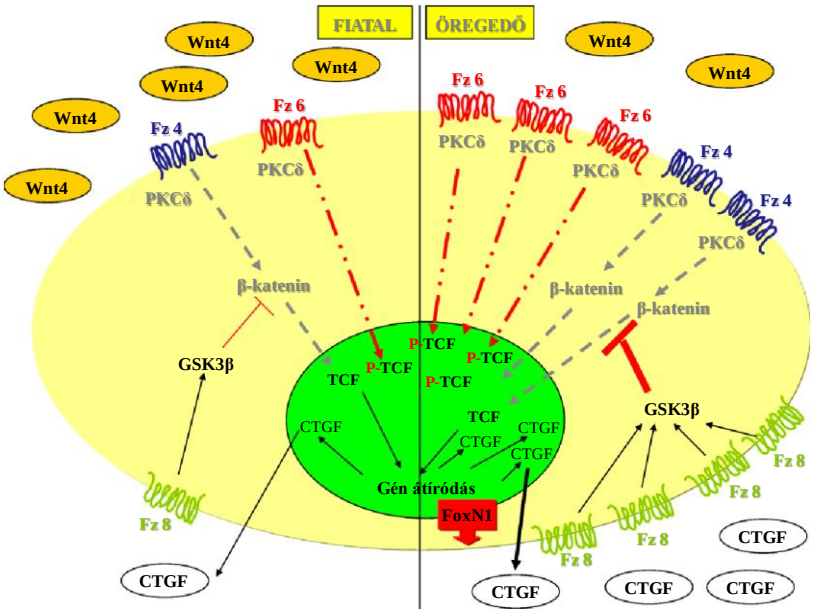
65. ábra: CTGF és Fz8 receptor expresszió változása öregedés során
(Varecza, Kvell et al. 2011)

(A) és (B) CTGF és Fz8 gén expressziójának vizsgálata Q-RT-PCR analízissel 1 és 9 hónapos egerek tímuszából tisztított tímusz epitéliumban. Belső kontrollnak β-aktint alkalmaztunk. A statisztikailag szignifikáns különbségeket csillag jelöli.

Az eredmények alátámasztani látszottak a kezdeti feltételezéseinket. Kezdetben az öregedés során a Fz4 receptor szintje nő és ezzel párhuzamosan megnövekszik a Wnt4 célgének átíródása. E mellett növekszik a negatív visszacsatolásban résztvevő jelátviteli molekulák jelenléte is a rendszerben, gátolva ezzel a kanonikus Wnt jelátvitelt. Ennek eredményeként hiába erősödik a kanonikus Wnt jelek extracelluláris jelenléte, az intracelluláris szabályozás nem engedi a kanonikus Wnt jelek által indukált proliferációs és egyéb, a sejtek túléléséért felelős gének átíródását. Ezzel a tímusz epiteliális őssejtek proliferációs képessége, végül száma is lecsökken, amely az öregedő epiteliális hálózat regenerációjának gátlásához vezet.

A jelátviteli rendszerek összegzéseképpen (66. ábra) megállapíthatjuk, hogy az öregedési folyamat félidejében, amikor még a Wnt4 expresszió csökkenése nem kifejezett, megindul a Wnt4 receptorok expressziós szintjének növekedése. Ez a folyamat nem csak a korábban ezeket a receptorokat expresszáló epiteliális alcsoportokra jellemző, hanem a tímusz epiteliumra általában. A megnövekedett receptorexpresszióból származó jelátvitel megindulása értelemszerűen növeli a kanonikus célgének átíródását. Ennek a folyamatnak azonban a lineáris logika ellentéte az eredménye, hiszen a célgének közül a negatív visszacsatolási rendszerben játszik szerepet például a CTGF, amely szekrécióját követően a Fz8 receptorhoz is kapcsolódhat. Feltehetően egy PKC ζ indukálta mechanizmuson keresztül aktiválja a GSK3β enzimet, amely a β-katenin molekulát foszforilálja, és így a β-katenin érzékennyé válik a proteoszomális degradációra. Ezzel csökken a β-katenin szint, amellyel a kanonikus jelátvitel célgénjeinek átíródása is csökken. Ezzel párhuzamosan erősödnek a Fz6

gátló receptorból származó jelek is. Ez a folyamat annak a következménye, hogy a megnövekedett receptor expresszióhoz megnövekedett PKC δ szint társul, amely preferenciálisan a Fz6 receptorhoz kapcsolódik, növelve ezzel a negatív jelek továbbításának arányát. Így a kanonikus Wnt jelátvitel két úton is gátlás alá kerül: a β-katenin szint csökkenésével és a TCF transzkripciós faktor foszforilációjával. Így, ha a β-katenin be is jut a magba, az aktív transzkripciós komplex összeállásának esélye rohamosan csökken, hiszen a TCF foszforilált állapotban nem képes a β-kateninhez kötődni. Ennek a jelátviteli folyamatnak a következménye, hogy a kanonikus jelátviteli jelekre érzékeny FoxN1 transzkripciós faktor átíródása drasztikusan lecsökken. A lecsökkent FoxN1 szint lehetővé teszi az epiteliális sejtek dedifferenciációját, majd fibroblaszt-jellegű átalakulását. Az epiteliális-mezenhimális tranzíción átesett sejtekben, ahol a zsírsejt irányú átalakulást szabályozó molekulák (LAP2α, PPARγ, ADRP) szintje növekszik, az adipoid transzdifferenciáció már visszafordíthatatlanná válik.



66. ábra: A tímusz öregedésének molekuláris modellje (Varecza, Kvell et al. 2011)

Fiatal tímuszban a Wnt4 szint magas, és mindkét receptora, a Fz4 és a Fz6 receptorok is közel azonos szinten expresszálódnak. Az öregedés megindulásával csökkenni kezd a Wnt-4 expresszió és növekszik a receptor, különösen a gátló receptor, Fz6 expresszió, amelynek kapcsán a kanonikus Wnt jelátvitelt negatívan befolyásoló folyamatok indulnak meg. Ezek hatására csökken a FoxN1 szint, ami először EMT-hez, majd adipoid transzdifferenciációhoz vezet.

6. ÖSSZEFOGLALÁS

A hagyományos kísérleti megközelítési módszerek a jelátviteli folyamatok fizilógiás hatásainak felderítésére egyre kevéssé alkalmazhatók. A szervezetben lezajló jelátviteli folyamatok nem érthetők meg ugyanis kizárólag sejtvonalakon végzett kutatásokkal, hiszen minden fiziológiás válaszreakció a szövetek és sejtek reciprokális egymásra hatásának következtében keltett, receptor-ligand kapcsolatból származó intracelluláris jelek eredőjeként jön létre. Ezért az immunrendszer speciális sejtjeinek perifériális aktivációja, avagy fejlődése közben végbemenő osztódási vagy differenciálódási, illetve öregedési jelátviteli folyamatairól csak komplex szöveti rendszerek felállítása mellett kaphatunk átfogó képet. A komplex szöveti rendszereken túlmenően nagyon fontos a primer sejtek alkalmazása is, hiszen a fiziológiás folyamatokról csak primer szövetek sejtjei szolgáltatnak megbízható adatokat. Az általános jelátviteli folyamatok megismerésén túlmenően a szövetspecifikus jelátviteli rendszerek megismerése igen nagy fontossággal bír, hiszen terápiás célzattal csak a jól ismert folyamatok manipulálhatóak biztonsággal.

Ezért munkám legnagyobb értékének tartom, hogy a jelátviteli folyamatokat primer sejtekből álló szöveti modellekben, illetve *ex vivo* kultúrákban végeztem. A szöveti modellezés lehetővé tette számos egyidejű jel eredőjének fiziológiai hatásvizsgálatát, mely kiemelkedő fontosságú összefüggések felismeréséhez vezetett:

1. Kollégáimmal megállapítottuk, hogy pusztán a PKC család tagjainak manipulálása nem lehet hatékony terápiás célpont, hiszen az enzimek közötti hasonlóság és funkcionális redundancia nem teszi ezt lehetővé. Arra is rávilágítottunk, hogy egyedi PKC molekulák számos, akár ellentétes előjelű jelátviteli folyamatban párhuzamosan is részt vehetnek, ezért funkcióikat csak a jelátviteli rendszerek eredőjeként értelmezhetjük.

2. A fejlődést szabályozó fő jelátviteli családok vizsgálatával demonstráltuk, hogy a Notch és Wnt jelátviteli hálózatok és a köztük fellépő interakciók fontos szerepet játszanak a tímusz mikrokörnyezetének kialakításában és a T-sejtek tímuszon belüli fejlődésének szabályozásában.

3. Kimutattuk, hogy a fejlődő timociták TCR és MHC-függő szelekciós jelei ko-receptorok és sejten belüli jelátviteli folyamatok eredményeként módosítja a különféle TCR-ral rendelkező T-sejtek túlélési folyamatait. A sejtes interakciók megértésére új eljárást dolgoztunk ki a

tímusz epitélium sejtfelszíni molekula-repertoárjának módosítására. Ehhez rekombináns retro- és adenovírusokat használtunk. Rámutattunk, hogy a környezeti tényezők illetve receptor-ligand interakciók megváltozása vezethet a TCR-ből származó „high avidity" azaz apoptotikus jelek módosításához, amely végül a perifériás TCR repertoár összetételét meghatározza.

4. Úttörő jellegű kutatásokkal kimutattuk, hogy a korábbi feltételezésekkel ellentétben az öregedő tímuszban nem az epitéliális sejtek apoptotikus pusztulása és az epitélium helyére migráló zsírsejtek okozzák a tímusz adipoid atrófiáját, hanem azok a speciális molekuláris változások, amelyek lehetővé teszik a tímusz epitélium de-differenciációját, epitéliális-mezenhimális tranzícióját és zsírsejtekké történő átalakulását. Ebben a folyamatban a Wnt4 szintek csökkenése fontos szerepet tölt be és Wnt4 expresszió szinten tartásával megakadályozható a tímusz involúcióra jellemző molekuláris változások megindulása.

Kísérleteink eredményei kapcsán a tímusz öregedésére vonatkozó általános dogmák váltak megkérdőjelezhetővé. A kísérletekkel is alátámasztott következtetéseink teljesen új alapokra helyezik a tímusz adipoid atrófiájának molekuláris hátterét. A kutatásainkban azonosított folyamatok olyan komplex jelátviteli szabályozás alatt állnak, amelyet más kutatócsoportoknak még nem sikerült azonosítaniuk. Ennek megfelelően a mi munkánk úttörő jellegű nem csak a tímusz, de az egész immunrendszer öregedésének megértése szempontjából is.

7. AZ EREDMÉNYEK GYAKORLATI JELENTŐSÉGE

Az immunrendszer jelátviteli folyamatainak megértése nagy jelentőséggel bír, hiszen az egészséges szervezetben lezajló fiziológiás folyamatok leírása képezi az alapját minden, a betegségekben megváltozott jelátviteli szabályozás megismerésének, terápiás megközelítési módok kidolgozásának, illetve farmakológiai célpontok azonosításának és gyógyszerhatóanyagok kifejlesztésének.

Az immunológiai folyamatok megértésére tett erőfeszítések különösen fontosak az öregedés szempontjából. Nem csak Magyarország, de az egész fejlett világ népessége rohamosan öregszik, melynek az egyénre és a társadalomra nehezedő anyagi terhei a megnövekedett egészségügyi költségek terén is jelentkeznek. Az immunrendszer öregedésének lassítása, az idősek hatékonyabb immunizálhatóságát tenné lehetővé, és párhuzamosan növelné az immunológiai válaszkészséget. Ezzel csökkenthetővé válna a fertőző betegségek terjedése, súlyos daganatos és autoimmun kórképek kialakulása, melynek közvetlen következménye a költséges kórházi kezelést és ápolást igénylő betegek számának csökkenése is.

Már a jelátviteli alapfolyamatok, mint például az enzimaktivitás módosítási lehetőségeinek megismerése az immunrendszerben közvetlenül hasznosítható eredményekhez vezetett. A PKC β aktivitását gátló 13-HODE-ra, mint természetes metabolikus termékre vonatkozó kísérleti eredményeink, pl. a vaszkuláris biokompatibilitás szabályozására benyújtott amerikai szabadalom igénypontjait támasztotta alá (Van Gorp, Buchanan et al. 1999).

A T-sejtek fejlődési lépéseinek megismerése mellett a sejttípusok egymásba való átalakíthatóságának jelentőségét sem szabad alábecsülni. Az öregedés kapcsán további jelátviteli vizsgálatainkban az epitéliális-mezenhimális tranzíció fordítottjának, a mezenhimális-epitéliális átalakulás szabályozásának molekuláris hátterét kívánjuk felderíteni. Ehhez a saját kísérleteinkben felismert szabályozó molekulákra koncentrálunk elsősorban, melyhez sikerült megnyernünk magyar és finn kis és középvállalatokat is. Elsősorban a Wnt4 molekula tímusz epitéliumra kifejtett protektív hatását vizsgáljuk tovább, illetve olyan fehérje interakciók kidolgozására igyekszünk koncetrálni, amely a LAP2α adipoid transzdifferenciációt indukáló hatását csökkenti. A terápiás célpontok meghatározása nem csak az immunrendszer fiziológiás öregedésének sebességét csökkenthetné, hanem reményt

adhat krónikus betegségek szteroid kezelése következtében felgyorsult öregedési folyamatok lassítására vagy visszafordítására is.

A kísérletek kivitelezéséhez használt komplex szöveti rendszerek kidolgozása kapcsán a „tissue engineering” területén is hasznosítható újításokat eredményeztek munkáink. Kísérleteink alátámasztották, hogy az általunk kidolgozott kísérleti rendszerek és a szövetek közötti interakciót szabályozó fő jelátviteli családok szervezett együttműködése magasan szervezett szövetek előállítását teszi lehetővé *in vitro* körülmények között. Az így előállított szöveti rendszerek mind jelátviteli kísérletek kivitelezésére, mind gyógyszerek tesztelésére, mind transzplantáció-kész szövetek előállítására alkalmasak. Ezekkel az eredményekkel kapcsolatosan jelenleg három szabadalmi beadvány és négy kutatási cikk várja a bírálók döntését.

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9.2. Az értekezés alapjául szolgáló közlemények

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Az első és utolsó szerzős közlemények összesített impakt faktora: 45,286

Az összes közlemény összesített impakt faktora: 110,182

9.5. Könyvek, könyvfejezetek

9.5.1. Tankönyv

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10. AZ ÉRTEKEZÉS ALAPJÁUL SZOLGÁLÓ KÖZLEMÉNYEK KÜLÖNLENYOMATAI

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Multiple suppression pathways of canonical Wnt signalling control thymic epithelial senescence

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ABSTRACT

Members of the Wnt family of secreted glyco-lipo-proteins affect intrathymic T-cell development and are abundantly secreted by thymic epithelial cells (TECs) that create the specific microenvironment for thymocytes to develop into mature T-cells. During ageing, Wnt expression declines allowing adipoid involution of the thymic epithelium leading to reduced naïve T-cell output. The protein kinase C (PKC) family of serine-threonine kinases is involved in numerous intracellular biochemical processes, including Wnt signal transduction. In the present study, PKCδ expression is shown to increase with age and to co-localise with Wnt receptors Frizzled (Fz)-4 and -6. It is also demonstrated that connective tissue growth factor (CTGF) is a Wnt-4 target gene and is potentially involved in a negative feed-back loop of Wnt signal regulation. Down-regulation of Wnt-4 expression and activation of multiple repressor pathways suppressing β-catenin dependent signalling in TECs contribute to the initiation of thymic senescence.

1. Introduction

During ageing of the immune system the gradual loss of naïve T-cells is associated with the rate of thymic adipose involution that correlates with significant destruction of the epithelial network. As impaired T-cell production leads to weakened immune responses, understanding the mechanism of thymic involution has high physiological and medical importance.

In our recent studies of thymic involution Wnt-4 secretion was significantly reduced in TECs while LAP2α expression concomitantly increased triggering epithelial–mesenchymal transition (EMT) and then pre-adipocyte-differentiation (Kvell et al., 2010).

As Wnt-4 is the primary regulator of FoxN1 expression and consequently TEC identity, understanding Wnt-4 signalling carries particularly high importance (Balciunaite et al., 2002). The difficulty of signalling studies, however, stems from the general complexity of Wnt pathways (Kuhl and Pandur, 2009). Wnt-4, for example, has been described as activator of both β-catenin dependent canonical (Lyons et al., 2004) and JNK/PKC dependent non-canonical (Cai et al., 2002; Du et al., 1995) signalling pathways that interact at multiple levels. Apart from specific, there are also shared signalling elements in Wnt pathways including the main cell surface receptors Frizzleds (Fz) (Schulte and Bryja, 2007) as well as intracellular signalling molecules including G proteins (Malbon et al., 2001), Dishevelleds (Dvl) (Kuhl et al., 2001; Schulte and Bryja, 2007) and PKCs α (Kuhl et al., 2001), ζ (Ossipova et al., 2003), and δ (Kinoshita et al., 2003). PKCδ appears particularly important as this serine-threonin kinase can phosphorylate and therefore activate Dvls (Kinoshita et al., 2003) to relay ligand induced signals towards down-stream elements of Wnt cascades.

From the ten known mammalian Fz receptors, Fz-4 (Lyons et al., 2004) and Fz-6 (Lyons et al., 2004) have been confirmed to bind Wnt-4. Interestingly, while Fz-4 is an activator of the β-catenin dependent canonical pathway, signals from Fz-6 inhibit β-catenin dependent target gene transcription (Golan et al., 2004) indicating that regulation of Wnt-4 signalling might also begin at receptor level in the thymus.

As thymic involution is a complex physiological process and appears to be initiated by suppression of Wnt signals, understanding of receptor associated regulatory mechanisms can lead to target molecule recognition in the quest for re-juvenate the ageing thymus. To investigate the hypothesis, TECs of young and ageing adult Balb/c mice as well as a thymic epithelial cell line, TEP1 were used in the studies. Our experiments demonstrate that expression of Wnt receptors increase with age and that Frizzleds co-localize

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with signalling molecules PKCδ and Dvl. Examination of Wnt-4 target gene expression provides evidence for the existence of additional negative regulatory loops suppressing β-catenin dependent signalling that aids repression of thymic epithelial maintenance and provides opportunities for EMT and consequent adipocyte type differentiation.

2. Materials and methods

2.1. Cell culture

Tep1 (thymic epithelial) (Tanaka et al., 1993), 293 and Phoenix (PHX) human kidney epithelial cell lines were cultured in DMEM (Sigma–Aldrich) supplemented with 10% FCS and 100 μg of penicillin and streptomycin (Lonza Walkersville, Inc.).

2.2. Primary thymic epithelial cells

Balb/c mouse thymi (1 and 9 months) were the source of primary cell material. $CD45^-$ $EpCAM1^+$ TECs were isolated using magnetic cell sorting (Miltenyi Biotech) (purity was regularly above 90% – data not shown). Purified $EpCAM1^+$ TECs were cultured in medium alone, or in medium supplemented with Wnt4.

2.3. Antibodies

For western blot analysis rabbit polyclonal anti-PKCδ (C-17), anti-Dvl(C-19) (Santa Cruz), anti-Fz-6 and anti-Fz-4 (R&D systems Inc.) antibodies were used as primary and HRP-conjugated anti-rabbit and anti-goat (Santa Cruz) were used as secondary antibodies. For fluorescent microscopy studies primary Abs were anti-PKCδ (658-676) pAb (Calbiochem) (1:100), anti-PKCδ (C-17) (Santa Cruz) (1:100), anti-Fz-4, anti-Fz-6 (R&D systems Inc.) (1:100) and FITC labelled anti-EpCAM1 (clone G8.8) (American Type Cell Culture Collection)(1:50) and anti-Ly51-PE (BD Pharmingen) (1:50) antibodies. Secondary antibodies were NorthernLights donkey anti-goat IgG-NL493 and NorthernLights donkey anti-rat IgG-NL557 and anti-rat and anti-rabbit IgG-NL663 (all from R&D Systems Inc.). (Dilution factor for all secondary antibodies was 1:200).

2.4. Histology using fluorescent antibodies

Frozen thymic sections (9 μm thick) were fixed in cold acetone for 10 min, then dried for 15 min and rehydrated and blocked using 5% bovine serum albumin (BSA in PBS for 20 min) before staining with the appropriate antibodies. The primary antibody was applied at appropriate dilution in 100 μl on all sections for 30 min followed by 3 washing steps with PBS for 5 min each. Secondary Ab was applied for 30 min followed by 3 × 5 min wash with PBS as above. PBS-glycerol 1:1 mix was applied before covering with slide covers. The sections were analysed by an Olympus BX-61 Fluorescent microscope or by Olympus Fluoview 300 confocal microscope using the Olympus Fluoview FV1000S-IX81 software.

Staining controls were the following: primary Ab with no secondary Ab, no primary just secondary Ab and irrelevant primary Ab for isotype control in combination with secondary Ab. All the stainings were repeated for a minimum of three times.

2.5. Subcloning of Wnt-4 and full length PKCδ

Wnt-4 was purchased and subcloned from a commercially available vector (Origene), while the full length PKCδ was a kind gift of Jae-Won Soh, Tnha University, Korea. Both Wnt-4 and PKCδ sequences were subcloned into the MIGRI retroviral vector (gift from W.S. Pear, Department of Pathology and Laboratory Medicine, University of Pennsylvania, PA). Retrovirus was produced by transfecting the plasmid DNA into the Phoenix packaging cell line (American Type Cell Culture Collection) using Lipofectamine 2000 (Invitrogen).

2.6. Transient transfection of siRNA PKCδ

siRNA specific for PKCδ was supplied by Santa Cruz. Tep1 cells were grown to 80% confluency and then siRNA and control siRNA was delivered using Lipofectamine according to manufacturer's instruction.

2.7. Cell sorting

Tep1 cells were infected with recombinant retroviruses encoding GFP, Wnt-4-GFP or wild type- PKCδ-GFP then sorted based on GFP expression by FACSVantage Cell Sorter (BD). GFP positive cells were cultured further under conditions described in Section 2.1.

2.8. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (Q-RT-PCR)

RT-PCR was conducted as described previously (Kvell et al., 2010). Q-RT-PCR was performed using SYBR Green Q-RT-PCR reagents and random hexamer primers (Applied Biosystems) as recommended by the manufacturer using an ABI Prism 7900HT sequence detection system. Threshold cycles (C_T) for three replicate reactions were determined using Sequence Detection System software (version 2.2.2), and relative transcript abundance was calculated following normalization with a β-actin PCR amplicon. Quantitation of Q-RT-PCR products were based on a standard curve generated from untreated TEP1 cell line gene expression. PCR primer sequences are listed in Table 1.

2.9. PKCδ activation assay

Tep1 cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Sigma–Aldrich) and immunoprecipitated with rabbit anti-PKCδ (658-676) pAb (Calbiochem) and protein G resin (Sigma–Aldrich) overnight at 4 °C. Kinase assay was performed using an HTScan PKCδ Kinase–assay Kit (Cell Signaling Technology Inc.) with biotinylated substrate peptide in the presence of diluted PKCδ. Active PKCδ kinase GST fusion protein was supplied to the kit as positive control. PKCδ specific activity was quantified in a colorimetric ELISA Assay using 96-well streptavidin-coated plates (Institute of Isotopes, Budapest, and Soft Flow Hungary Ltd., Hungary). Phosphorylation level of biotynilated substrates from each kinase reaction mix were measured using a rabbit anti phosphoSer/Thr-antibody (1:1000) (provided with the kit) detected by a HRP-labelled anti-rabbit (1:1000) (Santa Cruz) in the presence of TMB substrate. Optical density (absorbance) was read in an iEMS Reader MF V2.9 (Thermo Scientific, Waltham, MA) spectrophotometer using a bi-chromatic measurement system at 450 nm and 620 nm as reference.

2.10. Purification of proteins from cell membrane and cytosol

Tep1 cells (1×10^6/condition) were treated with Wnt-4 and control supernatants for 30 min then cells were pelletted and cytosolic and membrane proteins were isolated as described previously (McMillan et al., 2003). Proteins of cytosolic and membrane fractions were separated in 10%SDS PAGE, blotted, blocked in 3% fat-free milk and probed for PKCδ protein. To ensure equal loading protein levels were visualised by Ponceau Red staining, when proteins were entering the separating gel.

2.11. Immunoprecipitation and Western blotting

Cell lysates were immunoprecipitated using anti-Fz-4 and anti-Fz-6 antibodies (R&D systems Inc.) and protein G resin (Sigma–Aldrich) in the presence of protease and phosphatase inhibitors (Sigma–Aldrich). Proteins were resolved in 10% SDS–PAGE, blotted onto nitrocellulose membranes, then blocked in buffer containing 3% fat-free dried milk and probed for the proteins of interest with primary then in the appropriate HRP-conjugated secondary antibodies. Proteins were visualized by enhanced chemiluminescence (Pierce) according to the manufacturer's instructions in a FUJI LAS4000 image station.

Table 1
PCR primers.

Gene	Accession nos.	Forward primer	Reverse primer
β-actin	NM_007393	TGG CGC TTT TGA CTC AGG A	GGG AGG GTG AGG GAC TTC C
Wnt-4 cloning primers	NM_030761	gaagatcttc ATGAGTCCCCGCTCGTGC	ccgctcgagcgg TCATCGGCACGTGTGCAA
Wnt-4 PCR primers	NM_030761	CTC AAA GGC CTG ATC CAG AG	TCA CAG CCA CAC TTC TCC AG
CTGF	NM_010217	GGCCTCTTCTGCGATTTCG	CCATCTTTGGCAGTGCACACT
PKC-δ PCR primers	NM_011103	AGGCCGTGTTATCCAGATTG	CGGTTCATGGTTGGAAACTT
Frizzled 4	NM_008055	TCTGCTTCATCTCCACCACCTT	GCGCTCAGGGTAAGAAAACCT
Frizzled 6	NM_008056	GCGGCGTTTGCTTCGTT	CACAGAGGCAGAAGGACGAAGT
Frizzled 8	NM_008058	TTCCGAATCCGTTCAGTCATC	GCGGATCATGAGTTTTTCTAGCTT

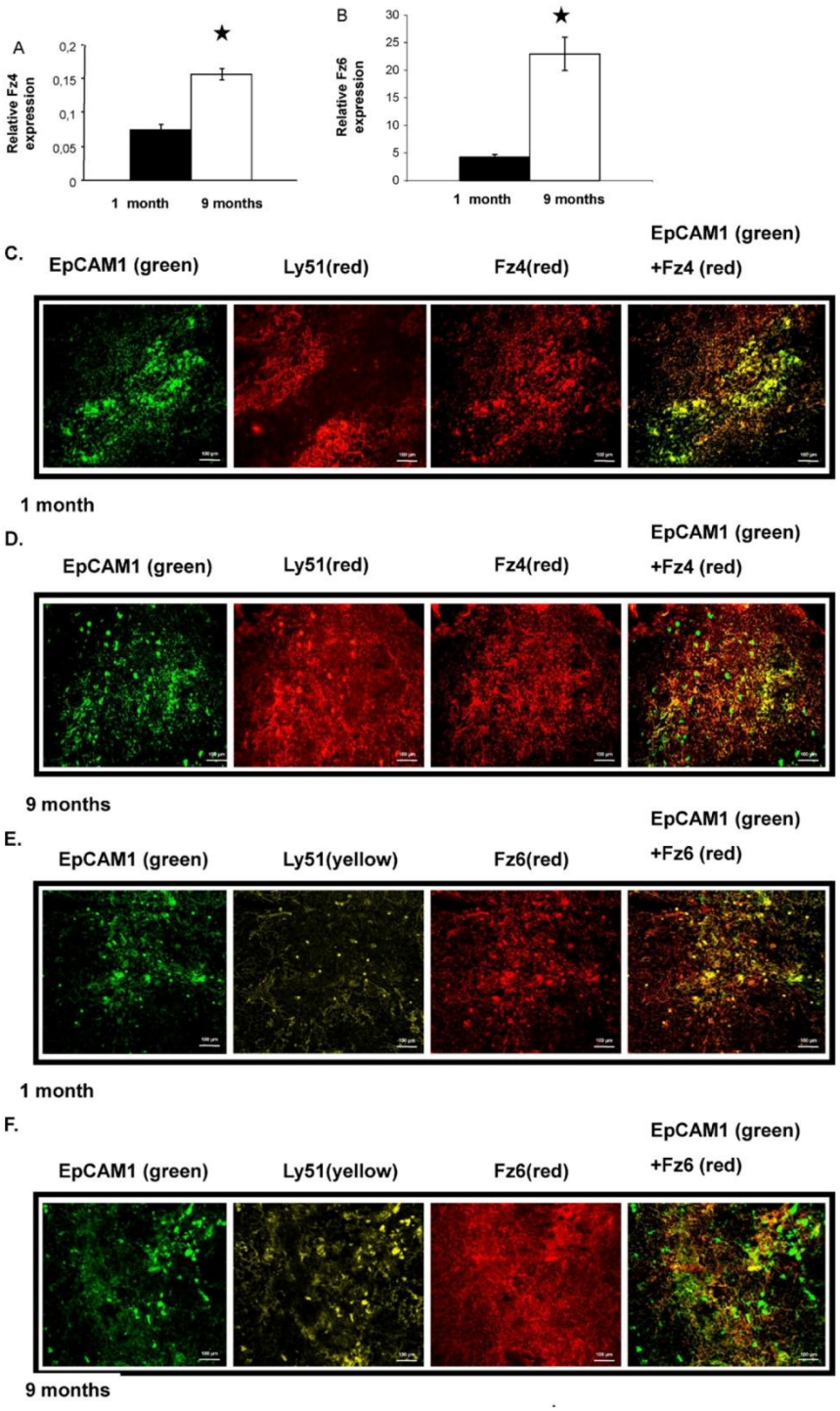

Fig. 1. Fz-4 and Fz-6 expression during thymic senescence. (A and B) Q-RT-PCR analysis of Fz-4 and Fz-6 expression in young (1month) and ageing (9 months) mouse thymic epithelium. Data was normalised to β-actin. Statistically significant differences are marked by asterisks. (C–F) The expression level and staining pattern of Fz-4 and Fz-6 was also assayed by histology using anti-Fz-4-NL663 and anti-Fz-6-NL663 antibodies, respectively. Thymic morphology was displayed via staining with anti-EpCAM1-FITC and anti-Ly51-PE TEC markers. Size marker is shown in the corner of the figure. Characteristic stainings are shown from a minimum of five repeats.

2.12. Microarray

Tep 1 cells were washed for one hour in FCS free medium then the cells were treated for 1 hr with Tep1 supernatants of control and Wnt4 over-expressing Tep1 cell lines, respectively. Following incubation cells were collected, RNA was purified, then mRNA were amplified and microarrays were performed in the Centre for Genomics, University of Debrecen, Hungary. Gene expression changes beyond 0.5x/2-fold cut off criteria were accepted. Genes demonstrating the largest fold differences were validated by qPCR then tested in an additional three independent experiments. A list of genes upregulated in the microarray experiment and selected for further analysis can be viewed in Supplementary Table 1s. In the present study CTGF (Connective Tissue Growth Factor) was selected as a read-out gene, as it reproducibly changed expression levels in additional experiments.

2.13. Statistics

Experiments were repeated at least three times and statistical significance was determined using the Student's t-test. $P < 0.05$ denoted statistically significant.

3. Results

3.1. Fz-4 and Fz-6 levels are affected by age

Expression levels of Wnt-4 receptors, Fz-4 and Fz-6 were analysed in thymi of 1 month and 9 month old mice. The two age groups were selected specifically, as 1 month old mice are considered to be young adults where thymic involution has not yet begun. At the age of 9 months age associated morphological changes are not substantial yet, but characteristic age associated molecular and morphological changes can already be detected. This is not surprising as the detection of small lipid droplet-expressing cells in the perivascular space of *FoxN1Cre mice* has recently been reported as early as 3 months of age (Youm et al., 2009). Q-RT-PCR analysis of purified EpCAM1$^+$ TECs of 1 and 9 month old mouse thymi showed increased expression of Fz-4 and Fz-6 mRNA (Fig. 1a and b) by 9 months of age. Immunohistochem-

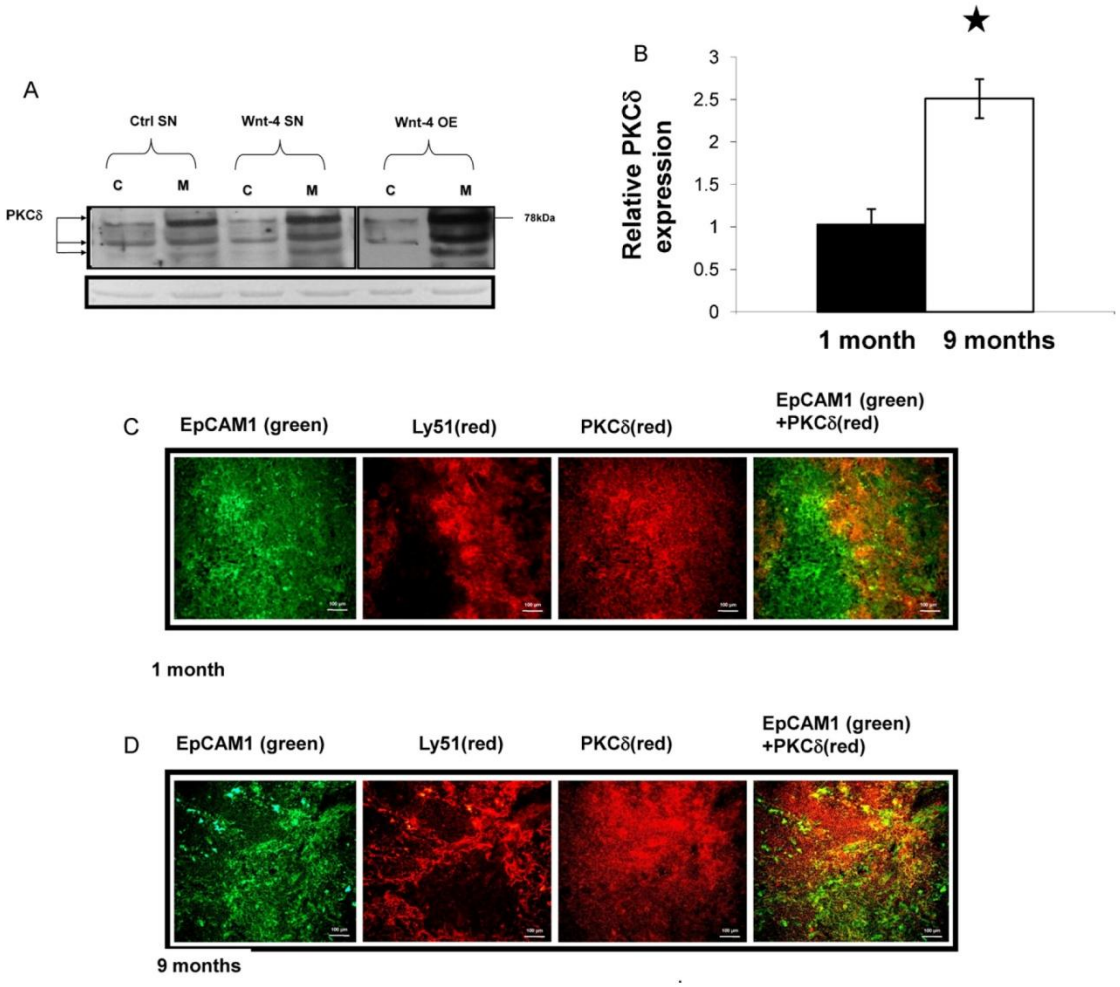

Fig. 2. (A) Intracellular translocation of PKCδ following Wnt-4 treatment. Cytosolic and membrane proteins were separated from control, Wnt-4 treated and Wnt-4 overexpressing Tep1 cells. Western blot analysis demonstrated PKCδ translocation. Loading controls are shown below the Western blot as Ponceau red stained total protein. Representative blots and stainings are shown from three repeats. (B) Changes of PKCδ expression with age by Q-RT-PCR. TECs were purified from young (1 month) and ageing (9 months) mice. Data were normalized to β-actin housekeeping gene. Statistically significant differences are marked by asterisks. (C and D) Age associated changes in PKCδ expression by histology. Cryostate sections of 1 month and 9 months old mouse thymi were stained with anti- PKCδ– NL663, anti-EpCAM1-FITC and anti-Ly51-PE. The overlay of the staining pattern is also shown. Characteristic stainings are shown from a minimum of five repeats.

istry using Fz-4 and Fz-6 specific antibodies confirmed elevated levels of both receptor proteins (Fig. 1c–f) in ageing thymi. Additionally, differential expression pattern of Fz4 and Fz6 was also observed in the thymic medulla and cortex. While in the young thymus the medulla ($EpCAM1^{++}/Ly51^{-}$) was preferentially stained for Fz4 and Fz6, the cortex ($EpCAM1^{+}/Ly51^{+}$) only faintly stained for this receptor. In the 9 month old thymus the medulla is less pronounced and in contrast to the young tissue and the whole section including the cortex appears increasingly positive for both receptors.

3.2. Active receptor signalling is indicated by PKCδ translocation

Active receptor signalling is invariably associated with modified phosphorylation of receptor associated signalling molecules. As protein phosphorylation depends on the activity of kinases, being an acknowledged activator of Dvl, PKCδ activity was investigated in Wnt-4 signalling. To test the involvement of PKCδ in Wnt-4 signal transduction, increased Wnt-4 presence was achieved by treatment using the supernatant of Wnt-4-transgenic thymic epithelial (Tep1) cells (Supplementary Fig. 1). Wild type Tep1 cells were exposed to SNs of control (Tep1-GFP) and Wnt-4 (Tep1-Wnt-4-GFP) cells for 1 h, then cytosolic and membrane fractions were isolated from cell lysates. Similarly to previous studies with Wnt-5a (Giorgione et al., 2003), Western blot analysis revealed that within one hour of Wnt-4 exposure PKCδ translocated into the membrane fraction (Fig. 2a) where the cleavage products (Kanthasamy et al., 2006) characteristic of active PKCδ were detected. Densitometric analysis of total and cleaved PKCδ demonstrated PKCδ activation (2 fold increase) upon Wnt-4 exposure. Additionally, increased membrane localisation of PKCδ was also detected (39 fold increase) in the Wnt-4-overexpressing cell line (Fig. 2a). As for Wnt-4 specific receptor expression, both Fz-4 and Fz-6 levels increased with age, therefore it was assumed that active receptor signalling might require more PKCδ during ageing. Indeed, apart from localisation of PKCδ to the membrane fraction (Fig. 2a), up-regulation of PKCδ was also detected at both mRNA (Fig. 2b) and protein level (Fig. 2c and d) in the ageing thymi. Interestingly, a characteristic cortico-medullary PKCδ pattern has also emerged. In both young and ageing thymi PKCδ was preferentially expressed in the cortex ($EpCAM1^{+}/Ly51^{+}$) (Fig. 2c and d).

3.3. PKCδ in Wnt-4 signalling

To specify the role of PKCδ in Wnt-4 signalling, it was necessary to identify a read-out gene that would reliably respond to Wnt-4 stimulus. Microarray (Supplementary Table 1) and subsequent Q-RT-PCR analysis of Wnt-4 exposed Tep1 cells identified connective tissue growth factor (CTGF) as such a target gene for Wnt-4 (Fig. 1s d). To investigate PKCδ involvement in Wnt-4 signalling, PKCδ activity was modified by overexpression of wild type PKCδ (Supplementary Fig. 2a–c) or by PKCδ gene specific silencing using commercially available siRNA for PKCδ (Santa Cruz) (supplementary Fig. 2d). Tep1 cells with increased or decreased PKCδ levels were exposed to control and Wnt-4 SNs for 1 hr, then CTGF expression was analysed. Surprisingly, although over-expression of PKCδ had no radical effect on Wnt-4 target gene transcription (Fig. 3a), even moderate down-regulation of PKCδ was able to significantly increase CTGF expression (Fig. 3b).

3.4. Co-immunoprecipitation of PKCδ, with Dvl, Fz-4 and Fz-6

To investigate whether PKCδ can associate with Fz-s, Fz-4 and Fz-6 co-immunoprecipitation patterns were analysed. Tep1 cells were treated with control and Wnt-4 SNs then proteins were

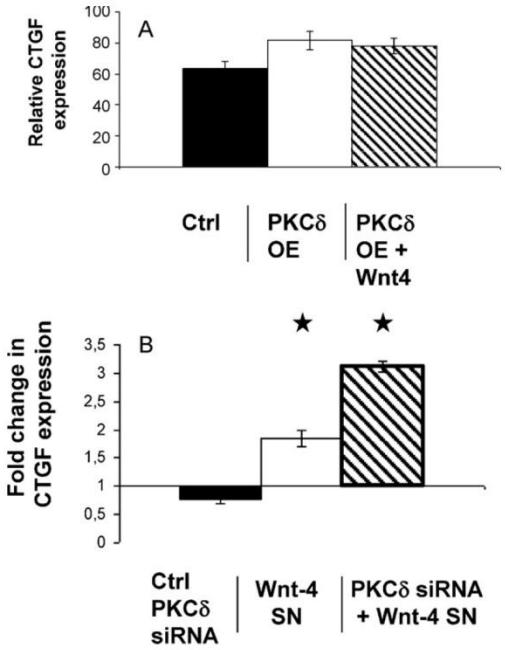

Fig. 3. (A) Analysis of Wnt-4 target gene expression. Q-RT-PCR analysis of CTGF expression in control and Wnt-4 treated PKCδ over-expressing Tep1 cell line. (B) Q-RT-PCR analysis of CTGF expression in control and Wnt-4 treated in Tep1 cell lines pre-treated with siRNA for PKCδ. Data were normalized to β-actin. Statistically significant differences are marked by asterisks.

immunoprecipitated using anti-Fz-4 and –Fz-6 antibodies, and probed for PKCδ and Dvl. Immunoprecipitation revealed increased association of PKCδ and its active cleavage products with both Fz-4 (1.2 fold, 2 fold and 1.4 fold, respectively) and Fz-6 (1.4 fold, 1.5 fold and strongly detectable over non-detectable, respectively) upon Wnt-4 treatment (Fig. 4a). To find out whether PKCδ co-localises with Fz-6 in primary thymic tissue, immunohistochemistry was performed. Experiments demonstrated age dependent increase of both Fz-6 and PKCδ as well as co-localisation of Fz-6 and PKCδ staining (Fig. 4c and d). While in the young thymus Fz-6 and PKCδ co-localisation is more pronounced in the thymic cortex (Fig. 4c), in the ageing thymus it is the medulla that exhibits stronger staining for both proteins (Fig. 4d).

3.5. Increased expression of CTGF and Fz-8

While increased expression and activity of the Fz-6 receptor, a suppressor of the canonical Wnt signalling pathway explains some aspects of uneven target gene transcription following manipulation of PKCδ activity, parallel changes like up-regulation of Fz-4 also occur during ageing that might add to the complexity of the signalling process. Increase in Fz-4 levels in ageing mice correlated with increased CTGF gene expression (Fig. 5a). If Fz-6 that also increases during senescence is truly a suppressor of β-catenin signalling then CTGF expression should have decreased or remained unchanged as Fz-4 transmitted signals would have been quenched by Fz-6 signalling. To test the above hypothesis, we have considered the following: CTGF has recently been reported to negatively regulate canonical Wnt signalling by blocking β-catenin stabilisation via GSK3β activation leading to phosphorylation and consequent degradation of β-catenin (Luo et al., 2004), indicating that CTGF might be part of a negative feed-back loop. As CTGF is a

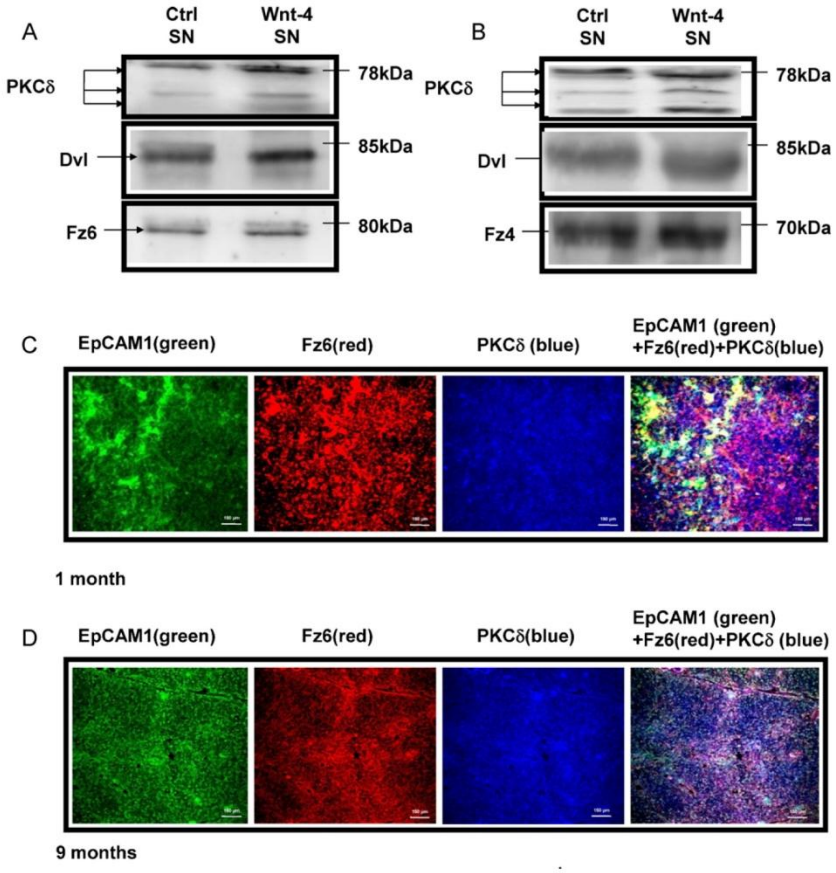

Fig. 4. Analysis of Fz-4, Fz-6, Dvl and PKCδ co-localisation. (A and B) Western blot analysis following immunoprecipitation using anti-Fz-6-antibody (A) or anti-Fz-4-antibody (B) shows increased association of Fz-6 with PKCδ and Dvl upon Wnt-4 treatment. Representative blots are shown from two repeats. (C and D) Histology of 1 month and 9 months old mouse thymi using anti- PKCδ– NL663, anti-Fz-6-NL557 and anti-EpCAM1-FITC antibodies. The overlay of the three stainings is shown. Size markers are shown in the corner of all histology figures. Characteristic stainings are shown from a minimum of five repeats.

secreted protein, expression of Fz-8 (Mercurio et al., 2004) a recently reported receptor for CTGF was analysed in purified TECs of 1 and 9 months old thymi using Q-RT-PCR reactions. As parallel with CTGF, Fz-8 mRNA levels increased (Fig. 5b) in ageing mice, while FoxN1 (data not shown) similarly to 12 months old mice reported previously (Kvell et al., 2010), a direct target of β-catenin dependent Wnt-4 signals (Balciunaite et al., 2002) was undetectable indicating the existence of an additional negative feed-back loop.

4. Discussion

Naïve T cell production is highest in young individuals and declines as thymic involution progresses with age. This impaired T-

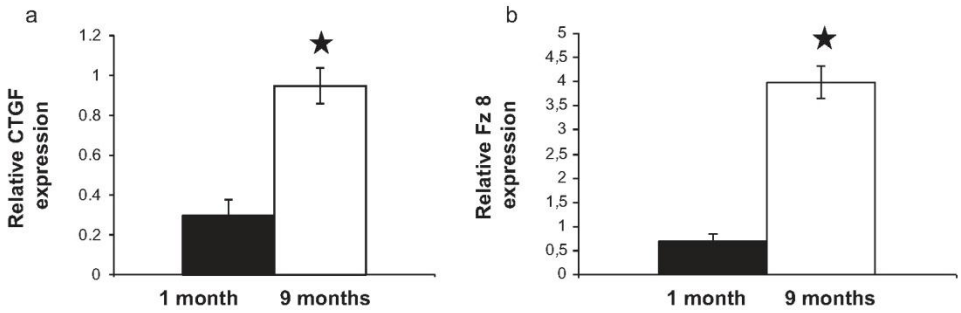

Fig. 5. CTGF and Fz-8 expression during thymic senescence. (A and B) Q-RT-PCR analysis of CTGF and Fz-8 expression in young (1 month) and ageing (9 months) mouse thymic epithelium. Data was normalised to β-actin. Statistically significant differences are marked by asterisks.

cell production leads to weakened immune responses especially against novel viral infections and increased incidence of autoimmune diseases.

In our previous work age-related down-regulation of Wnt-4 has been identified as a trigger for EMT and pre-adipocyte differentiation during thymic involution (Kvell et al., 2010). In the present work those Wnt receptor associated molecular mechanisms were investigated that can lead to structural and functional decline of the thymic epithelial network.

As in the mouse down-regulation of Wnt-4 expression is known to have occurred by the age of 12 months (Kvell et al., 2010), an earlier time-point was probed. At the age of 9 months Wnt-4 and FoxN1 decline was measured to be moderate (data not shown) along with further characteristic morphological and molecular changes. Interestingly, both Wnt-4 receptors, Fz-4 and Fz-6 are up-regulated at this age coinciding with increased receptor signalling. Apart from increased activation of the inhibitory receptor Fz-6, the expression of CTGF a recently identified potential negative regulator of canonical Wnt signalling is also up-regulated. It has been described that Fz-6 receptor activation can lead to increased kinase (NLK and TAK) activity resulting in phosphorylation of TCF (Luo et al., 2004), an important component of the β-catenin-TCF transcription complex. Once phosphorylated, TCF can no longer bind to β-catenin therefore gene transcription initiated via the canonical Wnt signalling pathway (for example via Fz-4) is inhibited. In contrast to Fz-6 signalling, CTGF uses a different way to interfere with β-catenin dependent signal transduction. CTGF can interact with Fz-8 as well as LRP6, an important co-receptor of Wnt signalling (Mercurio et al., 2004) and can trigger activation of GSK3β (Crean et al., 2004) via PKC ζ. Activation of GSK3β leads to increased phosphorylation and accelerated degradation of β-catenin which results in suppression of canonical Wnt signals.

Additional to increasing inhibitory pathways the loss of Wnt-4 levels can amalgamate age related decrease of FoxN1 and therefore loss of TEC identity that eventually leads to EMT and adipoid involution of the thymus. Our current hypothesis describing multiple mechanisms that lead to suppression of Wnt signalling is summarized in Fig. 6.

While it appears that active β-catenin signalling is absolutely essential for the maintenance of thymic epithelium, it is still not clear how imbalance in Wnt signalling affects de-regulation of thymic morphology. This is of note as recent experimental data where prolonged canonical Wnt signalling was enforced in TECs (Zuklys et al., 2009) describes similar changes in the thymic structure that is observed during both physiological and steroid induced ageing of the thymic epithelial network (Talaber et al., 2011). Importance of the balance in Wnt signalling is particularly well demonstrated in a Klotho deficient mouse model (Liu et al., 2007). The secreted protein Klotho can interact with various Wnt family members and suppresses biological activity of Wnts. Tissues and organs from Klotho-deficient animals show evidence of increased Wnt signalling resulting in accelerated cellular senescence both in vitro and in vivo. Based on the above model it has been postulated that depletion of the organ specific stem cell pool might be responsible for the accelerated ageing process in this setting.

Nevertheless, our studies focusing on the role of PKCs in Wnt associated receptor signalling have highlighted additional complexities at the intracellular level. In previous studies PKCα and PKCδ have been implicated to play an active role in disassembly of the nucleus in early apoptosis by phosphorylating nuclear lamin proteins (Dreger et al., 1999). Lamina-associated polypeptide 2 (LAP2) has been described to require phosphorylation via PKCs to fulfil its physiological function and LAP2α is up-regulated during ageing within TECs. While theoretically PKCδ could have a role within the nucleus during senescence, our current experiments support PKCδ activity in the cell membrane as transducer of negative Wnt signals. These findings support our earlier conclusions, namely thymic involution is not directly associated with PKCδ–dependent mass apoptosis and replacement of TECs by invading adipocytes, but rather a slow EMT process that results in

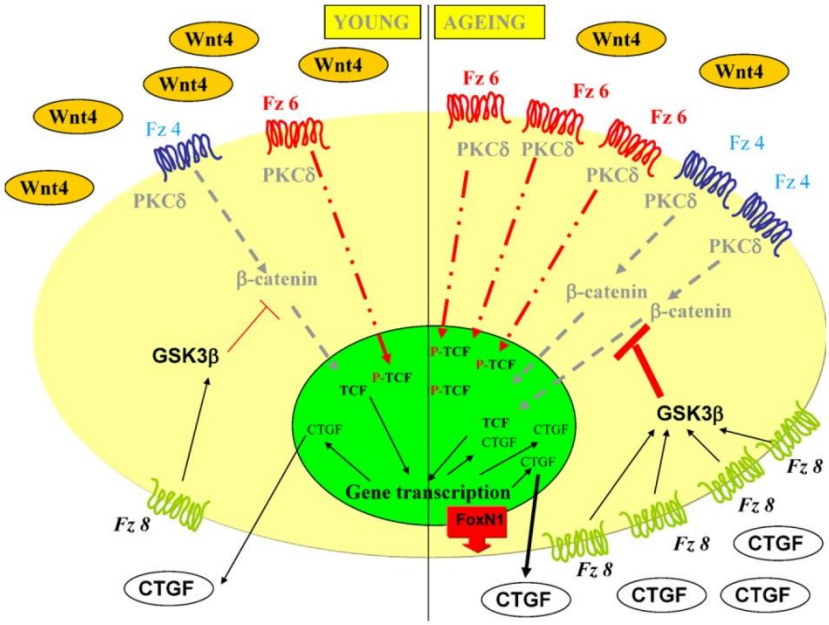

Fig. 6. Molecular model of thymic epithelial senescence. In young thymi Wnt-4 levels are high and both Fz-4 and Fz-6 receptors are expressed. Wnt-4 levels decrease with age, while receptor and receptor associated signalling molecule expressions increase triggering multiple suppression pathways of Wnt signalling.

adipocyte type trans-differentiation of resident TECs (Kvell et al., 2010; Youm et al., 2009).

Upon Wnt-4 signalling PKCδ seems to associate both with Fz-4 and Fz-6, latter being an inhibitory receptor of the canonical Wnt pathway. If Wnt-4 triggers CTGF gene transcription as a canonical target via Fz-4, then suppression of Fz-6 signalling by down-regulating PKCδ levels can up-regulate CTGF expression (Fig. 3a and b). Additionally, slight down-regulation of CTGF in the presence of PKCδ up-regulation also suits this hypothesis (Fig. 3a). Yet, there are still questions to be addressed. For example as PKCδ can also associate with Fz-4 and have other functions in intracellular signalling therefore the mere up-regulation of PKCδ would not halt β-catenin dependent signal transduction.

Further studies, however, are required and are on their way to identify the role of PKCδ in Fz-6 signalling and their combined task in thymic atrophy using in vitro cell line and in vivo transgenic animal studies.

5. Author contribution

ZV, KK and JEP have designed and performed experiments. ZV, KK and GM have participated in sub-cloning of Wnt-4 and PKCδ into viral plasmids, viral vector preparation, establishment of transgenic cell lines, performed Wnt-4 treatment and microarray experiments under the supervision of JEP. Also, ZV, KK and GT performed TEC purification, cDNA preparation and RT-PCR. ZV also performed microarray data analysis, PKCδ activity assays, western blotting and immunoprecipitation under the supervision of JEP. JEP and KK supervised the analysis of Q-RT-PCR data, VC and DB performed statistical analysis. JEP, GA and EJJ helped with the analysis of histology data. All authors discussed the results and commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mad.2011.04.007.

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Wnt-4 Protects Thymic Epithelial Cells Against Dexamethasone-Induced Senescence

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Abstract

Glucocorticoids are widely used immunosuppressive drugs in treatment of autoimmune diseases and hematological malignancies. Glucocorticoids are particularly effective immune suppressants, because they induce rapid peripheral T cell and thymocyte apoptosis resulting in impaired T cell–dependent immune responses. Although glucocorticoids can induce apoptotic cell death directly in developing thymocytes, how exogenous glucocorticoids affect the thymic epithelial network that provides the microenvironment for T cell development is still largely unknown. In the present work, we show that primary thymic epithelial cells (TECs) express glucocorticoid receptors and that high-dosage dexamethasone induces degeneration of the thymic epithelium within 24 h of treatment. Changes in organ morphology are accompanied by a decrease in the TEC transcription factor FoxN1 and its regulator Wnt-4 parallel with upregulation of lamina-associated polypeptide 2α and peroxisome proliferator activator receptor γ, two characteristic molecular markers for adipose thymic involution. Overexpression of Wnt-4, however, can prevent upregulation of adipose differentiation-related aging markers, suggesting an important role of Wnt-4 in thymic senescence.

Introduction

Autoimmune diseases and hematological malignancies are significant causes of morbidity and mortality world wide.[1,2] Although research is ongoing, treatment options are still often limited to high-dosage synthetic glucocorticoid (GC) analogs despite their nonspecificity and multiple side effects. Indeed, GCs are still applied in therapy for acute and chronic autoimmune diseases and hematological malignancies,[3,4] because they effectively promote apoptosis of leukemia cells[5] and trigger complex antiinflammatory actions by influencing both molecular and cellular components of the immune system.[6] Apart from triggering decreased expression of cytokines and major histocompatibility complex class II (MHC II), GCs also induce apoptotic death of peripheral[7] and developing T cells. In mouse models, GCs cause massive thymocyte depletion, especially in the $CD4^+CD8^+$ (double positive [DP]) thymocyte population,[8–12] blocking *de novo* T cell production.

Prior experiments have also demonstrated that high-dose GCs induce a dramatic[13] and apoptosis-associated[14] involution of the thymus, and not only thymocytes but also thymic epithelial cells (TECs) are seriously affected.[15] Additionally, a recent report by Fletcher et al.[16] has highlighted that TEC depletion appears reversible, and thymic epithelial stem cells play an important role in this process.

Because physiological steroids are implicated in the regulation of aging,[17,18] we theorized that GC treatment might affect thymic epithelial senescence. Although morphological similarities between physiological and induced thymic involution are striking, to date the process has not been studied in detail at the molecular level. One possible mechanism is that during physiological aging TECs undergo epithelial-to-mesenchymal transition (EMT) and then proadipose differentiation.[19,20] Our studies have recently provided evidence that this process is regulated by Wnt-4 and FoxN1 decline, leading to drastic reduction in TEC identity[21,22] and simultaneous upregulation of lamina-associated polypeptide (LAP) 2α as well as preadipocyte-related markers peroxisome proliferator activator receptor (PPAR) γ and adipose differentiation-related protein (ADRP).[20]

On the basis of the above studies, we theorized that GCs do not simply deplete thymocytes and the majority of TECs, but they also inhibit the function of the remaining epithelium via downregulation of characteristic TEC markers, leading to preadipocyte differentiation. In the present study, we provide evidence that both primary TECs and the primary TEC-derived TEP1 cell line express glucocorticoid receptors (GRs)

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and respond to GC treatment. Upon a single high-dose GC injection, gene expression levels of both Wnt-4 and FoxN1[21] become significantly downregulated, resulting in upregulation of preadipocyte differentiation markers (LAP2α, PPARγ). Regeneration of TECs—including normal FoxN1 level—occurs within 3 months after dexamethasone (DX) injection. However, if GC-treatment is continued with repeated small doses as in clinical applications, then Wnt-4 and FoxN1 transcription cannot recover and aging markers remain locked at elevated levels. Overexpression of Wnt-4 in the FoxN1-deprived TEP1 cell line is, however, able to protect against GC-induced adipose transdifferentiation, indicating Wnt-4 as the primary protector of thymic epithelium against adipose involution.

Materials and Methods

Mice and treatment of animals

Four-week-old BALB/c mice were used for the experiments. Animals received a single-dose (20 mg/kg) DX (Oradexon, Organon) injection intraperitoneally (i.p.) in phosphate-buffered saline (PBS) and then were sacrificed 24 and 168 h after injection; control animals received PBS. Another group of mice received PBS and DX for 3 months, respectively. There was also a group of mice receiving one high dose of DX, then a continuously low dose of DX (2 mg/kg) on every second day for a month, to mimic the therapeutic regimen of autoimmune diseases.[23,24] All animal experiments were carried out in accordance with the regulations set out by Pécs University's committee on animal experimentation (#BA 02/2000-2/2006).

Cell lines and in vitro *DX treatment*

The TEP1 cell line was maintained and used for experiments as described.[20] The Wnt-4-overexpressing TEP1 cell line was generated as described previously.[20] Cell lines were treated with DX (Sigma, dissolved in dimethylsulfoxide [DMSO] until use) with a final concentration of 1 μM for 1 week or solvent, respectively.

Preparation of TECs

Thymic lobes were digested with 3 mg/mL collagenase II (GIBCO) for 30 min, then washed with Dulbecco modified Eagle medium (DMEM) 10% fetal calf serum (FCS). Cell suspensions were then labeled with anti-EpCAM1-FITC (clone G8.8) and washed with magnetic cell sorting (MACS) buffer followed by incubation with anti-fluorescein isothiocyanate (FITC) microbeads (Miltenyi Biotec), the EpCAM1$^+$-cells were used for total RNA isolation and subsequent quantitative polymerase chain reaction (PCR) analysis. The cells were purified using MACS LS separation columns (Miltenyi Biotec).

Histology using fluorescent antibodies

Frozen thymic sections (7—10 μm thick) were fixed in cold acetone, then dried and blocked using 5% bovine serum albumin (BSA) in PBS for 20 min before staining with a-Ly51-PE (clone 6C3) and a-EpCAM1 (clone G8.8) antibodies (either indirectly coupled with a-rat Northern Light 637 secondary antibody or directly labeled with FITC). GR, ER-TR7, and Wnt-4 protein levels were detected using an a-GR-FITC mouse monoclonal antibody (clone 5E4B1 developed in our laboratory[25] and commercially available at Serotec), a rat monoclonal a-ER-TR7, and a polyclonal goat a-Wnt4 antibody (Abcam). Visualization was performed using an a-rat-Ig-PE secondary antibody and a-goat Ig Northern Lights 557 (RnD systems), respectively. Parallel with GR detection, corresponding sections were incubated with an irrelevant antibody. To detect GR expression in TECs, the Olympus Fluoview 300 confocal microscope with an Olympus Fluoview FV1000S-IX81 system was used. All the other sections were analysed using an Olympus BX61 microscope equipped with CCD camera and AnalySIS software.

RNA isolation, preparation of cDNA

Total RNA was isolated using RNeasy plus kit (Qiagen), following the manufacturer's instructions. Following RNA isolation, DNase digestion was performed using a DNase I digestion kit (Sigma) and cDNA was reverse transcribed using a high-capacity RNA to cDNA kit according to the manufacturer's instructions (Applied Biosystems).

Quantitative real-time and qualitative reverse transcriptase PCR analysis of purified TECs and cell lines

For real-time PCR analysis, we used an ABI 7500 Software system and ABI SYBR Green PCR master mix. The expression levels of LAP2α2, PPARγ, ADRP, and Wnt-4 were analyzed and normalized to the level of 18S rRNA. The qualitative expression of GR and CD45 in TEC was verified by PCR using ReddyMix (ABgene) according to the manufacturer's instructions, with 18S rRNA as an internal control. The PCR products were visualized on agarose gels. The sequences of primers are listed in Table 1. In the case of FoxN1 and AIRE, TaqMan chemistry was used for PCR reaction and analysis, and the levels of these genes were normalized to the TaqMan HPRT1 expression. PCR reactions were run for a maximum of 40 cycles.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD), and the effects between various experimental groups were compared with the Student t-test. $p < 0.05$ was considered as significant.

Results

GR expression in primary thymic epithelium and the TEP1 cell line

GCs regulate cellular function via GR, which belongs to the nuclear receptor superfamily[26] and is required for the regulation of development and homeostasis of various epithelial-like tissues.[27,28] Using reverse transcriptase (RT)-PCR analysis (Fig. 1A) and confocal laser scanning microscopy (Fig. 1B), it was demonstrated that apart from thymocytes[8,9] GR is also expressed in primary TECs and the TEP1 cell line, confirming their ability to respond directly to GC stimuli.

Effects of single-dose GC administration

To study how the thymic epithelial network is affected by GC, sections of DX-treated and control thymi were stained

TABLE 1. LIST OF GENE-SPECIFIC PRIMERS

Gene	*Forward primer*	*Reverse primer*
CD45	5′-CCG GAA TTC CGG ATG GGT TTG TGG CT-3′	5′-CCG CTC GAG CGG CTA ATC ACT GGG TG-3′
GR	5′-TGG TGT GCT CCG ATG A-3′	5′-AGG GTA GGG GTA AGC-3′
FoxN1	Applied Biosystems TaqMan probe PN4351272 (Mm00477457_m1)	
LAP2α	5′-TGA ACT GCA GGC AGC TAA GA-3′	5′-TCA TAG CTA GAC TCT GAG G-3′
PPARγ	5′-CCC AAT GGT TGC TGA TTA CAA A-3′	5′-AAT AAT AAG GTG GAG ATG CAG GTT CT-3′
ADRP	5′-CGC CAT CGG ACA CTT CCT TA-3′	5′-GTG ATG GCA GGC GAC ATC T-3′
Wnt4	5′-CTC AAA GGC CTG ATC CAG AG-3′	5′-TCA CAG CCA CAC TTC TCC AG-3′
18S rRNA	5′-GGG TCG GGA GTG GGT AAT TT-3′	5′-AGA AAC GGC TAC CAC ATC CAA-3′
HPRT1 Taqman	Applied Biosystems TaqMan probe Mm0046968_m1	
AIRE	Applied Biosystems TaqMan probe Mm00477457_m1	

GR, Glucocorticoid receptor; PPARγ, peroxisome proliferator activator receptorγ; ADRP, adipose differentiation-related protein; AIRE, autoimmune regulator.

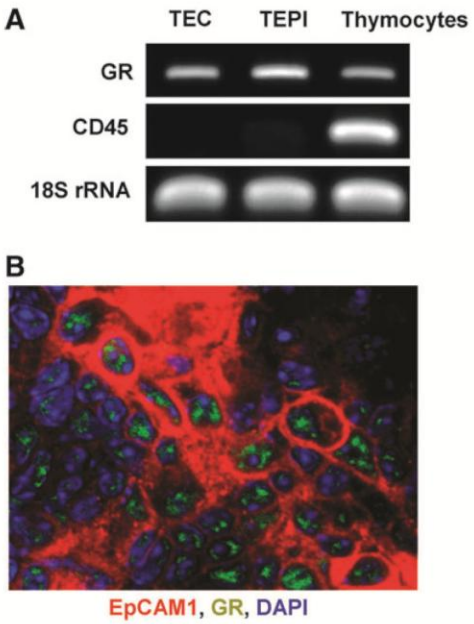

FIG. 1. Glucocorticoid receptor (GR) expression in the thymic epithelial cell (TEC) and TEPI cell lines. cDNA generated from mRNA of highly purified adult TECs and TEPI cells were tested in end point reverse transcriptase polymerase chain reaction (RT-PCR) analysis for GR expression. cDNA generated from thymocyte mRNA was used as positive control for GR expression, whereas TEC purity was tested for hematopoietic cell contamination using CD45 primers. 18S rRNA was used as an internal control (**A**). GR protein was detected by confocal microscopy using a-GR-FITC-labeled antibody (green) in frozen sections of untreated adult BALB/c thymi. TECs were detected using a-EpCAM1 antibody visualized by a-rat-Northern Light 637 secondary antibody (red). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) (**B**). Corresponding sections were probed with an irrelevant antibody as a staining control. Data shown above are representative of three separate experiments.

for the general epithelial cell marker, EpCAM1 and Ly51[29] to differentiate between medulla and cortex. Within 24 h, DX treatment induced marked reduction in epithelial cell-surface markers (Fig. 2B), particularly affecting the medullary ($EpCAM1^{++}Ly51^{-}$) thymic compartments. The remaining TECs were purified from GC-treated thymi based on EpCAM1 expression. In the purified TEC population, gene transcription was analyzed using quantitative real-time RT-PCR. During embryonic development, TEC maturation is regulated by Wnt-4[21]; therefore Wnt-4 levels were assayed. Quantitative real-time RT-PCR analysis and immunohistochemistry confirmed that both Wnt-4 mRNA (Fig. 2A) and protein levels (Fig. 2B) decreased significantly after 24 h of DX exposure. FoxN1, a transcription factor essential for thymic organogenesis and maintenance of TEC identity directly regulated by Wnt-4 expression,[30] was also found to be significantly decreased (Fig. 2A). FoxN1[30] and Wnt-4 mRNA levels were also measured a week later and appeared to remain significantly low (Fig. 2A).

As physiological thymic involution correlates with the upregulation of preadipocyte markers LAP2α[20] and PPARγ, the expression of these markers was also tested following DX treatment–induced involution. One day, 1 week, 1 month, and 3 months after DX exposure, TECs were purified and then analyzed using quantitative (q) RT-PCR. At day 1, the expression of LAP2α[20] and PPARγ was low (data not shown), but it became upregulated a week later (Fig. 2C), indicating that it takes several days of Wnt-4 and FoxN1 depletion to affect preadipocyte differentiation markers. By 1 month and even by 3 months after DX treatment, Wnt-4 mRNA expression only partially increased while FoxN1, LAP2α, and PPARγ mRNA levels returned close to normal levels (within standard deviation) supporting the time frame of recovery (Fig. 3A).

To analyze how thymic morphology is affected, thymic sections were stained for EpCAM1/Ly51. Three months after DX treatment, no significant differences were detected between DX-treated and age-matched controls (Fig. 3B), except for the medulla, which still appeared somewhat shrunken in DX-exposed animals (Fig. 3B). To determine thymic medullar mass, qPCR was performed for the medullary marker autoimmune regulator (AIRE) gene.[31] No significant difference

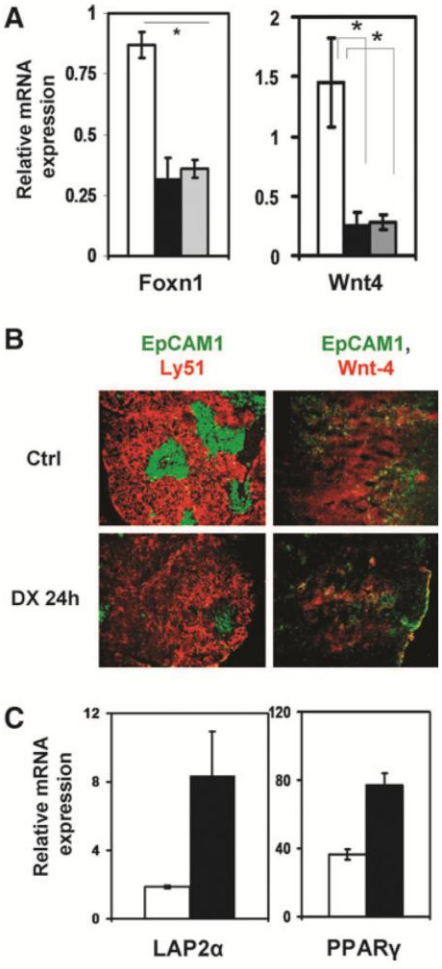

FIG. 2. Dexamethasone (DX)-induced effect on Wnt-4, Foxn1, adipocyte-related genes, and general morphology of the thymus. Foxn1 and Wnt-4 gene expression pattern (**A**) of purified control and DX-treated thymic epithelial cell (TEC) control (□), for 24 h (■). Gray bars (▩) represent gene expression 168 h after DX treatment and asterisks ($*p < 0.05$) indicate significant differences ($n = 3$ in each group). Note the scale differences for gene expression. Thymic sections of phosphate-buffered saline (PBS)- and DX-treated mice (24 h) were stained with a-EpCAM1-FITC (green) and a-Ly51-PE (red) monoclonal antibodies to reveal medullary and cortical compartments. The staining revealed depletion of mTECs following 24 h of DX administration. Images are representative of three independent experiments (**B**, *left*). Wnt-4 expression of control and DX-treated thymi is also shown (**B**, *right*). Wnt-4-Northern Lights 557 (red) and EpCAM1-FITC (green) in Ctrl and DX-treated thymi are presented (24 h). Images are representative of three independent experiments. Induction of adipose tissue-related genes in TEC following DX-treatment. (**C**) Expression of lamina associated polypeptide-2α (LAP2α), peroxisome proliferator activator receptor-γ (PPARγ), and adipose differentiation-related protein (ADRP) were tested in TECs 168 h after a single DX injection and was found to be elevated in DX-treated samples (■) (with an exception of ADRP), compared to control (□). Gene expression was normalized to 18S rRNA. All bars show means ± standard deviation (SD). Results are representative of three independent experiments ($n = 3$).

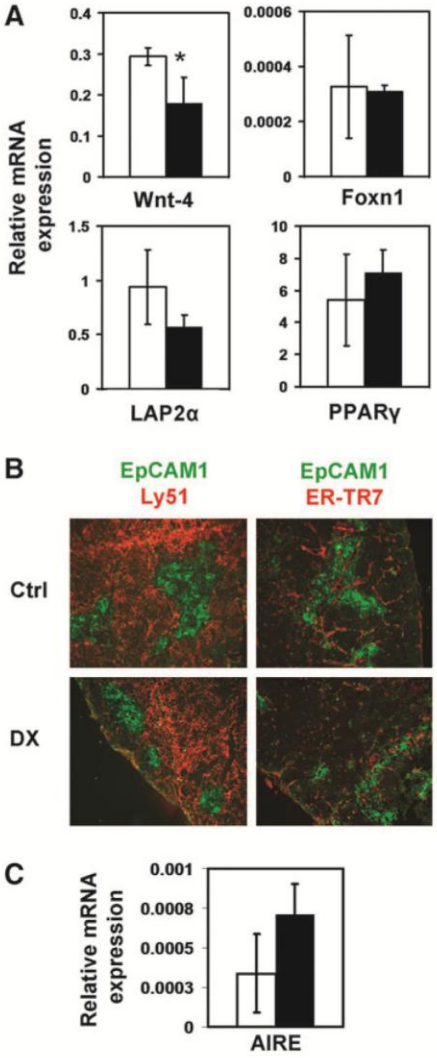

FIG. 3. Recovery of the thymic epithelial cell (TEC) compartment after dexamethasone (DX) treatment takes 3 months. (**A**) Bars show the gene expression changes of purified TEC: Control (□) and dexamethasone (DX) treated (■). The level of Wnt-4 remained significantly low even after 3 months ($*p < 0.05$), the levels of lamina associated polypeptide-2α (LAP2α) and peroxisome proliferator activator receptor-γ (PPARγ) were found to be unaltered, whereas adipose differentiation-related protein (ADRP) expression was found to be decreased. Note the scale differences for gene expression. Morphological analysis of age-matched control (Ctrl) versus DX-treated thymi after 3 months using EpCAM-Ly51 (TEC network) (**B**, *left*) and EpCAM-ER-TR7 (TEC and fibroblast) (**B**, *right*). In **B** (*left*), in the treated samples, the size of medulla appears to be smaller, but the TEC network is normal. In **B** (*right*), no remarkable differences are observed in general morphology using the ER-TR7 fibroblast marker staining on thymic sections of control and DX-treated mice. Images are representative of three independent experiments. In **C**, autoimmune regulator (AIRE) expression is shown in control (□) and DX- (■) purified TEC after 3 months, with no significant difference, indicating full mTEC recovery. All bars show means ± standard deviation (SD) ($n = 3$).

was detected in AIRE expression after 3 months of DX treatment (Fig. 3C), indicating the full competence of the mTEC compartment. Because EMT seems to precede preadipocyte transdifferentiation during physiological aging,[20] the staining pattern of fibroblast marker ER-TR7 was also examined following DX treatment. Apart from the scattered focal staining pattern of ER-TR7 in DX-treated thymi, no sign of significant EMT (Fig. 3B) was detected, supporting molecular data showing that 3 months is sufficient for TEC recovery.

Effects of sustained GC administration

To mimic the pattern of clinical applications of GCs, mice were injected with DX repeatedly for a time course of 1 month. TECs were examined and drastic downregulation of both Wnt-4 and Foxn1 mRNA levels was detected (Fig. 4A).

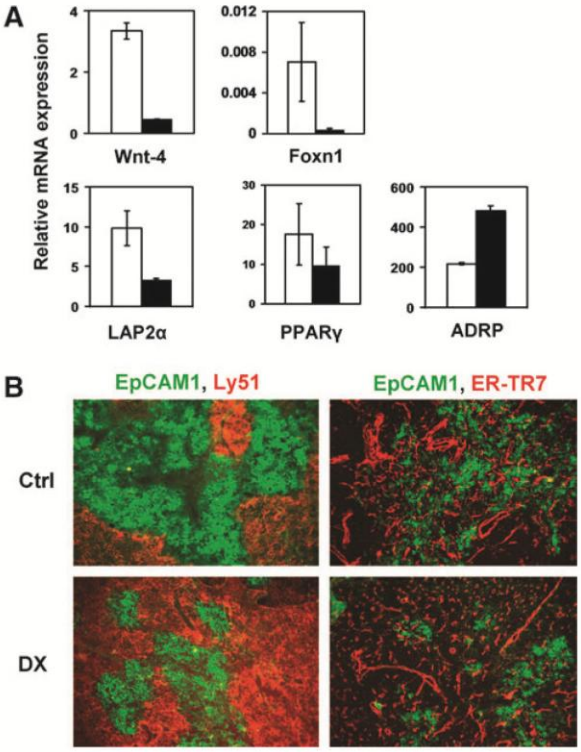

FIG. 4. Effect of continuous dexamethasone (DX) treatment on thymic epithelial cell (TEC) gene expression and architecture. (**A**) Bars showing the mean ± standard deviation (SD) ($n = 3$) of Wnt-4, Foxn1, lamina associated polypeptide-2α (LAP2α), peroxisome proliferator activator receptor-γ (PPARγ), and adipose differentiation-related protein (ADRP) expression of control (□) and DX-treated (■) TEC. The levels of Wnt-4, Foxn1, and LAP2α were found to be reduced after repeated *in vivo* DX injections, whereas PPARγ was unaltered. Additionally, ADRP was found to be elevated in DX-treated samples. Note the scale differences for gene expression. EpCAM1-Ly51 co-staining on thymus sections revealed that in DX-treated samples, the medullar area seems to be smaller compared to control (**B**, *left*). ER-TR7 staining showed no significant increase but became more punctuated in DX-treated samples. Images are representative of three independent experiments.

Although expression of early preadipocyte transdifferentiation markers LAP2α and PPARγ showed no significant alterations by this advanced time point, the downstream adipocyte differentiation factor ADRP was significantly increased in purified TECs of DX-treated animals (Fig. 4A), indicating that prolonged GC treatment pushed preadipocyte type transdifferentiation significantly further than a single-dose DX injection. In harmony with the molecular data, a strong decrease in the medullary compartment (Fig. 4B) was detected while the pattern of the fibroblast marker ER-TR7 (Fig. 4B) became punctuated in DX-treated thymi.

Wnt-mediated inhibition of steroid-induced adipose transdifferentiation

On the basis of our *in vivo* experiments, Wnt-4 and FoxN1 have a primary role in GC-triggered adipose involution. However, because thymocyte and TEC interactions are vitally important for the normal homeostasis of the thymus,[31] it was important to test whether mass depletion of thymocyte populations was the trigger to Wnt-4 and consequently FoxN1 downregulation, or was direct consequence of DX exposure. To answer this question, the TEP1 thymic epithelial cell line was also exposed to DX for 1 week and then molecular changes were analyzed. While Wnt-4 gene transcription was moderately downregulated LAP2α PPARγ and its downstream target ADRP were significantly increased (Fig. 5A), indicating that DX treatment directly induces changes leading to preadipocyte type transdifferentiation. To test the role of Wnt-4 in this process, a Wnt-4-overexpressing TEP1 cell line was created and then treated with DX for a week. True to our expectations, Wnt-4 was able to inhibit preadipocyte-type fate commitment of the cell line, and no significant changes were detected in preadipocyte-type transdifferentiation markers (Fig. 5B). Interestingly, because the TEP1 cell line does not express FoxN1 (unpublished observation), it has become evident that the absence of Wnt-4 alone is required and sufficient to allow preadipocyte-type transdifferentiation, and apparently FoxN1 has no significant role in the process.

Discussion

Our studies confirm that, beside T lymphocytes,[8,9] dendritic cells,[32,33] and TEC lines,[15] primary TECs also express GR-rendering TECs directly sensitive to GCs. Following GC exposure, and similar to physiological aging, DX treatment triggered Wnt-4 and FoxN1 downregulation, leading to increased expression of preadipocyte-type differentiation markers LAP2α and PPARγ.[34,20] LAP2α and PPARγ levels increased within a week following DX exposure, highlighting the accelerated rate of GC-induced aging compared to physiological senescence. This is in harmony with literature data, because the involvement of PPARγ has also been confirmed in the induction of thymic involution and ectopic adipogenesis by recent studies using Rosiglitazone,[35] an acknowledged PPARγ agonist.

The described increase of LAP2α, PPARγ, and ADRP expression is not likely to occur due to the enrichment of either mTEC or cTEC compartments. In untreated TECs, the LAP2α expression level is similar in both epithelial subsets (data not shown), indicating no difference in sensitivity to adipocyte transdifferentiation inducing factors.

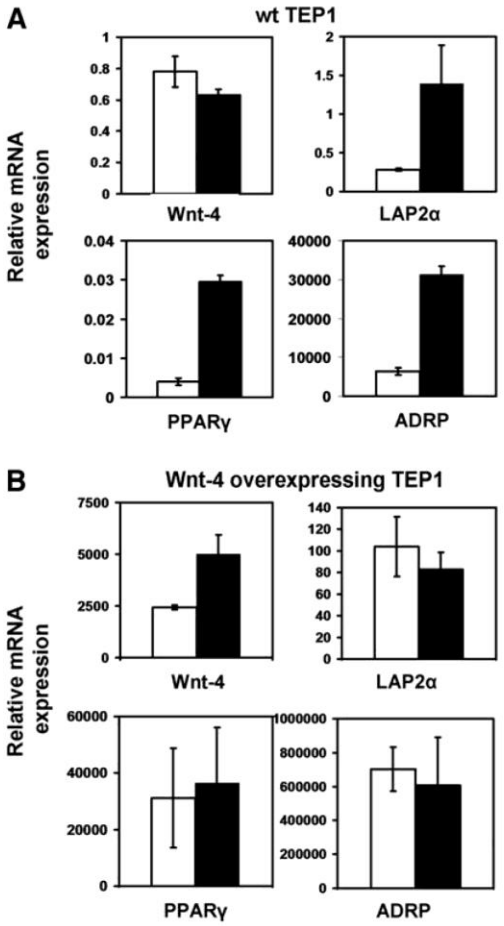

FIG. 5. Wnt-4 overexpression of the TEP1 cell line overcomes dexamethasone (DX)-induced adipocyte-related gene expression in TEP1 cells. Gene expression changes in TEP1-cell lines following 168 h of DX-treatment. The mRNA levels of Wnt-4, lamina associated polypeptide-2α (LAP2α), peroxisome proliferator activator receptor-γ (PPARγ), and adipose differentiation-related protein (ADRP) was tested in solvent- and DX-treated normal (**A**) and Wnt-4-overexpressing TEP1 (**B**). White bars indicate solvent-treated control and black bars show the values of DX-treated samples. Results are representative of three independent experiments and showing means ± standard deviation (SD). Note the different scales for gene expression.

Interestingly, as confirmed by histology, DX-treated thymi regained close-to-normal morphology 3 months following exposure, whereas DX-triggered effects were still detectable at the molecular level. Repeated DX administration resulted in drastic reduction of both Wnt-4 and FoxN1 expression and significant increase of ADRP expression, showing no sign of recovery at the mRNA level. Literature data are supportive, because prolonged decrease in FoxN1 and Wnt-4 expression have recently been demonstrated to lead to degeneration of the thymic epithelial network.[36] The reported phenotype was strikingly similar to age-associated involution of the thymus, except for the dramatically accelerated pace similar to that observed following GC administration. Others reported that ubiquitous deletion of FoxN1 in the postnatal thymus has also caused thymic atrophy and severe deterioration of the TEC network.[37] Because FoxN1 expression is Wnt-4 dependent,[21] Wnt-4 appears to be a realistic candidate molecule to defend the thymus against adipose involution. Using the Wnt-4-overexpressing TEP1 cell line, the working hypothesis was confirmed, because Wnt-4 overexpression could effectively block DX-induced increase of adipocyte markers and protect thymic epithelium-derived cells against DX-induced senescence at the molecular level.

In the present work, DX was shown to trigger accelerated thymic aging accompanied by thymic involution.[13,14,17] Using markers identified in our previous studies,[20] we demonstrate that the processes of physiological and GC-induced accelerated thymic senescence share similar molecular mechanisms yet operate at different time scales. Because GCs are used relatively often in clinical therapies for sustained periods to suppress actual flares of autoimmune diseases, our results call attention to a currently neglected potential side effect of sustained GC treatment, namely induced accelerated thymic epithelial aging. Accelerated thymic epithelial aging impairs TEC functions, including deletion of potentially autoimmune naïve T cells, as well as allows for accumulation of T cells proliferating in the periphery. Therefore GC-induced accelerated thymic epithelial senescence could provide permissive context for the development of T cell–mediated autoimmune diseases. This would mean that GC treatment could paradoxically lead to the emergence of immune pathologies, including certain autoimmune diseases.[38–40] Our findings concerning Wnt-mediated inhibition of GC-triggered accelerated thymic senescence are also particularly important, becasue Wnt-4 could potentially be a candidate molecule to inhibit adipose involution of the human thymus following GC treatment or even physiological senescence, although further *in vivo* experiments are required to clarify the potential application process.

Acknowledgments

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Author Disclosure Statement

There are no competing interests.

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Wnt4 and LAP2alpha as Pacemakers of Thymic Epithelial Senescence

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Abstract

Age-associated thymic involution has considerable physiological impact by inhibiting *de novo* T-cell selection. This impaired T-cell production leads to weakened immune responses. Yet the molecular mechanisms of thymic stromal adipose involution are not clear. Age-related alterations also occur in the murine thymus providing an excellent model system. In the present work structural and molecular changes of the murine thymic stroma were investigated during aging. We show that thymic epithelial senescence correlates with significant destruction of epithelial network followed by adipose involution. We also show in purified thymic epithelial cells the age-related down-regulation of Wnt4 (and subsequently FoxN1), and the prominent increase in LAP2α expression. These senescence-related changes of gene expression are strikingly similar to those observed during mesenchymal to pre-adipocyte differentiation of fibroblast cells suggesting similar molecular background in epithelial cells. For molecular level proof-of-principle stable LAP2α and Wnt4-over-expressing thymic epithelial cell lines were established. LAP2α over-expression provoked a surge of PPARγ expression, a transcription factor expressed in pre-adipocytes. In contrast, additional Wnt4 decreased the mRNA level of ADRP, a target gene of PPARγ. Murine embryonic thymic lobes have also been transfected with LAP2α- or Wnt4-encoding lentiviral vectors. As expected LAP2α over-expression increased, while additional Wnt4 secretion suppressed PPARγ expression. Based on these pioneer experiments we propose that decreased Wnt activity and increased LAP2α expression provide the molecular basis during thymic senescence. We suggest that these molecular changes trigger thymic epithelial senescence accompanied by adipose involution. This process may either occur directly where epithelium can trans-differentiate into pre-adipocytes; or indirectly where first epithelial to mesenchymal transition (EMT) occurs followed by subsequent pre-adipocyte differentiation. The latter version fits better with literature data and is supported by the observed histological and molecular level changes.

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Introduction

Thymic senescence

Thymic senescence begins early, around late puberty. This process is called adipose involution, as the thymus is invaded by adipose tissue [1]. Due to decrease in thymic epithelial tissue mass, the thymus can no longer support the same output of T-cell production [2]. Therefore peripheral blood T lymphocyte composition exhibits the dominance of memory T lymphocytes resulting in impaired responses towards novel, particularly viral infections [3,4,5]. Since the thymic epithelium has a key role in deleting auto-reactive T-cell clones, functional impairment increases the chances of developing auto-immune disease [6]. If we were able to slow down or even stop the loss of thymic epithelium the elderly would have a better chance to address late-onset autoimmune diseases and viral infections. However, despite studies of thymic senescence, the molecular mechanism of thymic aging remains elusive.

Signaling pathways of thymic epithelial cell development and maintenance

Understanding signaling mechanisms that regulate tissue development and maintenance of thymic epithelial cells might reveal the process of adipose involution. Certainly, maintenance and functional integrity of the thymic stroma requires stimuli through Notch, BMP, and Wnt signaling pathways [7,8,9,10,11]. Undoubtedly, the Wnt family of secreted glycoproteins is one of the best analyzed among the required ligands [12]. Most members of the nineteen known Wnt glycoproteins have been implicated in both the development of embryonic thymus and the maintenance of adult thymic epithelium [13]. In the thymus, Wnt ligands originate primarily from thymic epithelial cells and activate a highly complex signaling network via ten G-protein dependent receptors called Frizzleds (Fz), and their co-receptors of low-density lipoprotein receptor-related proteins 5/6 called LRP5/6 [14,15]. The actual constellation of ligands, receptors, co-receptors and further regulatory molecules define Wnt-mediated effects.

Recent studies have highlighted Wnt4 as responsible for the direct up-regulation of FoxN1, a key transcription factor responsible for the differentiation of thymic epithelial cells and the subsequent maintenance of thymic epithelial identity [13]. Interestingly, the Wnt/β-catenin pathway is known to efficiently block the adipocyte differentiation program in mesenchymal elements like fibroblasts [16,17,18,19].

Trans-differentiation of fibroblasts into adipocytes

Studies with fibroblast cells have also revealed that fibroblast to pre-adipocyte transformation is strongly connected to LAP2α, the member of the LAP2 protein family [17]. To date there are 7 classified intranuclear LAP2 polypeptides marked by the Greek alphabet. They are all splice variants of the same LAP2 gene previously called thymopoietin. While most splice variants associate with the nuclear envelope, LAP2α is involved in several nucleoplasmic activities including cell-cycle control and differentiation [20,21]. LAP2α is synthesized in the cytoplasm and is then transported into the nucleus by a PKC-dependent mechanism [22]. The mere over-expression of LAP2α in fibroblasts is known to directly up-regulate PPARγ expression, an acknowledged marker and key transcription factor of pre-adipocyte differentiation [17]. In pre-adipocytes PPARγ expression is followed by an increase of ADRP expression (adipose differentiation-related protein) a known direct target gene of PPARγ. Although LAP2α over-expression alone initiates pre-adipocyte differentiation in fibroblasts, it is not sufficient to complete the adipocyte differentiation program in the absence of additional stimuli [17].

Results and Discussion

Disintegration of epithelial network

Senescence exhibits characteristic histological changes in both the human and mouse thymus [1,23]. In order to demonstrate this process the thymic lobes of 1 month and 1 year old BALB/c mice were analyzed (see Figures 1A and 1B). In young adult mice, histology revealed strict segregation of epithelial cell compartments by staining for medullary (EpCAM1^{++}, Ly51^{-}) and cortical (EpCAM1^{+}, Ly51^{++}) epithelial cellular subsets (Figure 1A). This shows high level of morphological integrity just preceding puberty/early adulthood. However, the highly organized structure disintegrates and becomes chaotic by the age of 1 year (Figure 1B). By this age the previously shown strict cortico-medullary delineation becomes disintegrated, degenerative vacuoles appear surrounded by areas showing strong co-staining with both epithelial markers. There are also other large cellular areas that lack staining with either epithelial marker, a pattern completely absent at the young adult age.

Staining of extracellular matrix components of fibroblast origin (ER-TR7^{++}) was also performed on cryostate thymic sections of 2

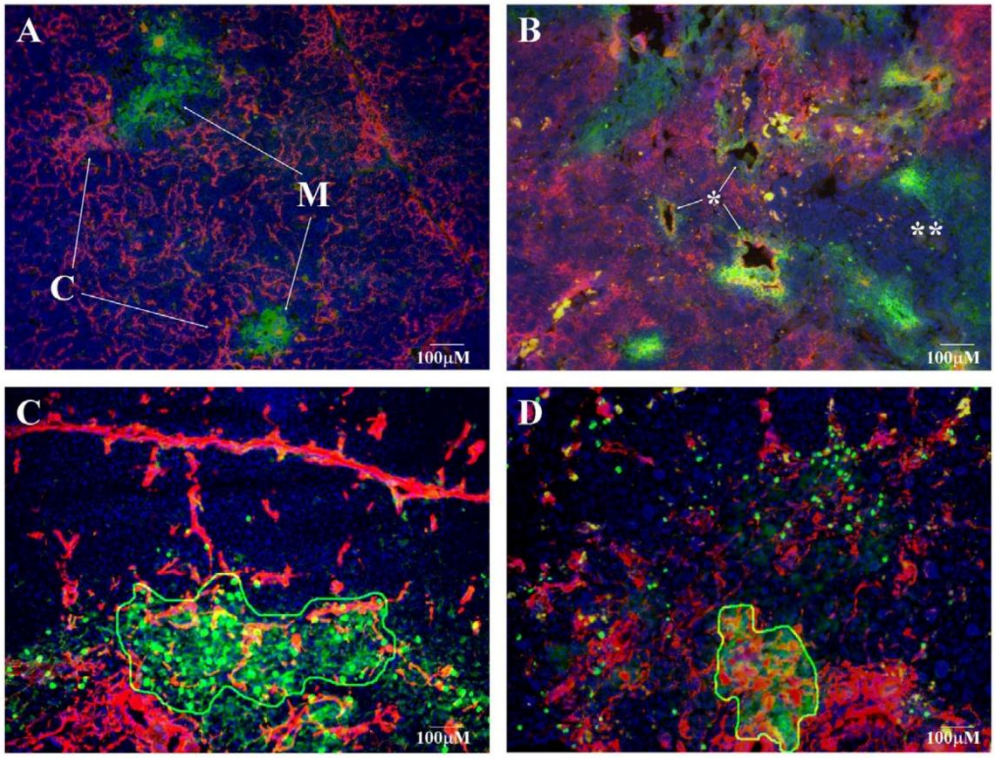

Figure 1. Disintegration of epithelial network. Figure 1A demonstrates cryostat section of 1 month, whereas figure 1B presents cryostat section of 1 year old BALB/c mouse thymus. Staining pattern: anti-EpCAM1-FITC (green), anti-Ly51-PE (red), DAPI (blue). 'M' marks medullary (EpCAM1^{++}, Ly51^{-}), while 'C' marks cortical (EpCAM1^{+}, Ly51^{++}) epithelial compartments on Figure 1A. Single asterisk (*) marks degenerative vacuoles, while double asterisk (**) mark the loss of epithelial staining on Figure 1B. Figure 1C (lower left) shows cryostate section of 2 month, whereas figure 1D (lower right) demonstrates cryostate section of 9 month old BALB/c mouse thymus. Staining pattern: anti-EpCAM1-FITC, ER-TR7-PE, DAPI (blue). The EpCAM1^{++} thymic medulla is outlined by continuous line on Figures 1C and 1D for easier visualization.
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month and 9 month old BALB/c mice to identify epithelial and mesenchymal elements in young adult and aging thymic lobes. The above ages were selected to check additional time points and more precisely map the timeframe of thymic physiological senescence (see Figures 1C and 1D). The staining patterns are strikingly different at the two ages examined. In the 2 month old thymic tissue section a-EpCAM1 and ER-TR7-staining show little tendency for co-localization. In stark contrast, by the age of 9 months a-EpCAM1 and ER-TR7-staining show significant overlap within the thymic medulla, a phenomenon completely absent at earlier ages.

Adipose involution

To demonstrate how the disorganization of thymic epithelial network is followed by the emergence of adipocytes, thymic sections of 1.5 year old GFP-transgenic BALB/c mice were analyzed. This mouse strain develops and reproduces exactly like control BALB/c mice, and the thymic epithelial function and thymocyte maturation is indistinguishable from wild type controls [24]. However, due to the ubiquitous and strong EF1 promoter-driven transgene transcription, bright GFP expression offers a native, green-colored, cytoplasmic staining for all the cells in these mice. Thymic sections of senescent GFP-transgenic mice were co-stained with LipidTox Red to identify adipocytes. Histology shows the presence of relatively large, inflated cells in which the green-colored (GFP-containing) cytoplasm is pushed to the periphery by red-staining neutral lipid deposits, a pattern characteristic of adipose cells (see Figure 2).

Molecular changes of thymic epithelium

Having presented structural changes of thymic epithelial senescence, we set out to investigate the underlying molecular events. In order to detect gene expression changes, thymic epithelial cells were purified from 1 month and 1 year old BALB/c mice based on EpCAM1 expression (MACS separation). Following cDNA synthesis, quantitative RT-PCR analysis was performed. Several genes including Wnt4, FoxN1, PPARγ, ADRP, lamin1 and LAP2α were tested (Table 1 lists primer sequences and characteristics, see Figures 3A–D for changes in gene expression). Figure 3A shows that the expression of both Wnt4 and FoxN1 decreases in thymic epithelial cells. Highly decreased level (or total absence in some cases) of FoxN1 could be the consequence of strong Wnt4 down-regulation by the age of 1 year, indicating that thymic epithelial cells can down-regulate FoxN1 expression while maintaining that of epithelial cell surface markers like EpCAM1 [13]. At the same time, mRNA levels of pre-adipocyte differentiation markers PPARγ and ADRP rise with age in the same, EpCAM1-positive cell population (Figure 3C). This finding is in harmony with histological data demonstrating the emergence of adipocytes in the thymic lobes of senescent mice (Figure 2). The expression of lamin1, a key component of the nuclear lamina remains unaffected during senescence in thymic epithelial cells; whereas, the expression of LAP2α increases significantly (see Figure 3B). This degree of dissociation between lamin1 and LAP2α expression is of note and suggests functional differences despite conventionally anticipated association of lamin1 and LAP2 molecular family members. The measured LAP2α up-regulation associated with age-related adipose involution is, however, in perfect agreement with other literature data suggesting the pre-adipocyte differentiation-promoting effect of LAP2α in fibroblasts [17]. This is the first report to show that such, normally fibroblast associated molecular changes occur in purified thymic epithelial cells. In the literature, epithelial-mesenchymal transition is associated with differential expression of E- and N-cadherin [25]. While E-cadherin decreases, N-cadherin normally compensates for the loss of E-cadherin expression. To investigate whether the first step towards pre-adipocyte differentiation is the epithelial-mesenchymal transition of epithelial cells, gene expression changes of E-cadherin and N-cadherin were measured (Figure 3D). While E-cadherin mRNA levels significantly decreased, N-cadherin gene expression showed a slight increase, indicating that EMT might be the initial step in epithelial cell transition to become pre-adipocytes.

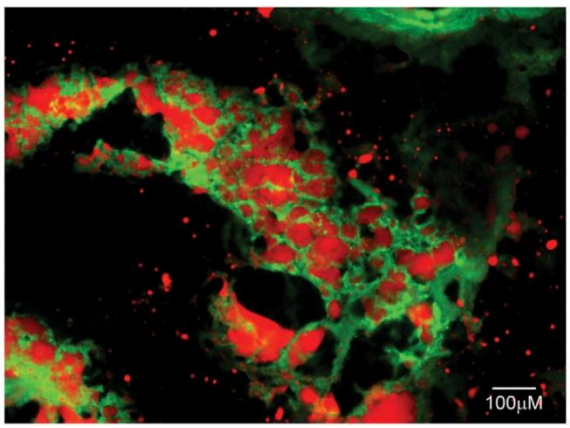

Figure 2. Adipose involution. Figure 2 shows adipose involution over cryostat section of 1.5 year old GFP-transgenic BALB/c mouse thymus. Staining pattern: GFP (green), LipidTox Red (red).
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Transgenic cell lines

Stable LAP2α over-expressing or Wnt4-secreting transgenic TEP1 cell lines were established using lentiviral transgenesis. The use of a primary-derived model cell line provides the advantage of absolute purity, the complete lack of other cell types that could potentially affect the gene expression profile of epithelial cells [26]. The established transgenic cell lines proliferated normally and did not show obvious signs of phenotypic changes (data not shown). In contrast to morphology, quantitative RT-PCR analysis revealed that LAP2α over-expression triggers an immense surge of PPARγ expression (Figure 4). Such an increase in mRNA level suggests that this is not a plain quantitative, but rather a qualitative change. ADRP a direct target gene of PPARγ was also up-regulated albeit to a lesser extent (Figure 4). On the other hand in Wnt4-secreting TEP1 cells the mRNA level of both PPARγ and ADRP was decreased (Figure 4). In the TEP1 cell line the expression of FoxN1 could not be addressed as it is very low/undetectable and remains as such with all the tested treatments (data not shown).

Transfected embryonic thymic organ cultures

To confirm the involvement of LAP2α and Wnt4 during adipogenesis through their direct effect on PPARγ expression in primary cells, murine thymic lobes were isolated from timed pregnancies at E12. Thymic lobes at the age of E12 provide an excellent experimental setting where the thymus has just been formed and there is no sign of aging. Furthermore, thymic lobes at this stage are also small enough to be both cultured and transfected as a whole, nutrients and virions have free access to most of the cells in the lobe without the need of disrupting any intercellular connection or tissue matrix [8,27]. The isolated lobes were therefore transfected with lentiviral vectors encoding GFP (mock), Wnt4 or LAP2α and were cultured for 4 days *in vitro*. Q-PCR was

Table 1. List of gene specific PCR primers.

Gene	Forward primer	Reverse primer
β-actin	5′-TGG CGC TTT TGA CTC AGG A -3′	5′-GGG AGG GTG AGG GAC TTC C - 3′
Wnt4	5′-CTC AAA GGC CTG ATC CAG AG - 3′	5′-TCA CAG CCA CAC TTC TCC AG - 3′
LAP2α	5′-TGA ACT GCA GGC AGC TAA GA-3′	5′-TCA TAG CTA GAC TCT GAG G-3′
Lamin1	5′ - TGA GTA CAA CCT GCG CTC AC -3′	5′ - TGA CTA GGT TGT CCC CGA AG -3′
PPARγ	5′ - CCC AAT GGT TGC TGA TTA CAA A -3′	5′ - AAT AAT AAG GTG GAG ATG CAG GTT CT -3′
ADRP	5′ - CGC CAT CGG ACA CTT CCT TA -3′	5′ - GTG ATG GCA GGC GAC ATC T -3′
E-cadherin	5′- AAG TGA CCG ATG ATG ATG CC -3′	5′- CTT CAT TCA CGT CTA CCA CGT -3′
N-cadherin	5′ - GTG GAG GCT TCT GGT GAA AT - 3′	5′ - CTG CTG GCT CGC TGC TT - 3′
FoxN1	Applied Biosystems TaqMan probe PN4351272 (Mm00477457_m1)	

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performed to confirm over-expression of LAP2α and Wnt4 in the embryonic thymic lobes as a result of lentiviral transgenesis (Figure 5A) and their effect on PPARγ expression was also analyzed (Figure 5B). The level of over-expression was confirmed following transfection with both LAP2α- and Wnt4-encoding viral vectors. Q-PCR analysis revealed that LAP2α over-expression triggers an increase of PPARγ expression, whereas additional Wnt4 secretion suppresses PPARγ level (Figure 5B). The latter Wnt4-mediated suppression of PPARγ expression in cultures of E12 thymic embryonic lobes was also confirmed by treatment with Wnt4-containing supernatants of Wnt4 over-expressing TEP1 cell line (data not shown). Interestingly, the expression of FoxN1 did not decrease in LAP2α over-expressing thymic lobes (data not shown), possibly due to high levels of Wnt4 in the embryonic thymic tissue preserving FoxN1 status. Our molecular studies using E12 thymic lobes confirmed our data obtained with the TEP1 cell lines, that even in embryonic thymic tissue pre-adipocyte differentiation markers can be up-regulated in the presence of LAP2α, indicating that the process can be dissected and controlled at a molecular level.

Conclusion

Here we show that with senescence, thymic epithelial Wnt4 secretion decreases, possibly below a threshold level that is required to maintain the identity of established thymic epithelial cells. This is measured by the loss of FoxN1 expression, a key transcription factor defining thymic epithelial cell identity. However, these epithelial cells still express cell surface markers

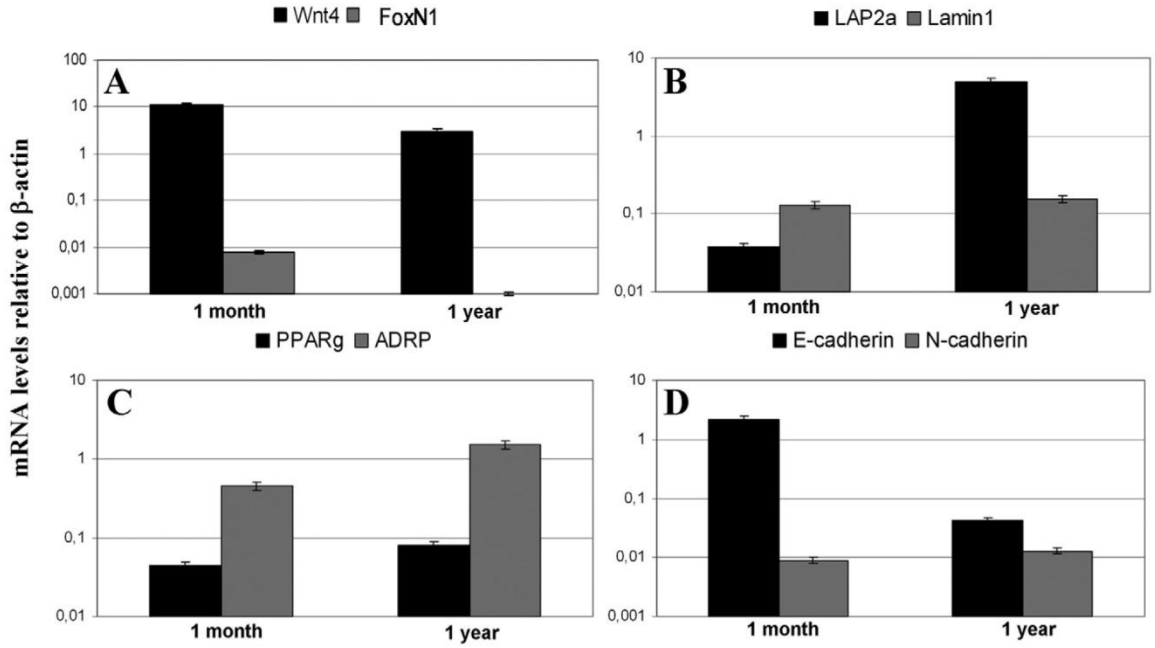

Figure 3. Molecular changes in thymic epithelium. Figures 3A–D demonstrate gene expression changes of MACS purified thymic epithelial cells measured by Q-PCR. Please note that the Y-axis scale is logarithmic. Error bars show ±1 SD.
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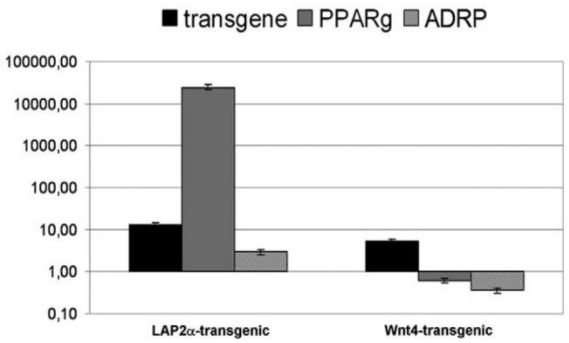

Figure 4. Confirmation in transgenic thymic cell lines. Figure 4 shows gene expression changes of LAP2α and Wnt4 over-expressing transgenic TEP1 cells measured by Q-PCR. Please note that Y-axis scale is logarithmic. Error bars show ±1 SD.
doi:10.1371/journal.pone.0010701.g004

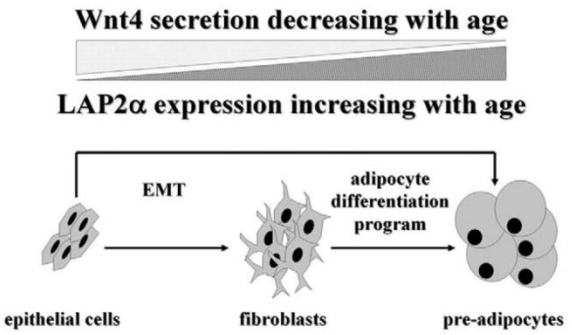

Figure 6. Model for thymic epithelial senescence. Figure 6 demonstrates our molecular level model of thymic adipose involution. Decreasing Wnt4 and increasing LAP2α levels promote epithelial cells to differentiate into pre-adipocytes either directly or indirectly via EMT.
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characteristic for thymic epithelial cells – i.e. EpCAM1. Wnt4 deprivation opens up an opportunity for trans-differentiation into pre-adipocytes. The simultaneous increase in LAP2α expression provides the necessary signal that pushes de-differentiated thymic epithelial cells to differentiate into pre-adipocytes, as detected by increased mRNA levels of PPARγ and ADRP.

We propose two different mechanisms for the process of adipose involution (see Figure 6). The first allows for the direct initiation of pre-adipocyte differentiation from de-differentiated thymic epithelial cells due to the down-regulation of Wnt4 and up-regulation of LAP2α. Although we cannot rule out this first model, we favor the second model where the process occurs indirectly: de-differentiation of thymic epithelial cells triggers EMT first, and then the resulting fibroblasts undergo the conventional route of differentiation program towards adipocyte-lineage commitment. The latter model certainly fits better with current literature of EMT [28] and is also supported by our histological and molecular results. Co-localization of a-EpCAM1 and ER-TR7-staining in the aging thymic medulla (Figure 1D) confirms that in the 9 month old thymus there are cells expressing the EpCAM1 marker as a legacy of their primary origin, and also secreting ER-TR7-positive extracellular matrix components, a function conventionally attributed to fibroblast cells. Moreover, Q-PCR data obtained with cDNA samples of MACS-purified thymic epithelial cells also demonstrate an age-related shift in cadherin expression levels characteristic for EMT (Figure 3D) providing additional evidence for the active process of EMT during thymic epithelial senescence.

Our model of thymic epithelial senescence is based on data obtained with mice undergoing physiological senescence. This is the first model for the molecular basis of the thymic epithelium to undergo adipose involution. This model withstands molecular level proof-of-principle using both a model cell line and primary embryonic thymic organ cultures rendered transgenic by lentiviral transgenesis.

Perspectives

Further experiments, however, are required. We plan using inducible, LAP2α-transgenic mice to allow us precise temporal-spatial over-expression of LAP2α in adult thymic epithelium to model and decisively verify the role of LAP2α in pre-adipocyte trans-differentiation *in vivo* exploiting our experience in establishing transgenic animals [24,29]. If LAP2α proves to be a master regulator of thymic adipose involution *in vivo* too, this knowledge appoints LAP2α as target molecule for directed rejuvenation of the thymic epithelial structure and function. This rejuvenation process could theoretically reinforce naïve T-cell output to reach young adult levels that could ameliorate senescence-related immunological disorders like impaired antiviral defense and late-onset autoimmune diseases.

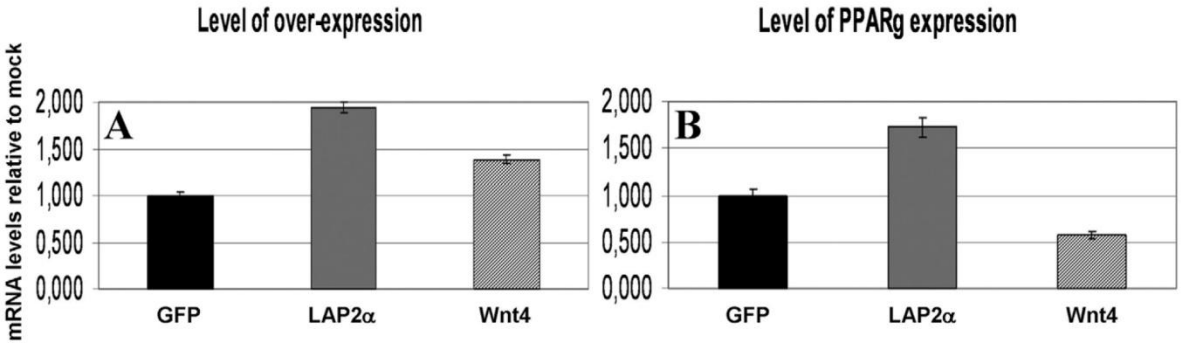

Figure 5. Confirmation in transfected thymic lobes. Figures 5A–B present gene expression changes measured by Q-PCR from cDNA of murine thymic lobes transfected at E12 and cultured for 4 days *in vitro*. Please note that Y-axis scale is linear. Error bars show ±1 SD.
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Methods

Cell lines and mice

The 293T (ATCC: CRL-11268) and TEP1 [26] cell lines were cultured in DMEM supplemented with 10% FCS, penicillin, streptomycin and β-mercapto-ethanol (Lonza Walkersville). For the experiments we used thymic lobes from timed pregnancies at E12, and also from adult BALB/c mice at 4 week and 1 year of age, and from 1.5 year old GFP-transgenic BALB/c-mice. Mice were bred in our animal facility; all animal work has been conducted according to relevant national and international guidelines following approval of ethics committee of the University of Pecs. Senescent animals developed and aged normally, without any treatment.

Transgenic cell, organ and animal models

The GFP-transgenic BALB/c model was created using lentiviral transgenesis as published by our group [24]. The Wnt4 sequence was purchased and subcloned from an Origene (Origene) vector containing human full-length Wnt4 cDNA. The full-length murine LAP2α cDNA containing plasmid was a kind gift of Dr. Simon Amos. The GFP (mock), LAP2α or Wnt4 over-expressing TEP1 cell lines or E12 thymic lobes were generated using lentiviral vectors that were prepared as described previously [30]. Following overnight lentiviral transfection the thymic lobes were transferred over Nucleopore Track-Etch Membranes (Whatman) and were cultured in DMEM supplemented with 20% FCS, penicillin, streptomycin, ciprofloxacin, amphotericin-B and β-mercapto-ethanol (Lonza Walkersville).

Histology using fluorescent antibodies, proteins and dyes

Sections (9 μm) of frozen thymic lobes of BALB/c mice were fixed in cold acetone, then dried and blocked using 5% BSA in PBS for 20 min before staining with a-Ly51-PE (clone 6C3), a-EpCAM-FITC (clone G8.8), ER-TR7-PE antibodies and DAPI. Thymic sections of GFP-transgenic mice were fixed in 4% paraformaldehyde before staining with LipidTOX Red following the manufacturer's instructions (Invitrogen). The sections were analyzed using an Olympus BX61 microscope equipped with a CCD camera and AnalySIS software.

Separation and enrichment of thymic epithelial cells

Thymic lobes were digested with type F collagenase from *C. hystolyticum* (Sigma) for 30 min, then washed with DMEM 10% FCS. Cell suspensions were then labeled with anti-EpCAM1-FITC (clone G8.8) and washed with MACS-buffer followed by incubation with anti-FITC micro-beads (Miltenyi Biotec), the $EpCAM^+$-cells were used for total RNA isolation and subsequent quantitative PCR analysis. The cells were purified using MACS LS separation columns (Miltenyi Biotec).

RNA isolation, preparation of cDNA, Q-PCR analysis

Total RNA was isolated the RNAII kit (Macherey-Nagel), including an on column DNA digestion step. cDNA was constructed using the high capacity RNA to cDNA kit (Applied Biosystems). For Q- PCR analysis, we used an AB7500 platform and either SYBR green or TaqMan PCR master mix (Applied Biosystems). Gene expression was normalized to β-actin. The sequences and data of primers are listed in Table 1.

Statistical analysis

All experiments were performed on three occasions, representative experiments are shown. Measures were obtained in triplicates; data are presented as mean ±1 SD by error bars.

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Author Contributions

Conceived and designed the experiments: KK GA EJJ JEP. Performed the experiments: KK ZV DB SH SP. Analyzed the data: KK ZV DB SH SP GA EJJ JEP. Contributed reagents/materials/analysis tools: KK GA EJJ JEP. Wrote the paper: KK JEP.

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Overexpression of ICAT highlights a role for catenin-mediated canonical Wnt signalling in early T cell development

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Transcription factors of the T cell factor/lymphoid enhancing factor (Tcf/Lef) family are key regulators in the development of T cell precursors to the $CD4^+8^+$ stage. These factors are known targets of the canonical Wnt signalling pathway, and regulate transcription of Wnt target genes following interaction with the armadillo repeat-containing protein β-catenin. However, as recent studies show normal thymocyte maturation in the absence of either β-catenin or its homologue γ-catenin, the role of Wnt signalling in Tcf/Lef activation during T cell development is controversial. To directly investigate the importance of catenin-mediated Wnt signalling in early thymocytes, we have compared the expression of β- and γ-catenin and analysed distinct stages of T cell precursor maturation following overexpression of inhibitor of β-catenin and Tcf (ICAT), which inhibits Wnt signalling by preventing binding of armadillo repeat-containing proteins to Tcf/Lef. By direct retroviral gene targeting of $CD4^-8^-$ and $CD4^+8^+$ precursors, we show that ICAT overexpression inhibits the $CD4^-8^-$-to-$CD4^+8^+$ transition, but not the $CD4^+8^+$-to-$CD4^+8^-$ or -$CD4^-8^+$ transition. Collectively, our data support a model in which canonical Wnt signalling influences T cell development in the thymus by playing an essential role in the maturation of $CD4^-8^-$ but not $CD4^+8^+$ thymocytes.

Key words: Cell differentiation · Cellular immunology · Thymopoiesis

Introduction

The generation of functionally competent T cells requires blood-borne migrant precursors to enter the thymus and undergo a series of developmental events that are regulated by the surrounding stromal microenvironment [1, 2]. Two important checkpoints during T cell development are regulated by cell-surface-expressed T cell receptor (TCR) complexes [3]. Thus, immature $CD4^-8^-$ T cell precursors which have undergone successful in-frame TCR β chain rearrangement receive signals through the pre-TCR complex to proliferate and express both CD4 and CD8 co-receptors [4, 5]. The fate of $CD4^+8^+$ cells is then determined by αβTCR interactions with self peptide/MHC complexes to eliminate potentially autoreactive cells and generate self MHC-restricted $CD4^+$ and $CD8^+$ T cells which exit the thymus for the periphery [6–8].

In addition to TCR-mediated signals, thymic epithelial cells are also known to be key mediators of thymocyte differentiation, and regulate several signalling pathways that play a role during thymocyte development. Thus, thymic epithelial cells express

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Abbreviations: DN: double-negative · **Fz:** frizzled · **ICAT:** inhibitor of β-catenin and T cell factor · **Lef:** lymphoid enhancing factor · **Tcf:** T cell factor

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Notch ligands, with Notch/Notch ligand interactions generating signals implicated in a number of developmental events, including commitment to the T cell lineage [9], and the αβ/γδ [10] and CD4/CD8 lineage choices [11]. In addition, thymic epithelial cells express genes encoding members of the Wnt family of secreted glycoproteins known to play a widespread role in development [12]. In conjunction with the expression of Wnt-binding frizzled (Fz) receptors by developing thymocytes, these findings suggest that Wnt production by thymic epithelium is another mechanism whereby the thymic stromal microenvironment regulates T cell precursor maturation [12]. Consistent with this, Wnt-1- and Wnt-4-deficient mice show a reduction in overall thymocyte numbers [13], while inhibition of Wnt binding to cell surface Fz receptors by retroviral expression of soluble Fz molecules has also been shown to inhibit the development of fetal-liver-derived precursor in fetal thymus organ cultures [14].

A known consequence of Wnt binding to Fz receptors is activation of the canonical Wnt signalling pathway resulting in hypophosphorylation and consequent stabilisation of β-catenin, which translocates to the nucleus where it complexes with the transcription factors of the T cell factor/lymphoid enhancing factor (Tcf/Lef) family to induce target gene transcription [15, 16]. Importantly, Tcf-1 and Lef-1 are expressed by T cell precursors and have been shown to play a key role during early stages of T cell development. Most notably, Lef-1-knockout mice that express low levels of a truncated form of Tcf-1 [$Tcf^{\Delta 5/\Delta 5}Lef\text{-}1^{-/-}$] show a profound block in thymocyte development, prior to the appearance of $CD4^+8^+$ cells [17, 18].

However, despite these findings supporting a role for Wnt signalling and Tcf/Lef activation during thymocyte development, the role of the canonical β-catenin-mediated Wnt signalling pathway in mediating these responses is controversial. Thus, deletion of β-catenin in $CD4^-8^-$ T cell precursors has been reported to result in either a partial blockade at the $CD4^-8^-$ stage [19], or to have no discernable effect on T cell development in the thymus [20]. One possibility for this discrepancy is that Wnt-mediated activation of Tcf/Lef in the absence of β-catenin is instead due to the homologue γ-catenin, which is also capable of activating members of this family of transcription factors [21]. Indeed, γ-catenin-deficient mice have recently been shown to have normal T cell development [22], suggesting the possibility of functional redundancy between β- and γ-catenin. Alternatively, the essential requirement for Tcf/Lef activation in thymocyte maturation may be mediated by non-canonical, β- and γ-catenin-independent pathways.

In this study we have attempted to distinguish between these possibilities and clarify the potential importance of canonical Wnt signalling in the thymus by comparing the availability of both β- and γ-catenin in developing thymocytes and by overexpressing inhibitor of β-catenin and Tcf (ICAT), a naturally occurring negative regulator of the canonical Wnt signalling pathway [23–25], at specific stages of T cell development. ICAT inhibits Tcf/Lef activation by binding to armadillo repeats present within both β-catenin and γ-catenin, but not other more distantly related members of the armadillo repeat-containing family of proteins [23–25], thereby preventing binding of these potential mediators of the canonical pathway to Tcf/Lef.

We show that both γ-catenin and β-catenin are present throughout the $CD4^-8^-$ double-negative (DN) 1–4 stages of T cell development and that overexpression of ICAT in immature T cell precursors results in a developmental block in T cell development prior to the $CD4^+8^+$ stage. In contrast, restricting overexpression of ICAT to $CD4^+8^+$ thymocytes does not prevent their ability to generate $CD4^+8^-$ and $CD4^-8^+$ single-positive thymocytes, indicating that canonical Wnt signalling is not essential for these later events. Collectively, our data provide direct evidence that mediation of Wnt signalling by β-catenin or its homologues plays an important but stage-specific role during T cell development.

Results

Both β- and γ–catenin are expressed throughout $CD4^-8^-$ stages of thymocyte maturation

Gene targeting studies have indicated that a normal programme of T cell development can occur in the absence of either β- or γ-catenin [20, 22]. However, these observations do not exclude the possibility of functional redundancy between these molecules, since both are able to activate members of the Tcf/Lef family, although the ability of γ-catenin to activate Tcf-1 is still unclear [21]. Although it has been suggested that γ-catenin, unlike β-catenin, is not readily detected in T cells [20], this has not been examined in detail in relation to developing thymocyte subsets during fetal thymus development. To ascertain whether both β-catenin and γ-catenin are available during the developmental stages when Tcf/Lef activation has been implicated in thymocyte maturation, we carried out RT-PCR analysis to analyse expression of these genes in purified DN thymocyte subsets. As shown in Fig. 1, mRNA for both molecules is detectable throughout the DN1 to DN4 $CD4^-8^-$ stages. Thus, these findings provide support for the possibility that Wnt-induced Tcf/Lef activation in early thymocyte development could be mediated by either β- or γ-catenin.

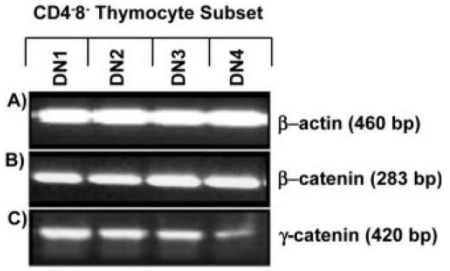

Fig. 1. β-Catenin and γ-catenin are expressed throughout the $CD4^-8^-$ stages of T cell development. Freshly purified $CD4^-8^-$ thymocytes were subdivided into $CD44^+25^-$ (DN1), $CD44^+25^+$ (DN2), $CD44^-25^+$ (DN3) and $CD44^-25^-$ (DN4) subsets, and analysed by RT-PCR for expression of β-catenin (B) and γ-catenin (C), with equal loadings of cDNA being assessed by analysing β-actin (A). Data shown are representative of three separate experiments.

Overexpression of ICAT blocks T cell development prior to the $CD4^+8^+$ stage

The naturally occurring negative regulator of the canonical Wnt signalling pathway, ICAT, has been shown to interact with armadillo repeats present in both β- and γ-catenin, thereby inhibiting interaction of these molecules with Tcf/Lef [23–25]. To investigate the effects of ICAT-mediated inhibition in primary developing thymocytes, we prepared retroviral constructs containing either GFP alone or GFP-ICAT to enable identification of successfully transfected cells and a measure of the level of construct expression. Exposure of E14 $CD4^-8^-$ thymocyte suspensions to retroviral supernatants followed by re-incorporation into reaggregate thymus organ cultures resulted in the appearance of clearly defined GFP^+ populations within 24 h (Fig. 2A, B). Importantly, when these GFP^+ populations were sorted and subjected to analysis by RT-PCR, overexpression of ICAT in the Mig-ICAT-infected cells was clearly evident as compared to the low levels of endogenous ICAT seen in cells transfected with GFP alone (Fig. 2C).

Moreover, to demonstrate the ability of ICAT overexpression to directly inhibit Wnt signalling in thymocytes, we analysed expression of the BMP and activin membrane-bound inhibitor (BAMBI) gene, which is regulated by catenin-mediated Wnt signalling [26]. RT-PCR analysis of control GFP-only and GFP-ICAT thymocytes showed that while mRNA for BAMBI is clearly detectable in control GFP^+ cells, expression is clearly down-regulated in GFP-$ICAT^+$ thymocytes (Fig. 2C). These findings indicate the feasibility of inhibiting canonical Wnt signalling through use of retrovirus-mediated overexpression of ICAT in primary T cell precursors.

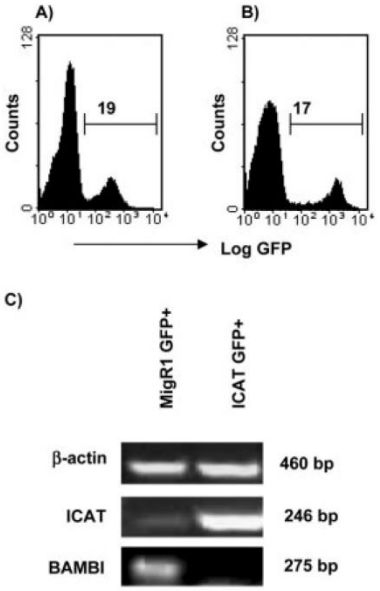

Fig. 2. Overexpression of ICAT in T cell precursors. Retroviral constructs encoding only GFP (MigR1-GFP) or GFP together with ICAT (Mig-ICAT) were used to infect $CD4^-8^-$ thymocytes. (A, B) Typical levels of GFP expression after overnight culture following infection with MigR1-GFP and Mig-ICAT, respectively. GFP^+ thymocytes identified after MigR1-GFP and Mig-ICAT infection were also sorted by MoFlo and analysed for ICAT expression by RT-PCR (C). Note the overexpression of ICAT in Mig-ICAT-treated cells, as compared to MigR1-GFP-treated cells. Panel (C) also shows a comparison of expression of the Wnt target gene, BAMBI, which is reduced in GFP-ICAT thymocytes as compared to GFP-only thymocytes. Data shown are representative of three separate experiments.

Utilising the approach outlined above, we next analysed the effects of ICAT overexpression on the ongoing maturation of T cell precursors. To ensure ICAT expression from the earliest stages of thymocyte maturation, freshly disaggregated fetal liver preparations were sorted to obtain $CD45^+$ T cell precursors at a purity >99% (not shown). Purified precursors were then infected with either MigR1-GFP or Mig-ICAT by resuspension in retroviral supernatants and cultured overnight in the presence of IL-7 and Flt-3 ligand. After overnight culture, cells were harvested and used as a source of precursors to repopulate alymphoid 2-deoxyguanosine-treated thymus lobes in hanging drop cultures as described previously [27]. Repopulated thymus lobes were organ-cultured for a further 11 days, and then teased apart to liberate thymocytes that were analysed for expression of GFP, CD4 and CD8 by flow cytometry.

Analysis of thymocytes harvested after infection with MigR1-GFP (Fig. 3A) or Mig-ICAT (Fig. 3E) showed

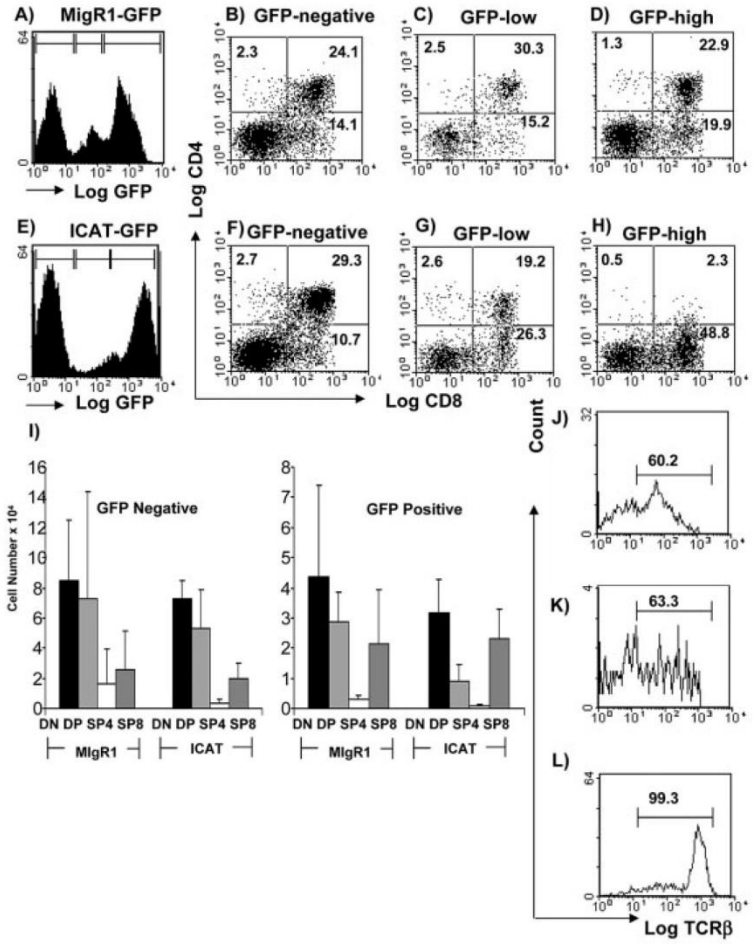

Fig. 3. ICAT overexpression blocks T cell development prior to the $CD4^+8^+$ stage. $CD45^+$ fetal liver precursors were infected with MigR1-GFP or Mig-ICAT retroviruses and then used to recolonise alymphoid thymus lobes. Cultures were harvested after 11 days and analysed by flow cytometry. (A, E) GFP expression in MigR1-GFP and Mig-ICAT cultures respectively, with populations of GFP^-, GFP^{low} and GFP^{high} cells being detectable (B–D, F–H). Each subset was also analysed for expression of CD4 and CD8 [(B–D) for MigR1-GFP, (F–H) for Mig-ICAT]. Also shown is analysis of TCRβ expression in $CD4^-8^+$ cells from MigR1-GFP (J) and Mig-ICAT (K) cultures, compared to $CD4^-8^+$ cells from adult thymus (L). In the experiment shown, 2.5×10^5 cells were obtained from MigR1-GFP thymus lobes, while 2.7×10^5 cells were recovered from Mig-ICAT thymus lobes. Note that in contrast to control cultures, overexpression of ICAT causes a block in the appearance of $CD4^+8^+$ cells. Data shown are representative of four separate experiments. Results shown in (I) are averaged from three independent experiments, and are presented with standard deviations (error bars).

comparable levels of GFP expression, with clear subsets of GFP^-, GFP^{low} and GFP^{high} cells being detected in both cases. Strikingly, in contrast to the normal pattern of T cell development seen in control (MigR1-GFP) cultures at all levels of GFP expression (Fig. 3B–D), overexpression of ICAT was found to have a negative effect on the progression of T cell precursors from the $CD4^-8^-$ to the $CD4^+8^+$ stage. Notably, increasing levels of ICAT overexpression as indicated by GFP expression level, resulted in a dose-dependent decrease in the generation of $CD4^+8^+$ cells (Fig. 3F–I).

Although overexpression of ICAT resulted in a decrease in the absolute cell number of $CD4^+8^+$ thymocytes, and a greater percentage of $CD4^-8^+$ was also observed compared to control cultures, no increase in absolute numbers of $CD4^-8^+$ cells was observed when ICAT was overexpressed (Fig. 3I). Whether this is due to an impact of ICAT overexpression on $CD4^-8^+$ cell survival is not clear. In addition, the $CD4^-8^+$ cells overexpressing ICAT do not appear to be typical mature $CD8^+$ cells, as although some cells were found to be expressing TCRβ (Fig. 3K), the level of expression is

clearly lower than that observed on mature $CD4^-8^+$ thymocytes (Fig. 3 L). That TCRβ expression was found to be similar in $CD4^-8^+$ cells from both control and ICAT cultures (Fig. 3J, K) is consistent with the notion that overexpression of ICAT inhibits development after the TCRβ selection checkpoint. Our results showing that thymocytes overexpressing ICAT fail to reach the $CD4^+8^+$ stage of thymocyte development yet are able to rearrange TCRβ are in agreement with the reported requirement for Tcf-1/Lef activation following pre-TCR signalling [17, 18], and provide support for a requirement for catenin-mediated canonical Wnt signalling in progression beyond this stage.

ICAT-mediated inhibition of Wnt signalling does not prevent $CD4^+8^+$ thymocyte maturation

Recent studies have reported that the partial block in the production of $CD4^+8^+$ thymocytes seen following conditional deletion of β-catenin in immature precursors is also accompanied by a reduction in the production of mature $CD4^+8^-$ and $CD4^-8^+$ single-positive cells [19]. In addition, expression of a stabilised form of β-catenin in $CD4^-8^-$ precursors deficient in either pre-TCR or αβTCR signalling results in the generation of both $CD4^+8^+$ and $CD4^+8^-/CD4^-8^+$ cells, respectively [28]. These studies in addition to analysis of β-catenin-transgenic mice [29] imply that as well as playing a role in the generation of $CD4^+8^+$ thymocytes, β-catenin signalling may be involved in positive selection to the single-positive stages. However, it is unclear from these studies on the established 'steady-state' thymus whether these effects on single-positive cell production are a reflection of the reduced availability of $CD4^+8^+$ precursors for positive selection, or whether they relate to effects on the survival of single-positive thymocytes. Alternatively, it is possible that the Wnt/β-catenin/Tcf pathway is directly involved in the regulation of changes in gene expression accompanying positive selection and differentiation to the $CD4^+$ and $CD8^+$ stages.

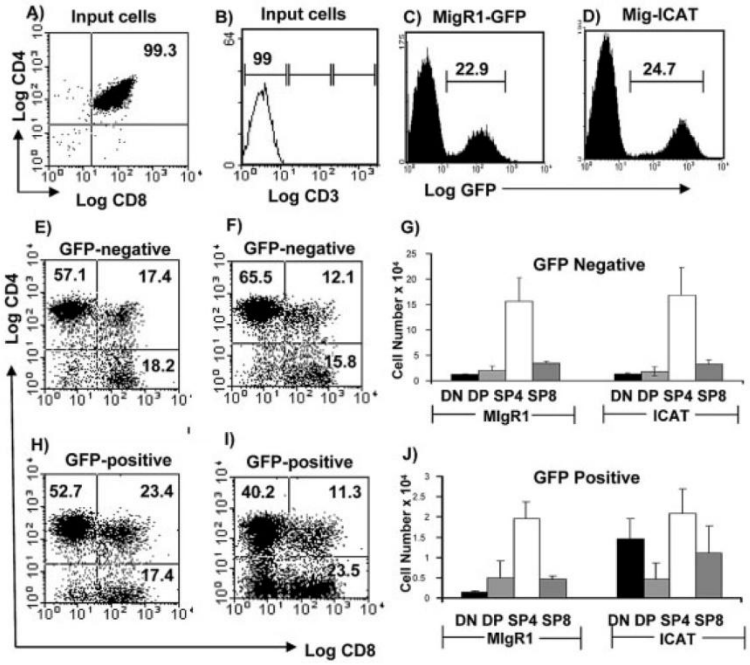

Fig. 4. Effects of ICAT overexpression on positive selection of $CD4^+8^+$ thymocytes. Freshly purified $CD4^+8^+CD3^-$ thymocytes [(A, B), gating in (B) to show $CD3^-$, $CD3^{low}$ and $CD3^{high}$ subsets] were infected with MigR1-GFP or Mig-ICAT retroviruses and incorporated into reaggregate thymus cultures. After 5 days, cultures were harvested and analysed by flow cytometry for GFP expression MigR1 (C) and Mig-ICAT (D) and CD4, CD8 (E–I). In the experiment shown, from an input of 7.5×10^5 $CD4^+8^+$ thymocytes, 2.7×10^5 thymocytes were recovered from MigR1 reaggregate thymus organ cultures, while 3.4×10^5 thymocytes were recovered from Mig-ICAT reaggregate thymus organ cultures. Note the presence of mature $CD4^+$ and $CD8^+$ single-positive thymocytes in the GFP^- and GFP^+ populations in MigR1-GFP- (E and H) and Mig-ICAT-treated cultures (F and I), indicating that ICAT overexpression does not inhibit positive selection of $CD4^+8^+$ thymocytes. Data shown are representative of three separate experiments. Results shown in (G, J) are averaged from three independent experiments, and are presented with standard deviations (error bars).

To explore these possibilities, and circumvent the block in development at the $CD4^-8^-$ stage, or any perturbations in the production/developmental potential of $CD4^+8^+$ cells resulting from inhibition of the Wnt/β-catenin/Tcf pathway in their $CD4^-8^-$ progenitors, we devised a strategy enabling us to overexpress ICAT directly at later stages of thymocyte development and allow analysis of the developmental progression of a defined cohort of $CD4^+8^+$ cells. Thus, early-stage proliferating $CD4^+8^+TCR^-$ thymocytes at a pre-positive selection stage of development were isolated to high purity (Fig. 4A, B) as described [30, 31], retrovirally infected with either MigR1-GFP or Mig-ICAT retroviruses, and used in 1:1 ratios with thymic stromal cells to make reaggregate thymus organ cultures. After 5 days, cultures were harvested and analysed by flow cytometry for GFP, CD4 and CD8 expression (Fig. 4). Successful infection of $CD4^+8^+$ cells, indicated by GFP expression (Fig. 4C, D), demonstrates the overall feasibility of using this approach to target specific developmental subsets to study T cell maturation.

Comparison of the GFP^+ population in MigR1-GFP control (Fig. 4 H) and Mig-ICAT reaggregate cultures (Fig. 4I) revealed the presence of populations of $CD4^+$ and $CD8^+$ cells in both cultures, although an increased population of $CD4^-8^-$ cells was present in the Mig-ICAT cultures, possibly reflecting increased susceptibility of catenin-inhibited $CD4^+8^+$ cells to apoptosis [28]. Overall, however, these results provide clear evidence that the generation of single-positive $CD4^+$ and $CD8^+$ cells does not have the same dependence on catenin-mediated Wnt signalling as that required for the maturation of $CD4^-8^-$ cells, and argue strongly against an essential requirement for Wnt/catenin/Tcf signalling in the maturation of $CD4^+8^+$ thymocytes.

Discussion

Despite evidence that activation of transcription factors of the Tcf/Lef family is crucial in early T cell development [17, 18], the role of the β-catenin-mediated canonical Wnt signalling pathway in regulating these activities remains controversial [19, 20, 28, 29]. Here we show that β-catenin and γ-catenin, both of which can activate Tcf/Lef, are expressed during early thymocyte development, suggesting that these two mediators of Wnt signalling may be functionally redundant. Moreover, by adopting a strategy to inhibit the interaction of both these molecules with Tcf/Lef by overexpression of ICAT, we provide evidence in support of this notion. Thus, overexpression of ICAT, which inhibits binding of both β- and γ-catenin to Tcf/Lef family members [23–25], causes a marked arrest in the development of $CD4^-8^-$ precursors to the $CD4^+8^+$ stage. This may reflect a block in further differentiation to the $CD4^+8^+$ stage or a failure of newly generated $CD4^+8^+$ cells to survive or expand their numbers in the absence of Wnt signalling, although the virtual absence of $CD4^+8^+$ cells at the highest levels of ICAT expression (Fig. 3) favours the first possibility.

In contrast to the block in the $CD4^-8^-$-to-$CD4^+8^+$ transition, using a novel strategy to achieve direct retroviral targeting of ICAT expression in pre-selection $CD4^+8^+$ thymocytes, we show that catenin-mediated Tcf/Lef activation is not an essential requirement for maturation to the $CD4^+$ or $CD8^+$ single-positive stage. These findings argue that, independently of any involvement of catenin-mediated signalling in the generation or survival of $CD4^+8^+$ cells [28, 32], this signalling pathway is not directly required during later stages of thymocyte development leading to the generation of $CD4^+$ and $CD8^+$ cells.

Overall, our results showing inhibition of T cell development are in favour of a role for catenin-mediated Wnt signalling in early thymocyte development, and we would suggest that the lack of effect on T cell development noted in the absence of either γ-catenin or β-catenin is a result of functional redundancy, as both these molecules are expressed in $CD4^-8^-$ T cell precursors (Fig. 1). Moreover, our findings fit well with studies on Tcf-1/Lef-deficient mice showing a stage-specific requirement for the activation of these factors in early T cell development [17, 18], and with thymus organ culture experiments where blocking of Wnt binding using soluble Fz receptors resulted in an arrest in T cell development at the $CD4^-8^+$ ISP stage [14]. However, in the latter case, the blockade in T cell development could be a consequence of perturbation of epithelial cell function, as recent studies demonstrate that Wnt signalling is also important in the functional development of thymic epithelium [33]. The direct targeting of ICAT expression specifically to T cell precursors in our studies excludes this possibility.

In conjunction with our previous observations on differential Wnt expression by thymocytes and thymic epithelial cells [12], our findings also suggest that Wnt production by thymic epithelium may be part of the essential role played by the latter in early T cell development. We have also previously shown that specific Fz receptors are expressed in a developmentally regulated pattern on developing thymocytes [12]. How individual Wnt and their binding to particular Fz receptors influence T cell precursor maturation is an important area for further study in understanding the role played by these molecules in the regulation of intrathymic T cell development.

Materials and methods

Animals

Balb/c (H-2^d) mice, housed under SPF conditions at the Biomedical Services Unit, University of Birmingham, were the source of fetal and neonatal thymic material. In the case of embryonic mice, the day of detection of a vaginal plug was designated as gestational day (E) 0.

Purification of thymocyte subpopulations

$CD4^-8^-$ T cell precursors were separated into DN1–4 subsets on the basis of expression of CD25 and CD44, using antibody-coated DynaBeads (Dynal, Wirral, UK) as described [34]. For prethymic precursors, fetal livers from E14 embryos were mechanically disaggregated and stained with allophycocyanin-conjugated anti-CD45 antibodies, and MoFlo-sorted (Dako-Cytomations) to a purity of greater than 99% (not shown). Pre-selection $CD4^+8^+TCR^-$ thymocytes were purified (>99% purity) by immunomagnetic bead selection to from neonatal thymus lobes, as described [30, 31].

Reverse transcription polymerase chain reaction

RT-PCR was conducted as described previously [35]. Samples were matched for β-actin to ensure equal cDNA loading. Primer sequences used in PCR reactions were: β-actin (5′): 5′-GTTACCAACTGGGACGACA-3′, (3′): 5′-TGGCCATCTCCTGC-TCGAA-3′, product size: 460 bp; ICAT (5′): 5′-ATGAACCGC-GAGGGAGCA-3′, (3′): 5′-CTACTGCCTCCGGTCTTCC-3′, product size: 246 bp; β-catenin (5′): 5′-CACAACCTTTCTCAC-CACC-3′, (3′): 5′-GCTTGCTCTCTTGATTGCC-3′, product size: 283 bp; γ-catenin (5′): 5′-GACCTGCAACAACAGCAAAA-3′, (3′): 5′-CTCCTCCATCCTCACACCAT-3′, product size: 420 bp; BAMBI (5′): 5′-AAACCGGTATCAGCATGACA-3′, (3′): 5′-TGCACTCCAAGTCCAACTTT-3′, product size: 275 bp.

Retroviral constructs and retroviral infection of T cell precursors

ICAT sequence was amplified by PCR reaction using forward (5′) 5′-ATGAACCGCGAGGAGCA-3′ and reverse (3′) 5′-CTACTGCCTCCGGTCTTCC-3′ primer sequences and cloned into the bi-cistronic MigR1 (a kind gift from W. S. Pear, Department of Pathology and Laboratory Medicine, University of Pennsylvania, PA) retroviral vector. Retrovirus was produced by transfecting the plasmid DNA into the Phoenix (PHX) packaging cell line (American Type Culture Collection, Rockville, MD) using Lipofectamine 2000 (Invitrogen). Retroviral supernatants were generated from retroviral vectors containing ICAT-GFP or MigR1-GFP in PHX cells.

$CD45^+$ fetal liver precursors or $CD4^+8^+$ thymocytes were resuspended in 250 μL of retroviral supernatant and 50 μL of DMEM containing 0.8 μg/mL polybrene at a density of 5×10^4 per well in 96-well plates. For fetal liver cells, medium was supplemented with Flt3 ligand and IL-7 (obtained from Peprotech) each at a final concentration of 5 ng/mL. Plates were centrifuged for 60 min at 2100 rpm at room temperature. For $CD4^+8^+$ thymocytes, cells were then recovered and used immediately for the formation of reaggregate cultures. In the case of fetal liver cells, cells were cultured overnight in 5 ng/mL Flt3 ligand and IL-7, and used to colonise alymphoid 2-deoxyguanosine-treated thymus lobe in hanging drop cultures as described [27]. After overnight culture, recolonised lobes were organ-cultured for 11 days [27].

Reaggregate thymus organ cultures

Thymocytes and thymic epithelial cells were mixed together by centrifugation at a ratio of 1:1, and the cell pellet was transferred to the surface of a 0.8-μm filter in organ culture [30, 31]. After the specified time period, cultures were teased apart, and recovered thymocytes were stained and analysed by flow cytometry.

Monoclonal antibodies and flow cytometry

Thymocytes harvested from reaggregate and fetal thymus organ cultures were immunostained with PE-conjugated anti-CD4 (clone GK1.5) and allophycocyanin-conjugated anti-CD8 (clone 53-6.7) antibodies (both eBioscience). Immunostained cells were analysed using an LSR flow cytometer (Becton Dickinson), with forward scatter and side scatter gates set to exclude non-viable cells. Both GFP^- and GFP^+ thymocytes were analysed for CD4 and CD8 expression. To analyse TCRβ expression, thymocytes harvested from control GFP-only and GFP-ICAT cultures were first depleted of $CD4^+$ cells using anti-CD4-coated Dynabeads (Dynal) to remove $CD4^+8^+$ thymocytes. Resultant suspensions were then stained sequentially with biotinylated anti-TCRβ (clone H57-597; eBioscience) and a cocktail of strepavidin-PE (eBioscience) and allophycocyanin-conjugated anti-CD8. TCRβ expression in retrovirally infected $CD4^-8^+$ thymocytes was then analysed by gating on GFP^+CD8^+ cells in MigR1-GFP and Mig-ICAT cultures.

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Modeling TCR Signaling Complex Formation in Positive Selection[1]

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T cell receptor signaling in the thymus can result in positive selection, and hence progressive maturation to the $CD4^+8^-$ or $CD4^-8^+$ stage, or induction of apoptosis by negative selection. Although it is poorly understood how TCR ligation at the $CD4^+8^+$ stage can lead to such different cell fates, it is thought that the strength of signal may play a role in determining the outcome of TCR signaling. In this study, we have characterized the formation of an active signaling complex in thymocytes undergoing positive selection as a result of interaction with thymic epithelial cells. Although this signaling complex involves redistribution of cell surface and intracellular molecules, reminiscent of that observed in T cell activation, accumulation of GM1-containing lipid rafts was not observed. However, enforced expression of the costimulatory molecule CD80 on thymic epithelium induced GM1 polarization in thymocytes, and was accompanied by reduced positive selection and increased apoptosis. We suggest that the presence or absence of CD80 costimulation influences the outcome of TCR signaling in $CD4^+8^+$ thymocytes through differential lipid raft recruitment, thus determining overall signal strength and influencing developmental cell fate. ***The Journal of Immunology,*** **2003, 171: 2825–2831.**

In addition to its role in T cell activation, TCR signaling plays a crucial role in regulating thymocyte selection at the $CD4^+8^+$ stage (1). Thus, high affinity/avidity TCR-MHC interactions lead to negative selection via induction of apoptosis, while low affinity/avidity interactions promote thymocyte survival and maturation (2, 3). This maturation results in the generation of MHC class I-restricted $CD4^-8^+$ and MHC class II-restricted $CD4^+8^-$ cells, which can be activated by foreign peptide/MHC complexes, but are tolerant to self peptide/MHC. How TCR signaling in $CD4^+8^+$ thymocytes results in such distinct cellular fates is poorly understood. However, current models emphasize the role of overall signal strength, reflecting the input from costimulatory and accessory molecules as well as the TCR, in determining the developmental outcome of TCR signaling (4, 5).

In mature T cells, TCR signaling is associated with the formation of a multimolecular complex, the immunological synapse, at the T cell/APC interface (6, 7). Association of TCR molecules and other signaling mediators with glycosphingolipid-enriched lipid rafts within these complexes is thought to facilitate the activation of signaling cascades. Thus, raft disruption has been shown to inhibit early signaling events in T cell activation (8). However, the relevance of signaling complex formation to TCR signaling during development is less clear. Recent studies have begun to analyze immunological synapse formation during thymic selection, using either lipid bilayers containing peptide/MHC complexes (9), or in the context of thymocyte responses in a negative selection system (10). However, this issue has not been addressed under conditions known to lead to positive selection, which under physiological circumstances is driven by interactions between thymocytes and cortical epithelium (11).

In contrast to positive selection, negative selection is normally mediated by dendritic cells and/or a subset of medullary epithelium (12–14). These two cell types differ from positively selecting cortical epithelium in their expression of costimulatory molecules of the B7 family (15). These molecules contribute to immunological synapse formation in mature T cells by generating signals important in the recruitment of lipid rafts, as a result of interactions with ligands such as CD28 (16, 17). Thus, differences in costimulatory molecule expression between positively and negatively selecting stromal cells may be important in determining the composition and duration of signaling complex formation, which may in turn play a crucial role in the developmental outcome of TCR signaling. Such a mechanism would be consistent with the notion that compartmentalization of costimulatory molecules within the thymus (18) is an important factor in controlling the outcome of TCR-driven thymocyte selection.

In this study, we have developed a model to study thymocyte responses as a consequence of interactions with epithelial cells using conjugates generated in vitro. We show that epithelial cell-$CD4^+8^+$ thymocyte conjugate formation occurs in a TCR-MHC-dependent manner, initiates a tyrosine kinase-dependent calcium flux, and results in the generation of phenotypically mature $CD4^+8^-$ and $CD4^-8^+$ cells, thus indicating the relevance of this approach to the study of positive selection. By analyzing the distribution of cell surface and intracellular molecules in preselection $CD4^+8^+$ thymocytes as a consequence of TCR-mediated interactions with thymic epithelium, we have defined the formation of a multicomponent molecular signaling complex in positive selection. Interestingly, while complex formation in positive selection induction shares some features with immunological synapse formation in T cell activation such as $p56^{lck}$ recruitment (19), strikingly, signaling complex formation in positive selection does not involve accumulation of GM1-containing lipid rafts. In contrast, providing additional costimulatory signals by introduction of CD80 to thymic epithelial cells promotes the recruitment of

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GM1-marked rafts in $CD4^+8^+$ thymocytes. Functionally, this recruitment correlates with a reduction in positive selection and increased thymocyte apoptosis. Thus, our data define for the first time the formation of an active signaling complex during the initiation of positive selection. Moreover, we suggest that the outcome of TCR signaling in thymocytes is determined by the costimulatory signals provided by thymic stromal cells.

Materials and Methods

Mice

BALB/c and MHC-deficient mice (Taconic, Germantown, NY) were bred and maintained at the Biomedical Sciences Unit, University of Birmingham. TCR-$\alpha^{-/-}$ mice were a gift of M. Owen (Imperial Cancer Research Fund, London, U.K.). Adult or neonatal BALB/c or adult MHC-deficient (4–6 wk) and neonatal TCR-$\alpha^{-/-}$ mice were used as a source of thymocytes. Thymuses from day 15 BALB/c embryos were used as a source of thymic stromal cells by culture for 5–7 days in 2-deoxyguanosine (Sigma-Aldrich, Poole, U.K.). Detection of the vaginal plug was designated day 0 of gestation.

Abs and fluorescence reagents

The following were used for flow cytometry: anti-CD4 PE (GK1.5), anti-CD8 FITC (53-6.7) (both BD PharMingen, San Diego, CA), and anti-human CD80 (MAB140; R&D Systems, Abingdon, U.K.), which was detected using anti-mouse FITC (Caltag, San Francisco, CA). Cells were analyzed using a BD Biosciences (Oxford, U.K.) LSR machine, with forward and side scatter gates set to exclude nonviable cells. Primary Abs for immunofluorescence were: anti-CD3 (KT3; Serotec, Oxford, U.K.), anti-CD45 FITC (I3/2, Sigma-Aldrich), anti-CD4 FITC, anti-CD8 FITC, anti-LAT[3] (linker for activation of T cells) (Upstate Biotechnology, Lake Placid, NY), anti-$p56^{lck}$ (Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated anti-phosphotyrosine (4G10; Upstate Biotechnology), and anti-CD80. The actin cytoskeleton was studied using phalloidin-rhodamine (Molecular Probes, Eugene, OR), and GM1 was detected using FITC-conjugated cholera toxin B (Sigma-Aldrich).

Preparation of thymocytes and thymic epithelium

In some experiments, thymocytes from BALB/c adult or neonatal mice were depleted of $CD3^+$ cells using anti-rat Dynabeads (Dynal, Wirral, U.K.) precoated with rat anti-mouse CD3 (clone KT-3; Serotec), followed by selection of $CD8^+$ cells using anti-CD8-coated Dynabeads. Removal of CD8 beads was conducted using Detachabead (Dynal, Great Neck, NY). Alternatively, as stated in figure legends, thymocytes were obtained from $MHC^{-/-}$ mice, or from TCR-$\alpha^{-/-}$ mice. Thymic epithelial cells were obtained by disaggregating 2-deoxyguanosine (2-dGuo)-treated 15-day BALB/c thymus lobes, as described (12).

Formation and flow cytometric analysis of thymocyte-epithelial cell conjugates

Thymocytes and thymic epithelial cells were mixed by centrifugation at a ratio of 1:1, and the resultant cell pellet was incubated at 37°C. Analysis of cell pellets immediately following centrifugation was designated time 0. In some experiments, before conjugate formation, thymocytes were labeled with PKH26 (Sigma-Aldrich), according to manufacturers' instructions, and thymic epithelium was labeled with CFSE (Molecular Probes) at a concentration of 10 nM, for 10 min at 37°C. Conjugate formation was performed, as described above, with cell pellets being resuspended in 200 μl PBS at the indicated time points, with immediate analysis by flow cytometry.

Cytochalasin D treatment of cells

Thymocytes and epithelial cells were labeled with PKH26 and CFSE, respectively, and then treated with 10 μM cytochalasin D (Sigma-Aldrich) for 10 min at 37°C. Cells were then used to study conjugate formation, as described above.

Measurement of changes in cytosolic Ca^{2+} concentration

Thymocytes were loaded with 5 μM Indo-1 AM (Sigma-Aldrich) at 37°C for 45 min. Thymocytes and epithelial cells were then labeled with PKH26 and CFSE, respectively, and mixed by centrifugation at a ratio of 1:1. Changes in cytosolic Ca^{2+} levels were monitored in free thymocytes and in thymocytes bound to epithelial cells, by gating on unbound thymocytes and thymocyte-epithelial cell conjugates and plotting the ratio of Ca^{2+}-bound Indo-1 to free Indo-1 against time. Where stated, thymocytes were incubated with 50 μM tyrphostin A9 (Calbiochem, San Diego, CA) for 10 min before mixing with epithelial cells.

Immunofluorescent analysis of thymocyte-epithelial cell conjugates

Thymocyte-Epithelial conjugates were allowed to form over 30-min incubation, and then allowed to adhere to poly(L-lysine)-coated slides (Sigma-Aldrich) at room temperature. Slides were fixed in 3.7% paraformaldehyde, permeabilized in 0.1% saponin, and blocked for 2 h at room temperature or at 4°C overnight in 1% FCS containing 0.1% saponin. Binding of Abs to LAT and $p56^{lck}$ was detected by biotinylated anti-rabbit IgG (Amersham, Bucks, U.K.), followed by streptavidin-FITC (Amersham), while binding of anti-CD3 was revealed using anti-rat FITC (Caltag). Labeling with biotinylated anti-phosphotyrosine was revealed by streptavidin-FITC. For detection of the lipid raft component GM-1, thymocytes were labeled with FITC-conjugated cholera toxin B before incorporation into conjugates with either control or CD80-infected thymic epithelial cells. CD80 expression in conjugates was performed using sequential incubations in mouse anti-human CD80 (R&D Systems) and anti-mouse rhodamine (Chemicon, Temecula, CA). Analysis was performed using a Zeiss (Oberkochen, Germany) Axioplan fluorescence microscope with Digital Scientific (Cambridge, U.K.) camera and software with a total magnification of ×1000.

Reaggregate thymus organ cultures

Thymocytes and thymic epithelial cells were mixed together by centrifugation at a ratio of 1:1, and the cell pellet was transferred to the surface of a 0.8-μm filter in organ culture (11, 12). After the specified time period, cultures were teased apart, and recovered thymocytes were analyzed by flow cytometry.

Adenoviral infection of thymic epithelium

Adenoviral supernatant generated from adenoviral vectors containing cDNA encoding either human CD80 or green fluorescence protein (GFP) was obtained from Qbiogene (Carlsbad, CA). Freshly trypsinized 2-dGuo-treated thymuses were used as a source of thymic epithelium. Aliquots (3×10^5) of cells were resuspended in 96-well plates in a volume of 50 μl RPMI with the addition of 0.5 μl of adenoviral supernatant, and then centrifuged for 60 min at 2100 rpm at room temperature. Cells were then recovered and used immediately for the formation of reaggregate cultures. In experiments analyzing the effects of CD80 expression on the kinetics of conjugate formation and lipid raft formation, freshly infected stromal cells were cultured overnight before use to allow expression of the introduced gene.

Results

Isolated thymocytes and thymic epithelial cells form conjugates in a TCR-MHC-dependent manner

Thymic epithelium promotes positive selection of thymocytes in a TCR-MHC-dependent manner (1). Conjugate formation has been used previously to study membrane-associated molecular redistribution in the interaction of T cells and APC (reviewed in Ref. 7). To explore the possibility of using this approach to study immune complex formation in thymocyte selection, we examined conjugate formation between preselection $CD4^+8^+$ thymocytes and thymic cortical epithelial cells, the cell type normally responsible for driving positive selection under physiological conditions. To study thymocyte selection on a molecular basis, we sought to recreate thymocyte-epithelial cell interactions in vitro. Thus, equal numbers of fluorescently labeled $CD4^+8^+$ thymocytes (PKH26) and thymic epithelial cells (CFSE) were associated by centrifugation, and then conjugate formation was identified by two-color events using flow cytometry. In contrast to cell pellets analyzed immediately following centrifugation (time 0, Fig. 1*a*), incubation of cell mixtures for 30 min revealed the formation of thymocyte-epithelial cell conjugates appearing as

[3] Abbreviations used in this paper: LAT, linker for activation of T cells; GFP, green fluorescence protein; 2-dGuo, 2-deoxyguanosine.

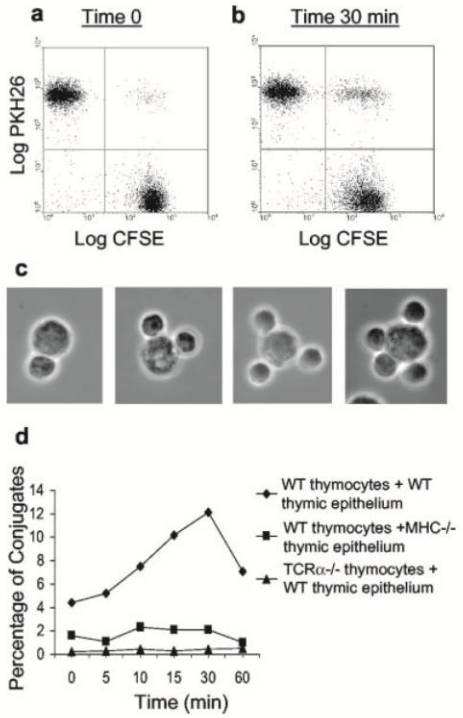

FIGURE 1. $CD4^+8^+$ thymocytes form conjugates with thymic epithelial cells in a TCR-MHC-dependent manner. $CD4^+8^+$ thymocytes from $MHC^{-/-}$ mice were labeled with PKH26, and thymic epithelial cells with CFSE. Cells were mixed at a ratio of 1:1 and analyzed by flow cytometry immediately (*a*) or after 30-min incubation at 37°C by flow cytometry (*b*) or microscopy (*c*). The proportion of conjugates formed at time points between 0 and 60 min was determined, with $PKH26^+CFSE^+$ events representing conjugates (*d*, diamonds). Alternatively, $MHC^{-/-}$ thymic epithelial cells were mixed with wild-type thymocytes (*d*, squares), or TCR-$\alpha^{-/-}$ thymocytes mixed with wild-type thymic epithelium (*d*, triangles), and conjugate formation between 0 and 60 min was analyzed. Similar data were obtained from three separate experiments.

$CFSE^+PKH26^+$ events (Fig. 1*b*), which consisted of a single epithelial cell bound by thymocytes (Fig. 1*c*). Importantly, immunofluorescence analysis routinely showed that greater than 99% of the thymocytes bound to epithelial cells in conjugates were of a $CD4^+8^+$ phenotype (data not shown), ruling out the possibility that thymocytes at other maturational stages were being studied. To investigate the relevance of these thymocyte-epithelial cell interactions to thymic selection events, we assessed the importance of TCR-MHC interactions in conjugate formation, using combinations of MHC-deficient epithelium and wild-type thymocytes or TCR-deficient thymocytes and wild-type epithelial cells. In contrast to conjugate formation between TCR-$\alpha\beta$- and MHC-expressing cells, use of either MHC-deficient thymic epithelium or TCR-$\alpha\beta$-deficient (TCR-$\alpha^{-/-}$) thymocytes resulted in abrogation of conjugate formation (Fig. 1*d*). Thus, conjugate formation between thymocytes and epithelial cells (in this system) is TCR-MHC dependent, demonstrating the potential of this system to study molecular events in TCR-triggered thymocyte selection.

Conjugate formation leads to active signaling in thymocytes

Within lymphocytes, polymerization of the actin cytoskeleton provides the initial cellular polarization necessary for signaling molecule recruitment, and hence intracellular response to the TCR signal (20). Thus, as a functional measure of signaling, we analyzed accumulation of polymerized actin in thymocytes bound to thymic epithelium. First, polymerization of the actin cytoskeleton at the point of epithelial cell contact (Fig. 2*a*) was detected in thymocytes in response to thymic epithelium. Moreover, disruption of the actin cytoskeleton in either thymocytes or thymic epithelium by prior treatment with cytochalasin D abrogated conjugate formation (Fig. 2*b*), suggesting that actin redistribution in both cell types is functionally important for stable conjugate formation.

In mature T cells, TCR ligation by peptide/MHC complexes triggers a complex signaling cascade leading to the activation of signaling mediators through tyrosine phosphorylation. To further define the signaling response of thymocytes interacting with epithelial cells, we analyzed tyrosine phosphorylation in thymocyte-epithelial cell conjugates. In contrast to unconjugated thymocytes, which showed diffuse staining (data not shown), accumulation of tyrosine phosphorylation at the focus of cell-cell contact was observed in thymocytes interacting with epithelial cells (Fig. 3*a*, Table I). A change in intracellular calcium levels is also a key downstream indicator of membrane-proximal signaling events, and therefore could be used as an early indicator of thymocyte signaling as a consequence of thymocyte-epithelial cell interactions. Thus, $CD4^+8^+$ thymocytes were loaded with Indo-1 before incorporation into conjugates (21). Again, PKH26 (thymocytes) and CFSE (thymic epithelium) labeling was used to detect thymocyte-epithelial cell interactions. As calcium mobilization represents a rapid intracellular response to signal induction, cells were monitored at an early time point following mixing by centrifugation (5 min) (Fig. 3*b*). As shown in Fig. 3*c*, gating on unbound thymocytes showed that these cells maintain an unchanging basal level of intracellular calcium in the absence of epithelial cell interactions. In contrast, when calcium levels were measured in thymocytes involved in conjugates, 65% demonstrated a significant and sustained

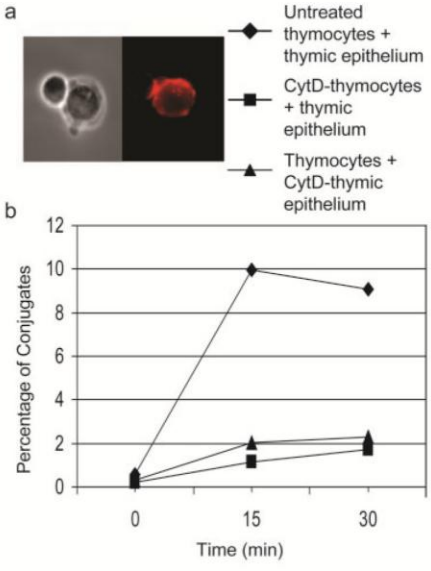

FIGURE 2. Thymocyte-Epithelial cell interactions are dependent upon polymerization of the actin cytoskeleton. $CD4^+8^+$ thymocytes from $MHC^{-/-}$ mice were incubated with thymic epithelial cells for 30 min, adhered to slides, and permeabilized to enable analysis of actin cytoskeleton polymerization using phalloidin (*a*). Analysis of conjugate formation using flow cytometry was used to compare interactions between cytochalasin D-treated thymocytes and untreated thymic epithelial cells (squares), or between untreated thymocytes and cytochalasin D-treated epithelium (triangles), to conjugate formation between untreated thymocytes and untreated epithelial cells (diamonds) (*b*).

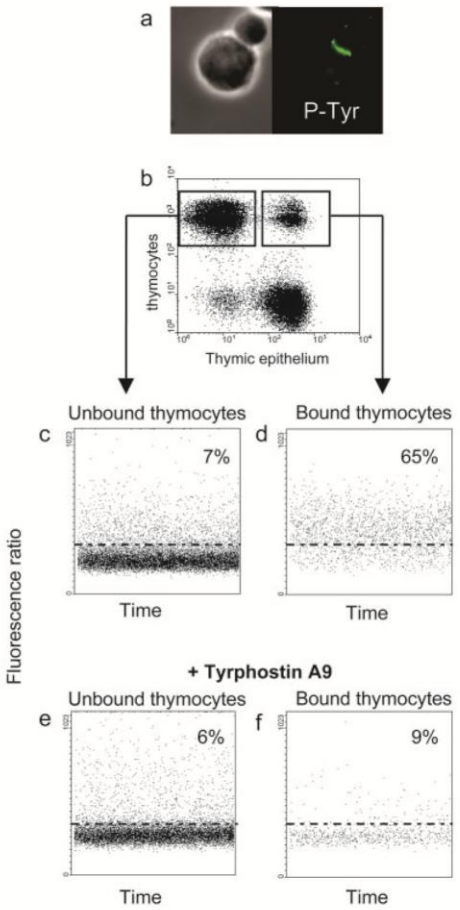

FIGURE 3. Thymocyte-Epithelial cell interactions increase thymocyte intracellular calcium levels, and induce phosphotyrosine accumulation. $CD4^+8^+$ thymocytes from $MHC^{-/-}$ mice were incubated with thymic epithelial cells for 30 min, adhered to slides, permeabilized, and labeled with Abs to phosphorylated tyrosine residues (*a*). $CD4^+8^+$ thymocytes from $MHC^{-/-}$ mice were loaded with Indo-1 and then labeled with PKH26. Thymic epithelial cells were labeled separately with CFSE. Cells were mixed at a ratio of 1:1 and incubated for 5 min, and conjugate formation was analyzed by flow cytometry (*b*). The cytosolic calcium concentration of free (*c*) and epithelial cell-bound thymocytes (*d*) was then analyzed by flow cytometry for 204 s. To see whether the calcium flux was a direct consequence of tyrosine kinase activation, Indo-1/PKH26-loaded thymocytes were incubated with 50 μM tyrphostin A9 before conjugate formation, in which calcium flux was again measured in free (*e*) and epithelial cell-bound (*f*) thymocytes.

increase in intracellular calcium concentration (Fig. 3*d*), an early indication of signal transduction. Of note, pretreatment with the tyrosine kinase inhibitor tyrphostin A9 (22) did not affect conjugate formation, but effectively blocked this calcium flux in epithelial-bound thymocytes (Fig. 3*f*). Thus, calcium elevation as a result of thymocyte-epithelial cell interactions is consistent with a tyrosine kinase-mediated TCR-induced activation of intracellular calcium release.

Signaling induced in thymocyte-epithelial cell conjugates can lead to positive selection

Although it is clear from the above data that interactions between thymic epithelial cells and thymocytes in conjugates generate a cellular response, in terms of both actin polymerization and intracellular signaling, it was important to clarify the developmental response of thymocytes to this stimulus, because TCR signaling in $CD4^+8^+$ cells can lead to either negative selection through the induction of apoptosis or positive selection leading to functional maturation. Thus, conjugates formed between MHC-expressing thymic epithelium and TCR-$\alpha\beta$-expressing thymocytes were purified and reassociated in reaggregate thymic organ cultures, able to support the positive selection process (11, 12). These cultures were harvested after 7 days and analyzed for the generation of mature $CD4^+8^-$ and $CD4^-8^+$ cells. As shown in Fig. 4*b*, such cultures efficiently support the appearance of single-positive thymocytes derived from $CD4^+8^+$ cells associated with epithelial cells in conjugates. Thus, thymocyte-epithelial cell conjugates provide a model in which to study signaling-associated molecular events involved in positive selection driven by thymocyte/epithelial cell interaction.

Table I. *Polarization of thymocyte cell surface and intracellular signaling molecules*

Molecules Studied	30 min
CD3	35 ± 5%
CD4	51 ± 2%
CD8	38 ± 3%
CD45	28 ± 7%
$p56^{lck}$	78 ± 3%
LAT	65 ± 5%
Phosphotyrosine	83 ± 3%
Actin	75 ± 5%

Thymocyte-Epithelial cell interactions induce polarization of key cell surface and intracellular signaling mediators

Numerous reports now indicate that TCR-MHC-mediated activation of mature T cells is associated with the redistribution of the TCR and accessory molecules to form an immunological synapse at the point of T cell contact with APC (7, 23). This signaling complex formation is now known to include accumulation of molecules such as CD45 (24), CD3 (25), and exclusion of CD43 (24). In contrast, little is known about molecular redistribution in thymocytes associated with the delivery of positive selection signals by thymic epithelium. Having established a model to look at individual cell-cell interactions leading to positive selection, we used immunofluorescence labeling to analyze the influence of interactions with thymic epithelial cells on the distribution of cell surface and intracellular molecules in thymocytes in comparison with that

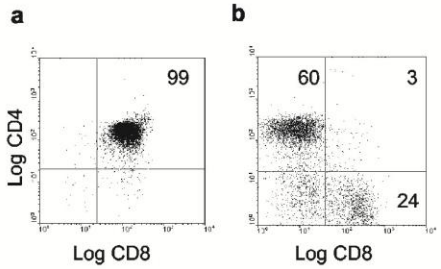

FIGURE 4. Thymocyte-Epithelial cell conjugates represent positive selection induction. Conjugates between $CD4^+8^+$ thymocytes purified from BALB/c mice (*a*) and wild-type thymic epithelium were purified and placed in reaggregate organ culture. After 7 days, thymocytes were harvested and analyzed for CD4, CD8, and TCR expression by flow cytometry. Shown are the CD4/CD8 profiles for TCR^+ cells (*b*). In the experiment shown, an input of 3×10^5 thymocytes gave a recovery of 6×10^4 thymocytes. Similar data were obtained from three separate experiments.

seen in signaling complex formation in mature T cell activation. For this purpose, conjugates formed between $CD4^+8^+$ thymocytes and thymic epithelial cells after 30 min of incubation, when conjugate formation is optimal (Fig. 1*d*), were adhered to slides and permeabilized to allow analysis of both cell surface and intracellular molecules. Using fluorescently labeled Abs, we found redistribution of CD3, CD4, CD8, and CD45 to the point of contact with epithelial cells in up to 80% of thymocytes forming conjugates (Fig. 5, *a–d*; Table I). Thymocytes not involved in interactions with epithelium showed an unbroken ring of staining in the cell membrane (data not shown).

Using this method to study intracellular signaling molecule distribution, we also observed accumulation of the tyrosine kinase $p56^{lck}$ (Fig. 5*e*) and the adapter protein LAT in thymocytes interacting with thymic epithelial cells (Fig. 5*f*; Table I), which contrasted with diffuse labeling in unconjugated thymocytes (data not shown). These findings correlate with the evidence of thymocyte signaling activation described above, and further characterize the composition of the signaling structure formed in prepositive selection thymocytes upon interactions with epithelial cells.

Thymocyte-positive selection is not associated with accumulation of GM1-marked lipid rafts

T cell activation involves the localization of cholesterol- and glycosphingolipid-rich membrane microdomains to the site of TCR ligation, forming the now well-studied lipid raft, which is thought to enhance recruitment of key raft-associated signaling molecules (26–28). Strikingly, using FITC-conjugated cholera toxin B to detect GM1, we found that $CD4^+8^+$ thymocytes bound to thymic epithelial cells failed to show accumulation of GM1 at the point of cell-cell contact (Fig. 6*a*). One possible reason for the lack of GM1 accumulation in thymocytes could be lower levels of expression of this molecule in these cells. To investigate this, we analyzed $CD4^+8^+$ thymocytes and mature T cells for levels of expression of GM1. As shown in Fig. 6*b*, GM1 surface expression in thymocytes

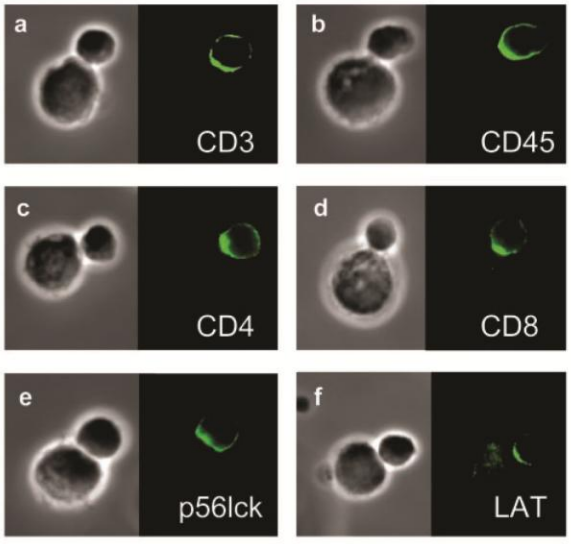

FIGURE 5. Induction of molecular redistribution in thymocytes by thymic epithelium. $CD4^+8^+$ thymocytes from $MHC^{-/-}$ mice were incubated with thymic epithelial cells for 30 min, adhered to slides, permeabilized, and labeled with Abs to CD3 (*a*), CD45 (*b*), CD4 (*c*), CD8 (*d*), $p56^{lck}$ (*e*), and LAT (*f*). Polarization to the point of contact with epithelial cells was observed for all molecules examined, while unbound thymocytes did not show evidence of molecular polarization (data not shown).

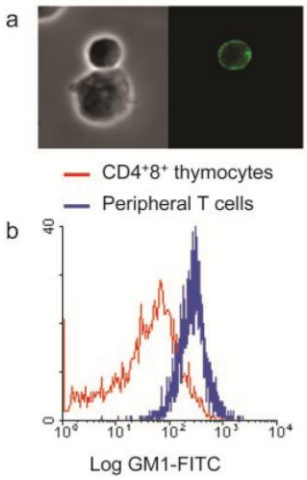

FIGURE 6. The initiation of thymocyte-positive selection does not involve accumulation of GM1 lipid rafts. $CD4^+8^+$ thymocytes from $MHC^{-/-}$ mice were labeled with cholera toxin FITC, and then incubated with thymic epithelium for 30 min. Cells were adhered to slides for analysis of GM1 polarization in thymocytes (*a*). $CD4^+8^+$ thymocytes and peripheral T cells were labeled with cholera toxin FITC and analyzed by flow cytometry (*b*).

and T cells appears to be developmentally regulated, with $CD4^+8^+$ cells expressing lower levels than mature peripheral T cells. Thus, the absence of lipid raft accumulation in positive selection initiation could be due to insufficient levels of GM1 expression in $CD4^+8^+$ thymocytes, rather than an intrinsic inability to mediate this response. An additional possibility is that the absence of GM1 polarization in thymocytes is due to the lack of appropriate costimulatory molecule expression on thymic epithelium. Costimulation has been shown to play a role in raft accumulation in peripheral T cells (29–31), although a recent publication indicates that lipid raft accumulation can occur independently of the CD28/B7 system (32). Less is known about requirements for lipid raft accumulation in immature thymocytes, however. Interestingly, Ebert et al. (29) analyzed the responses of thymocytes to Ab-coated beads and reported a lack of GM1 polarization in thymocytes even when CD28 was cross-linked along with TCR and CD4. However, whether such interactions between thymocytes and Ab-coated beads are representative of more physiological cell-cell interaction is unclear. Unlike mature APC, cortical epithelial cells capable of mediating positive selection do not normally express costimulatory ligands CD80 and CD86. Thus, to explore the functional consequences of differences in costimulatory molecule expression in relation to signaling complex formation and lipid raft aggregation, we set out to induce defined modifications in the costimulatory profile of positively selecting thymic epithelium.

Thymic epithelial cell suspensions from 2-dGuo-treated thymus lobes were infected with adenoviral supernatant encoding cDNA for either human CD80, which has previously been shown to interact with mouse ligands (33), or GFP, which served as a control vector. In the experiment shown, 64% of epithelial cells were found to be GFP^+ (Fig. 7*a*), with 60% of epithelial cells demonstrating cell surface expression of CD80 (Fig. 7*b*). Moreover, the CD80 molecules introduced into these cells are functionally competent, as adenoviral infection of thymic epithelium with CD80 enables these cells to act as effective APCs in stimulation assays with mature T cells (data not shown). Interestingly, interactions with $CD80^+$ thymic epithelium caused a dramatic accumulation of GM1 in thymocytes at the point of

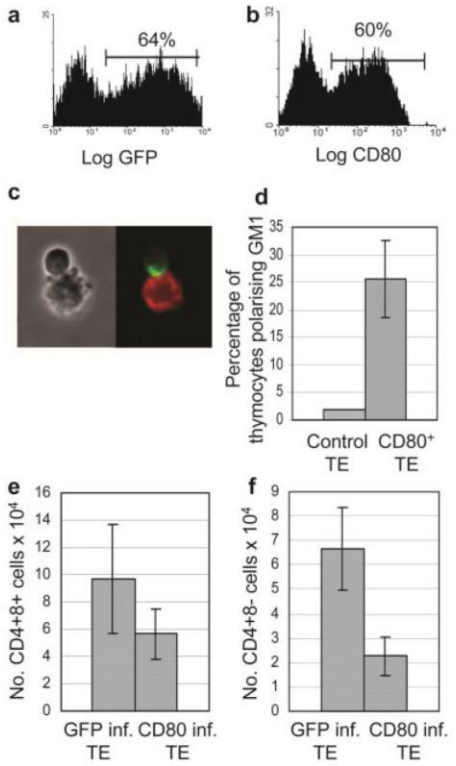

FIGURE 7. Enforced expression of CD80 by thymic epithelium promotes lipid raft accumulation and a reduction in thymocyte-positive selection. Thymic epithelial cells from 2-dGuo-treated fetal thymic lobes were infected with CD80 adenoviral supernatant or GFP-only adenoviral supernatant. Infected cells were analyzed by flow cytometry for expression of GFP and CD80 (*a* and *b*, respectively). $CD4^+8^+$ thymocytes from $MHC^{-/-}$ mice were labeled with cholera toxin FITC, and then incubated with thymic epithelium infected with CD80 adenovirus for 30 min. Cells were adhered to slides, and labeled with anti-CD80, followed by anti-mouse rhodamine. Quantitation of the number of thymocytes showing GM1 polarization in thymocytes bound to $CD80^+$ (*c*) or $CD80^-$ thymic epithelium is shown in Fig. 7*d*, in which a minimum of 50 conjugates was counted per experiment. Reaggregates were made of 1×10^6 $CD4^+8^+$ thymocytes and either GFP-infected epithelial cells or CD80-infected epithelial cells. Thymocytes were harvested after 5 days and analyzed for CD4 and CD8 expression. Average numbers of $CD4^+8^+$ (*e*) and $CD4^+8^-$ (*f*) cells were calculated from three separate experiments.

cell contact (Fig. 7*c*), with a 13-fold increase in the number of thymocytes displaying GM1 polarization compared with thymocytes interacting with control thymic epithelium (Figs. 6*a* and 7*d*). Thus, despite their lower levels of GM1 expression, thymocytes are able to accumulate GM1 when appropriate costimulation is available, as would be the case for thymocytes interacting with bone marrow-derived APC expressing CD80/CD86.

To assess the impact of CD80 expression by thymic epithelial cells on positive selection, we compared the generation of $CD4^+8^-$ cells in reaggregate cultures formed from $CD4^+8^+$ thymocytes and either GFP- or CD80-infected thymic epithelium. After 5 days, cultures were harvested and thymocytes were counted and stained for CD4 and CD8 expression. A 2- to 3-fold reduction in the generation of $CD4^+8^-$ thymocytes was noted in cultures in which thymic epithelial cells expressed CD80, as compared with development in control GFP-infected reaggregates (Fig. 7*f*). Moreover, this decrease in the generation of $CD4^+8^-$ cells correlated with a decrease in the number of $CD4^+8^+$ precursors in CD80-expressing reaggregate cultures (Fig. 7*e*). Importantly, the reduction in positive selection observed in CD80-expressing reaggregate cultures was not due to a reduction in the efficiency of thymocyte-epithelial cell interactions, as flow cytometry showed an increase in the frequency of conjugate formation with CD80-infected thymic epithelium when compared with control thymic epithelium (data not shown). Moreover, in cultures harvested at the earlier time point of 2 days, an increased proportion of thymocytes binding annexin V was observed in reaggregates of CD80-expressing thymic epithelium compared with control reaggregate thymic organ culture (data not shown). This correlated with a decrease in overall cell yield at this earlier 2-day time point, consistent with a role for apoptosis in determining $CD4^+8^+$ thymocyte numbers.

Discussion

TCR signaling as a result of interactions between thymocytes and thymic epithelial cells is essential for the induction of positive selection and the development of mature T cells from $CD4^+8^+$ cortical thymocytes. In this study, we have recreated the initial cellular interactions involved in thymocyte-positive selection in vitro. The formation of thymocyte-epithelial cell conjugates in this system is dependent upon TCR-mediated recognition, inducing rapid reorganization of the thymocyte actin cytoskeleton, and an increase in levels of intracellular calcium. Importantly, we also show that such interactions lead to thymocyte-positive selection, with $CD4^+8^+$ cells within epithelial cell conjugates developing into $CD4^+8^-$ and $CD4^-8^+$ cells in reaggregate culture. Interestingly, evidence shown in this work also supports involvement of the epithelial cell cytoskeleton in positive selection initiation, reminiscent of the requirement for dendritic cell cytoskeletal reorganization in peripheral T cell activation (34). Redistribution of cell surface and intracellular molecules has been shown to be a key feature of mature T cell activation by APC interactions. Thus, recent reports show the polarization of molecules such as CD45 (24), CD3, and CD4 (25) following activation, although the exclusion of CD43 has also been noted (24). Similarly, intracellular signaling mediators such as LAT and protein kinase C-θ have also been shown to accumulate at the point of interaction (35, 36). However, little is known about molecular redistribution in thymocytes undergoing selection; thus, our observations that polarization of CD3, CD4, CD8, and CD45, together with key signaling molecules, to the point of thymocyte-epithelial cell contact also takes place during the initial stages of positive selection provide new evidence for similarities between thymocyte selection and T cell activation. This redistribution plays an integral role in the formation of a multimolecular signaling complex at the thymocyte-epithelial cell interface, also involving accumulation of phosphotyrosine. In mature T cells, this synapse formation involves the clustering of a number of molecules in an ordered fashion, to form both a central and peripheral supramolecular activation cluster (6). Whether such segregation occurs during thymocyte/epithelial cell interactions described in this work remains to be determined. Nevertheless, our findings provide direct evidence that thymocyte-epithelial cell interactions leading to positive selection result in the redistribution of cell membrane-associated signaling molecules to the thymocyte-epithelial cell interface in a manner analogous to that seen in mature T cell-APC interactions.

A notable exception to this similarity is that the initiation of positive selection in $CD4^+8^+$ thymocytes does not involve accumulation of lipid rafts marked by GM1 labeling. Lipid raft accumulation is thought to be important in enhancing the recruitment of signaling molecules to the TCR complex and is a key feature of TCR signaling in peripheral T cells, although recent studies in human T cells also show that raft recruitment is not required for

CD8 activation (37). However, the involvement of costimulatory signals in raft recruitment, delivered as a result of interaction between CD28 and costimulatory ligands of the B7 family expressed on professional APC, is controversial (30–32). Although we show GM1 levels to be lower on $CD4^+8^+$ thymocytes than on peripheral T cells, we conclude that the lack of GM1 lipid raft accumulation during positive selection initiation, either by thymic epithelial cells as shown in this work, or previously by Ab-coated beads (29), is not due to an intrinsic inability of thymocytes to facilitate raft accumulation, because targeting expression of CD80 to thymic epithelial cells does induce lipid raft aggregation in $CD4^+8^+$ thymocytes. Interestingly, GM1 accumulation in thymocytes interacting with CD80-expressing thymic epithelium correlates with a reduction in positive selection and increased apoptosis in reaggregate cultures of $CD4^+8^+$ thymocytes and CD80-transfected epithelium. We speculate that thymic epithelium engineered to express CD80 converts positive selection of thymocytes to negative selection as a result of enhanced signaling through costimulatory-dependent raft recruitment. In this context, the failure of anti-CD28-coated beads to induce lipid raft accumulation (29) raises the possibility that CD80 may interact with a receptor other than CD28 to promote thymocyte raft aggregation. We are currently investigating the CD80 receptor expressed by $CD4^+8^+$ thymocytes, which is involved in the formation of lipid rafts as a result of interaction with CD80-expressing thymic epithelium. Interestingly, Abs to CTLA-4, another receptor for CD80, have been found to inhibit negative selection of thymocytes (38), perhaps implicating CTLA-4 in the experiments performed in this study. In contrast to our observations, some studies have shown that coligation of TCR, CD4, and CD28 can induce apoptosis in the absence of raft accumulation (29, 39). One possible explanation for this difference is that cells bearing very high affinity TCRs may be able to be triggered to undergo apoptosis without the need for signaling enhancement by raft accumulation, while cells with more moderate affinity TCRs may require raft accumulation to generate adequate signaling levels for apoptosis induction. Overall, our observations suggest a possible mechanism whereby the presence or absence of costimulation normally associated with professional APC and a subset of medullary epithelium, and absent from cortical epithelium, can determine the outcome of thymocyte selection through differential lipid raft recruitment, leading to quantitative or qualitative variations in TCR signaling.

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Thymic epithelial cells provide Wnt signals to developing thymocytes

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Interactions with thymic stromal cells are known to be critical for the development of T cells from progenitors entering the thymus, yet the molecular mechanisms of stromal cell function remain poorly understood. Accumulating evidence has highlighted the importance of β-catenin-mediated activation of T cell factor (TCF)/lymphoid enhancer factor (LEF) transcription during thymocyte development. As regulation of this signaling pathway is controlled by binding of soluble Wnt proteins to cell surface Frizzled (Fz) receptors, we studied components of Wnt/Fz-mediated signaling in the context of stromal cell regulation of thymocyte development. We show that mRNA for a variety of Wnt family members, notably Wnt-4, Wnt-7a and 7b, and Wnt-10a and 10b, are expressed by thymic epithelium rather then by thymocytes, while thymocytes demonstrate a developmentally regulated pattern of Fz receptor expression. Collectively these findings suggest (1) a functional role for Wnt-producing thymic epithelium in determining TCF/LEF-mediated transcriptional regulation in Fz-bearing thymocytes, and (2) a role for defined Wnt-Fz interactions at successive stages of thymocyte maturation. In support of this we show that separation of thymocytes from Wnt-producing epithelial cells and the thymic microenvironment, triggers β-catenin phosphorylation and degradation in thymocytes. Thus, sustained exposure to Wnt in the context of an intact stromal microenvironment is necessary for stabilization of β-catenin-mediated signaling in thymocytes.

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Key words: Thymic epithelium / Wnt / Frizzled / Thymocyte development

1 Introduction

T cell precursors entering the thymus undergo a program of proliferation, differentiation and selection that is regulated by interaction with the thymic microenvironment [1–3]. Some of the molecular mediators of these microenvironmental interactions have now been defined and their expression mapped to thymic epithelial cells, which are known to play a key role in supporting thymocyte development [3–5]. These include production of the growth factors IL-7 and SCF [6–8] and expression of Notch ligands [9] capable of activating the Notch signaling pathway recently defined as an essential requirement for the induction of T cell development [10–12]. Recent evidence has also highlighted the importance of the transcription factors T cell factor (TCF)-1/lymphoid enhancer factor (LEF)-1 and their co-activator, β-catenin, at both the pre-TCR regulated developmental checkpoint, controlling the transition from the $CD4^-CD8^-$ (double-negative, DN) to the $CD4^+CD8^+$ (double-positive, DP) stage of thymocyte maturation [13], and for survival at the DP stage, allowing positive selection and progression to the $CD4^-CD8^+$/$CD4^+CD8^-$ (single-positive, SP) stage [14]. However, little is known of the way in which this pathway of transcriptional regulation is controlled as thymocyte maturation proceeds, and whether this involves autocrine or paracrine effects mediated by the thymic stroma.

Activation of the TCF/β-catenin pathway is dependent upon interaction of members of the Wnt family of secreted glycoproteins with members of the Frizzled (Fz) family of transmembrane receptors [15]. Signaling through Fz receptors as a consequence of Wnt binding results in the stabilization of β-catenin, which on association with TCF/LEF converts it from a transcriptional suppressor to a transcriptional activator [16]. Although both Fz and Wnt proteins are known to be expressed in the thymus, the pattern of expression and functional role

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Abbreviations: Fz: Frizzled **DN:** Double-negative **DP:** Double-positive **SP:** Single-positive **TCF:** T cell factor **LEF:** Lymphoid enhancer factor **RT:** Reverse transcription

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of individual family members in relation to the various lymphoid and stromal components of the thymus is not clear. Thus defining these parameters is fundamental to understanding the microenvironmental regulation of transcriptional control by TCF/LEF in thymocyte maturation.

Here we have carried out spatial and temporal mapping of Wnt and Fz expression in conjunction with functional studies, to investigate the regulation of Wnt-mediated β-catenin signaling by defined thymocyte and stromal cell interactions. We show that Wnt genes, notably Wnt-4, are expressed by thymic epithelial cells rather than thymocytes, favoring a model in which Wnt production by thymic epithelial cells influences β-catenin stabilization and hence TCF/LEF-mediated transcription during thymocyte maturation. Correspondingly, analysis of Fz receptor expression on thymocytes has shown a strict, developmentally regulated pattern, which may suggest a role for particular Wnt-Fz interactions during defined stages of T cell development.

2 Results

2.1 Differential expression of Wnt genes by thymocytes and thymic stromal cells

Several lines of evidence point toward β-catenin-mediated activation of TCF/LEF transcription as being an important mechanism during differentiation of DN thymocytes [17–19]. As binding of Wnt molecules to membrane-located Fz receptors is known to play a key role in β-catenin-mediated signaling [20–22], we first analyzed intrathymic expression of a panel of Wnt and Fz family members in highly purified populations of thymocytes and thymic stromal cells.

Relative to thymic epithelial cells, Wnt gene expression detected by reverse transcription (RT)-PCR was minimal in DN stage thymocytes (Fig. 1A) and in all subsequent (DP and SP) thymocyte populations (Fig. 1B and data not shown). In contrast, purified thymic epithelial cells both freshly isolated at E15 (Fig. 1A) and functionally mature cortical epithelial cells (Fig. 1B), expressed a range of Wnt genes. These included Wnt-4, Wnt-7a and 7b and Wnt-10a and 10b with relative expression being highest for Wnt-4 (Fig. 1A, B). Supporting the PCR data, Western blot analysis showed readily detectable Wnt-4 protein expression in CD45-depleted thymic stroma and undetectable levels in DN and DP thymocyte subpopulations (Fig. 1C). Collectively, these data emphasize the importance of paracrine epithelial/thymocyte rather than autocrine or thymocyte/thymocyte effects in Wnt mediated

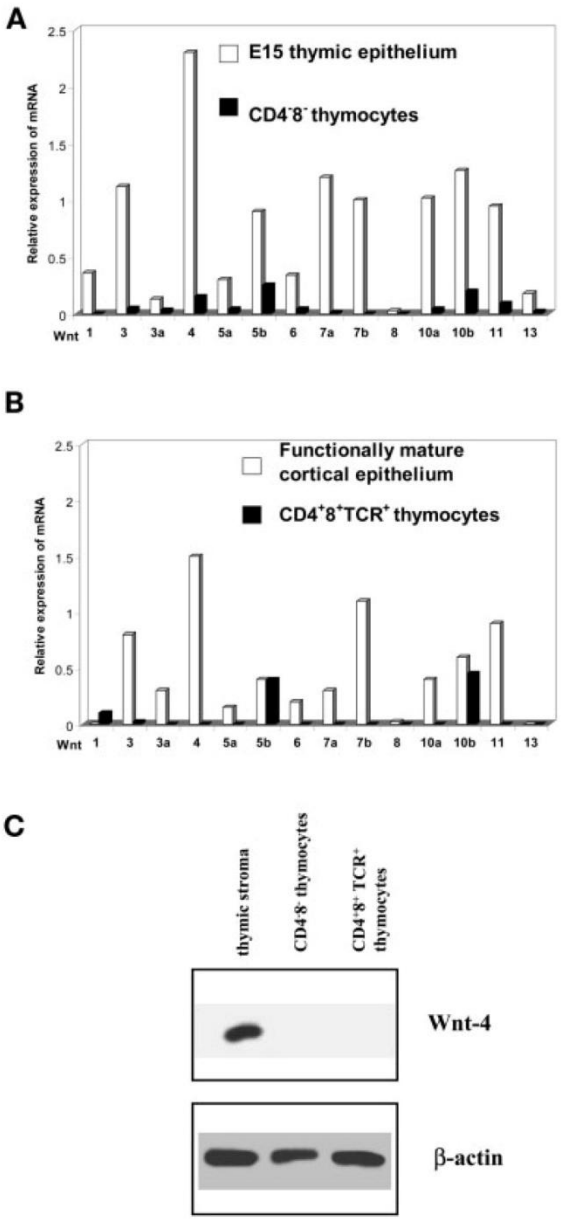

Fig. 1. Wnt family members are selectively expressed in thymic epithelial cells rather than thymocytes. (A) Relative expression of Wnt in E15 thymic epithelium and DN thymocytes or (B) in functionally mature cortical epithelial cells and DP thymocytes was determined by fixed-point RT-PCR using specific primers. PCR reactions were terminated at 35 cycles, the products were scanned by densitometry and expressed relative to β-actin level as an internal control. (C) Wnt-4 protein expression in CD45-depleted thymic stroma, DN and DP thymocytes was determined by Western blot analysis using a specific antibody against mouse Wnt-4. Equal protein loading was determined using anti-β-actin antibody.

regulation of β-catenin-TCF/LEF-dependent transcription in thymocytes.

2.2 Selected Fz receptors are expressed on thymocytes in a developmentally regulated pattern

Although there is some evidence for Fz receptor expression in whole thymus preparations [23], the expression and functional significance of individual Fz family members at different stages of thymocyte maturation has not been defined. Studies using soluble dominant-negative Fz receptors [23] have suggested the importance of Fz expression in thymocyte maturation. However, these studies were carried out with whole fetal thymic lobes and do not exclude the possibility of indirect effects through perturbation of thymic epithelial function since the possible expression of Fz receptors by these cells is not known.

To address these issues, we carried out RT-PCR analysis of Fz expression in defined thymocyte and epithelial cell preparations. As shown in Fig. 2A, Fz6 is the predomi-

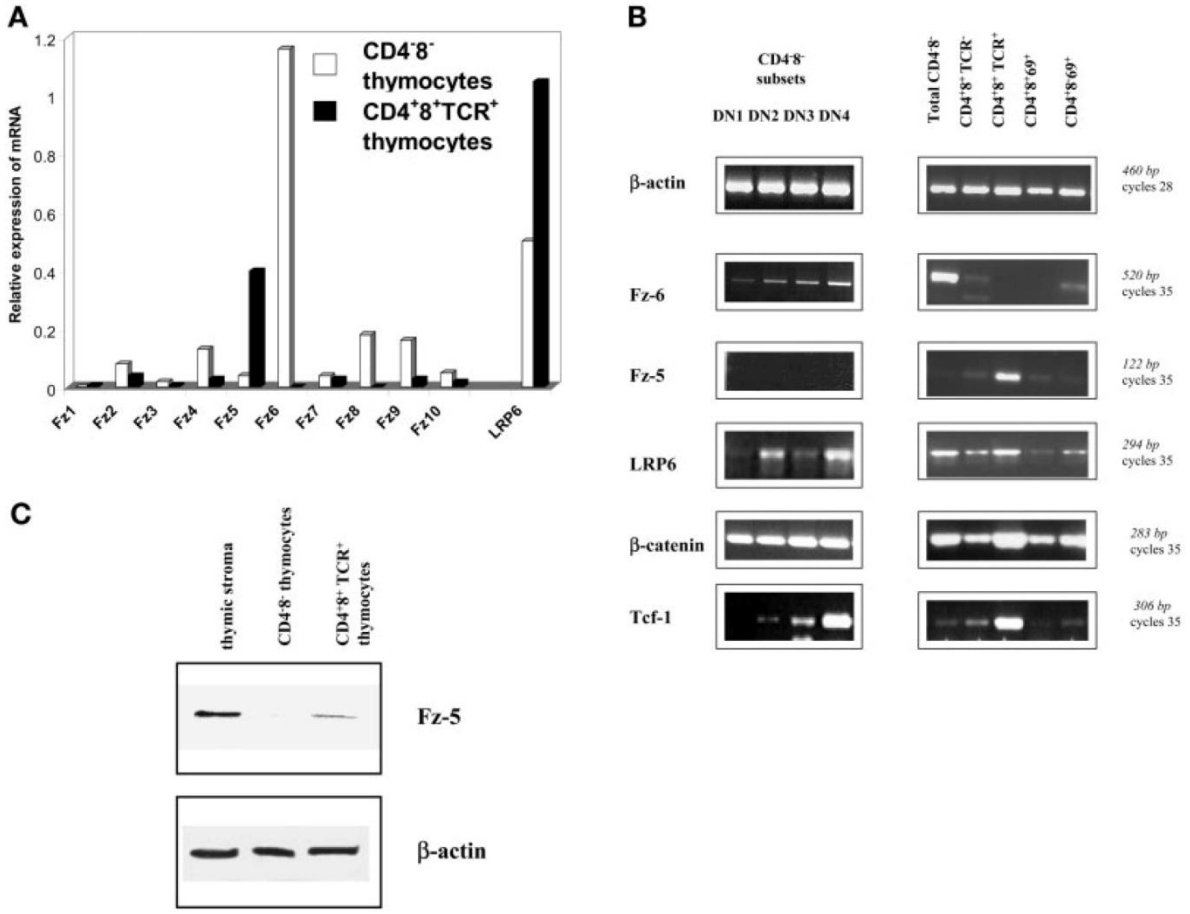

Fig. 2. (A) Expression of Fz receptors and the Fz accessory molecule, LRP6, in developing thymocytes. Relative expression in highly purified DN and DP TCR+ thymocyte populations was determined by fixed-point RT-PCR reactions which were terminated at 35 cycles. Products were scanned by densitometry and expressed relative to β-actin as internal control. (B) Fz5 receptor protein is expressed in thymic stroma cells and DP but not in DN thymocytes. Fz5 receptor expression in CD45-depleted thymic stroma, DN and DP thymocytes was determined by Western blot analysis using an Fz5 antibody. Equal protein loading was determined by using anti-β-actin antibody. (C) Developmentally regulated expression of Fz5 and Fz6 receptor expression during thymocyte maturation. Fixed-point RT-PCR analysis was performed from cDNA of highly purified thymocyte subpopulations. The reactions were terminated at 35 cycles, except for β-actin, which was terminated at 28 cycles. Equal sample loading is demonstrated by comparability of β-actin level.

nant Fz family member expressed in DN cells, whilst Fz5 is predominant in DP cells. Fz5 protein expression on DP thymocytes is also supported by Western blot analysis (Fig. 2B). Further analysis of Fz expression in DN subsets defined by CD25 and CD44 expression (Fig. 2C) showed that Fz6 expression increases during thymocyte maturation from the DN1 ($CD4^-CD8^-CD25^-44^+$) to the DN4 ($CD4^-CD8^-CD25^-44^-$) stage, but then dramatically decreases at the early DP stage ($CD4^+CD8^+TCR^-$). Conversely, Fz5 message (Fig. 2C), and Fz5 protein (Fig. 2B), which is not detected in DN thymocytes, become detectable at the DP stage and is the dominant Fz receptor expressed by $CD4^+CD8^+TCR^+$ cortical thymocytes (Fig. 2C). Fz5 mRNA expression is subsequently dramatically down-regulated at the $CD4^+CD8^+CD69^+$ stage, suggesting that Fz expression may be down-regulated as a result of positive selection. Thus these results suggest that the expression of Fz family members is developmentally regulated and that different family members are functionally important at different stages of maturation.

Consistent with a functional role for Fz signaling through the β-catenin-TCF-1 pathway at both DN and DP stages, other components of this pathway are expressed in both DN and DP cells (Fig. 2C). These include β-catenin itself and the recently defined LRP6 molecule, which is expressed in association with Fz molecules in the cell membrane and appears to be important in the formation of a functionally competent Fz receptor complex [24–26].

Importantly, expression of Fz6 (Fig. 3) and other Fz family members, including Fz2, Fz4, Fz5, Fz7, Fz8, and Fz10 (data not shown), is also readily detectable in thymic epithelial cells. This suggests that functional studies involving Fz inhibition or the effects of added Wnt proteins on thymocyte development in the intact thymus must take into account the possibility of indirect effects due to the modulation of epithelial cell maturation or function.

2.3 Stability of β-catenin in thymocytes is regulated by the thymic microenvironment

The above data predict that β-catenin-mediated signaling in thymocytes is influenced by Wnt production by thymic epithelial cells. In the absence of Wnt-induced signaling through Fz receptors, β-catenin is targeted for degradation as a result of glycogen synthase kinase-3-mediated phosphorylation [27, 28]. Thus, the phosphorylation status of β-catenin can be used as a read-out of β-catenin stability and hence Fz-mediated Wnt signaling. To obtain functional evidence for the influence of epithelial products on β-catenin stability in thymocytes, and hence on the activation of TCF-1/LEF-1-regulated target genes in these cells, we examined β-catenin phosphorylation in thymocytes in contact with thymic epithelial cells, or following their removal from the thymic microenvironment.

As shown in Fig. 4, DN thymocytes freshly isolated from 15-day thymus lobes express readily detectable levels of β-catenin protein (Fig. 4) with no detectable levels of phosphorylation (Fig. 4) as indicated by Western blotting with an antibody specific for the phosphorylated form of β-catenin. Similarly, thymocytes maintained in culture for 5 h in the presence of epithelial cells in intact thymus organ cultures also showed no evidence of β-catenin phosphorylation (Fig. 4). In marked contrast, DN thymocytes removed from thymic lobes and cultured in isolation showed detectable phosphorylation within a similar 5-h culture period (Fig. 4). Thus, these data provide functional evidence that β-catenin stability in DN thymocytes is dependent upon contact with the thymic microenvironment and correlates well with the production of Wnt proteins by thymic epithelial cells.

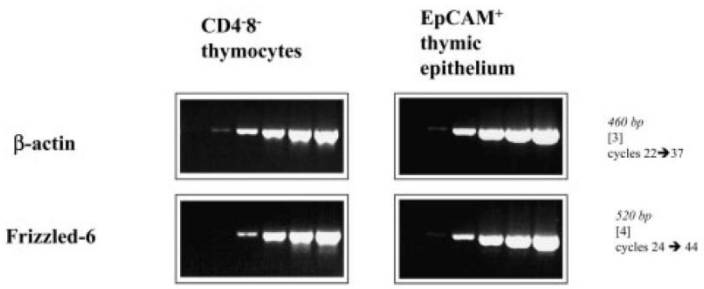

Fig. 3. Fz6 receptor genes expressed by thymocytes are also expressed by thymic epithelial cells. Semiquantitative RT-PCR analysis of Fz6 receptor message in DN thymocytes and E15 thymic epithelium showing comparable levels of expression by both cell types.

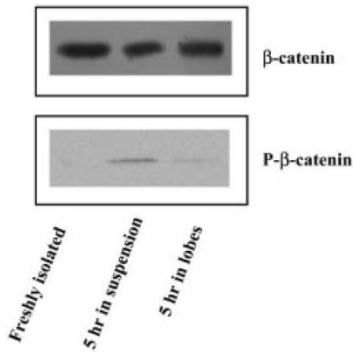

Fig. 4. Stability of β-catenin in $CD4^-CD8^-$ thymocytes is regulated by the thymic microenvironment. Phosphorylation status of β-catenin in $CD4^-CD8^-$ thymocytes was analyzed by Western blotting with antibodies specific either for β-catenin or phosphorylated β-catenin (P-β-catenin). E15 thymocytes were purified and either lysed immediately, or incubated for 5 h *in vitro* either in isolation or within intact thymus lobes. Harvested cells were selected on CD45-coated DynaBeads before analysis to exclude stromal contamination. Note the appearance of β-catenin phosphorylation in thymocytes cultured in isolation as compared to those maintained in intact thymus lobes.

3 Discussion

Recent findings have emphasized the importance of the β-catenin-TCF-1/LEF-1 pathway in thymocyte development [13, 14, 23]. In this study, we have defined the expression of Wnt proteins and Fz receptors, known to be important regulators of this pathway, in thymocyte and stromal cell populations of the developing thymus. Our findings provide evidence that developing thymocytes are not self sufficient in the production of Wnt proteins, and suggest that Wnt proteins produced by thymic epithelial cells are key to the regulation of β-catenin stability and hence TCF-1/LEF-1-dependent transcription in developing thymocytes. In terms of relative expression, we found that Wnt-4 is the most abundantly expressed Wnt family member in both E15 thymic epithelium and functionally mature cortical epithelial cells at subsequent stages of development (Fig. 1a, b). This finding correlates well with recent evidence for a functional role for Wnt-4 in thymocyte development from studies in mice double-deficient for Wnt-1 and Wnt-4, which show reduced thymus size and cellularity [29]. Interestingly, Wnt-1 expression appears to be associated with medullary epithelium (unpublished observations), suggesting that there may also be specialization of Wnt protein production by different epithelial subsets.

In addition to Wnt expression by epithelial cells, we have shown that thymocytes express Fz receptors in a developmentally regulated pattern. In DN thymocytes Fz6 is the predominant Fz family member expressed, but is replaced by Fz5 during the transition from the DN to the DP stage. In turn, Fz5 is down-regulated in cells undergoing positive selection and transition from the DP to the SP stage. Although the functional significance of individual Fz receptors and Wnt proteins is still to be elucidated, this complex pattern of expression argues for a role for specific Fz receptors at different stages of thymocyte development. In this context, there is evidence that β-catenin-mediated events are involved in the maturation of DN to DP cells [13] and in the survival of DP cells pending TCR-mediated selection [14]. Thus signaling through Fz6 may be important for DN cell maturation whilst Fz5 provides signals for DP cell survival. Moreover, changes in Fz expression at two key developmental checkpoints regulated by signaling through the TCR complex raises the possibility that changes in Fz expression at sequential stages of thymocyte maturation are regulated by signaling through the pre-TCR and TCR complexes, respectively.

As well as expression by thymocytes we have also found expression of a range of Fz receptors on thymic epithelial cells. Thus it is possible that epithelial cell proliferation and differentiation are also influenced by Wnt proteins. Our current studies are aimed at investigating the role of Wnt proteins in the development of the thymic microenvironment.

4 Materials and methods

4.1 Animals

BALB/c ($H\text{-}2^d$) mice, housed under SPF conditions at the Biomedical Services Unit, University of Birmingham, were the source of fetal, neonatal and adult thymic material. In the case of embryonic mice, the day of detection of a vaginal plug was designated as gestational day 0.

4.2 Purification of thymocyte subpopulations

Thymocyte subpopulations were purified using antibody-coated DynaBeads, exactly as described [30]. DN $CD4^-CD8^-$ thymocyte subsets were further subdivided into DN1–DN4 subsets using a combination of anti-CD44- and anti-CD25-coated DynaBeads. Briefly, $CD44^+CD25^-$ (DN1) thymocytes were obtained by depletion of $CD25^+$ thymocytes by anti-CD25-coated DynaBeads from 14-day BALB/c fetal thymocyte suspension. DN2 and DN3 subsets were purified from 15-day BALB/c thymocyte suspensions using anti-CD25- and anti-CD44-coated magnetic beads (Dynal, Wirral, GB). Rosetted, $CD44^+25^+$ (DN2) thymocytes were snap-frozen for RT-PCR analysis. DN3 ($CD44^-CD25^+$) thymocytes were puri-

fied by depleting CD44$^+$ cells, using anti-CD44-coated DynaBeads. CD25$^-$CD44$^-$ (DN4) thymocytes were sorted from 17-day BALB/c thymocyte suspensions using multiple round of magnetic beads coated with anti-CD4, -CD8, -CD44 and -CD25 antibodies. Purity of preparations was routinely tested by flow cytometric analysis, with typical purities being greater than 95% (data not shown).

4.3 Thymic epithelial cell preparations

For E15 thymic epithelial cells thymus lobes from 15-day BALB/c embryos were digested with 0.25% trypsin in 0.02% EDTA, and then washed in RPMI + 10% FCS. Thymic epithelial cells were then purified from the resultant cell suspension using DynaBeads coated in anti-EpCAM antibody (clone G8.8, a kind gift of Dr A. Farr, University of Washington, Seattle). Cells were then immediately snap frozen for RT-PCR analysis. Functionally mature cortical epithelial cells were prepared from disaggregated deoxyguanosine-treated lobes by selection on anti-MHC class II-coated beads as described previously [31].

4.4 Western blotting

Stromal cell preparations and thymocyte subpopulations were lysed in lysis buffer (20 mM Hepes pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 137 mM NaCl, 50 mM β-glycerophosphate, 1% Triton X-100 supplemented with 1 mM dithiothreitol, 2 mM PMSF, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 2 μg/ml pepstatin, 2 mM Na_3VO_4, 0.1 M sodium pyrophosphate, 1 μM β-microcystin and 10 mM NaF, RNase and DNase) on ice for 20 min, then snap frozen in liquid nitrogen and stored at –70°C until used. Just before loading on 10% SDS-PAGE, the samples were boiled in 2× SDS sample buffer. Gels were blotted onto polyvinylidene difluoride membrane, blocked in 1% BSA and probed with anti-phospho-β-catenin (Upstate Biotechnology), anti-β-catenin

Table 1. Primer sequences used

	5′	3′	Product size
β-actin	5′-GTTACCAACTGGGACGACA	5′-TGGCCATCTCCTGCTCGAA	460
Wnt1	5′-GAACATAGCCTCCTCCACGA	5′-GGAATTGCCATTTGCACTCT	187
Wnt3a	5′-ATGGCTCCTCTCGGATACCT	5′-GGGCATGATCTCCACGTAGT	201
Wnt3	5′-ACCTGGAGAAGGCTGGAAGT	5′-CTTGTCCTTGAGGAAGTCGC	280
Wnt4	5′-CTCAAAGGCCTGATCCAGAG	5′-TCACAGCCACACTTCTCCAG	293
Wnt5a	5′-GTCTACCTGTGGCTGCAGC	5′-GCACACAGTAGTCCGGACTG	451
Wnt5b	5′-AGTGCAGAGACCGGAGATGT	5′-GACAGATGTGTTGTCCACGG	217
Wnt6	5′-AAGACTGGGGGTTCGAGAAT	5′-GATTGCAAACACGAAAGCTG	133
Wnt7a	5′-GGCTTCGCCAAGGTCTTCG	5′-CATGAGGTCACAGCCACTGG	440
Wnt7b	5′-TGCCCGTGAGATCAAAAAG	5′-CTGCGTTGTACTTCTCCTTG	200
Wnt8d	5′-GGTGGAATTGTCCTGAGCAT	5′-CCCTTTCTCCAAACTGTCCA	287
Wnt10a	5′-CCTGGAGACTCGGAACAAAG	5′-AACCGCAAGCCTTCAGTTTA	157
Wnt10b	5′-GATACCCACAACCGCAACTC	5′-GGCTCACCTTCATTTACACACA	323
Wnt11	5′-GCTCCATCCGCACCTGTT	5′-CGCTCCACCACTCTGTCC	331
Wnt13	5′-CACCCGGACTGATCTTGTCT	5′-TGTTTCTGCACTCCTTGCAC	252
Fz1	5′-TGCCAGCCATCAAAACTATAAC	5′-AGAGGACACTGAAGACTCCG	270
Fz2	5′-CGGCTCTATGTTCTTCTCGC	5′-AGCCGGACAGAAAGATGATG	170[a)]
Fz3	5′-TATTTCGTTCTCGCTCCCC	5′-CATGTCTTTTTGCTTCCAACC	395
Fz4	5′-GCTTCATCTCCACCACCTTC	5′-TCAGTTCATCGGCATCCAC	431
Fz5	5′-ACCTGTGTGTGTCACTGGGA	5′-ACTTGACACTGGGGATGAGC	295
Fz6	5′-CACTTCTCGGTTGCTATGTCTATG	5′-GGTCTGCTCGTCCCTCTTTG	520
Fz7	5′-CTTCCTGCTAGAGGACCGTG	5′-ATGGCCAAAATGGTGATTGT	295
Fz8	5′-CCAGAGCCTTGACAACCTAC	5′-AGAAAAGGCAGGCGACAAC	233
Fz9	5′-TTCACCGTGTTCACCTTCC	5′-AGAGTCAAAACCACCCACC	266
Fz10	5′-CGGCTCTATGTTCTTCTCGC	5′-CTTTCTTAGTGCCCTGCACC	297
LPR6	5′-GGTGTCAAAGAAGCCTCTGC	5′-GCTCGAGGACTGTCAAGGTC	294
β-catenin	5′-CACAACCTTTCTCACCACC	5′-GCTTGCTCTCTTGATTGCC	283
Tcf1	5′-ACCAGTCCCACAGTGTCCTC	5′-GGAGCAGCAGTGTCAATGAA	306

[a)] Alternative spliced form for Fz10

(Upstate Biotechnology), anti-mouse-Wnt-4 (R&D Systems), anti-Fz5 (Upstate Biotechnology) and anti-β-actin (Sigma) antibodies. Second-step antibodies were horseradish peroxidase-labeled anti-rabbit or anti-sheep or anti-goat Ig. Blots were visualized using the chemiluminescent Supersignal kit (Pierce) and scanned densitometrically for quantification (Syngene).

4.5 RT-PCR

RT-PCR was conducted with β-actin as an internal standard, as described previously [32]. Samples were matched for β-actin expression, then amplified with the relevant primer pair, scanned densitometrically and expressed as relative to β-actin. Primer sequences used in PCR reactions are given in Table 1. Fixed-point RT-PCR were performed at an annealing temperature of 55°C, using 1.5 mM Reddy Mix (Sigma) for 35 cycles, except for β-actin: annealing temperature 50°C, 2 mM Reddy Mix (Sigma), 28 cycles.

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Con A activates an Akt/PKB dependent survival mechanism to modulate TCR induced cell death in double positive thymocytes☆

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Abstract

While low avidity ligation of the T cell receptor (TCR) leads to positive selection and further maturation of developing thymocytes providing the immune system with mature $CD4^+$ and $CD8^+$ (single positive) T cells, high avidity ligation triggers negative selection by apoptotic cell death and therefore the TCR repertoire is purged of autoreactive T cells. On peripheral T cells, however, high avidity ligation of the TCR triggers activation and survival not death. In the present study we used concanavalin A (Con A) and α-CD3ε antibody to investigate a possible survival mechanism in connection with TCR ligation. Con A and α-CD3ε were used in the study for the following reasons: (1) they both mimic the effects of high avidity TCR ligation by activating peripheral T cells, and (2) they trigger distinctively different physiological changes in developing thymocytes. While Con A supports events associated with cellular survival, α-CD3ε induces apoptotic cell death. In our experimental system the TCR was cross-linked by Con A and α-CD3ε in thymocytes of major histocompatibility complex (MHC) deficient thymus organ cultures, where signals from the TCR can be triggered on zero background signal level. We have found that TCR cross-linking by Con A and not by α-CD3ε decreases the gene and protein expression of the pro-apoptotic molecule, Bad; and that Con A is capable of the activation of the survival signalling pathway including protein kinase B (Akt/PKB) independently of phosphatidyl inositol kinase (PI3K).

Keywords: Con A; Akt/PKB; Thymocytes

1. Introduction

The effects of concanavalin A (Con A) on activation and proliferation of mature T cells are well known and similar to those induced by α-CD3ε antibody, mimicking the effects of high avidity T cell receptor (TCR) ligation. Apart from T cell activation in the periphery, TCR engagement also plays an important role during T cell development in the thymus. Whilst low avidity TCR ligation drives $CD4^+8^+$, double positive (DP) thymocytes through positive selection, further maturation and therefore survival; high avidity TCR ligation induces negative selection and apoptotic cell death (Nossal, 1994). Despite the similar effects they trigger in mature T cells, by cross-linking the TCR with high avidity, Con A and α-CD3ε affect the development of immature thymocytes with strikingly different results. Whilst cross-linking of the TCR with α-CD3ε antibody (Smith et al., 1989) simulates the effects of negative selection by down-regulating CD4 and CD8 and culminating in apoptotic cell death (reviewed in Nossal, 1994); Con A is capable of inducing positive selection (Lovatt et al., 2000), therefore survival (Anderson et al., 1996) and further maturation of DP thymocytes. The above findings clearly indicate that high avidity TCR ligation, which is thought to be sufficient to trigger negative selection of DP thymocytes, can be modified by activating cellular survival mechanisms, which can override death signals.

One possible candidate in the involvement of the above signal modulation is the serine/threonine kinase protein kinase B (Akt/PBK), which mediates cell survival in a variety of systems, by phosphorylating and therefore inhibiting the function of pro-apoptotic members of the Bcl family (Datta et al., 1997), members of the Forkhead-related transcription factors (Brunet et al., 1999) and the death protease caspase-9 (Cardone et al., 1998). Studies involving over-expression of

Abbreviations: Akt/PKB, protein kinase B; JNK1, c-Jun N terminal kinase 1; MAPK, mitogen activated protein kinase; PI3K, phosphatidyl inositol kinase; PIP2, phosphatidyl inositol phosphate; PKA, protein kinase A; PKC, protein kinase C.

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constitutively active form of Akt/PKB has also prolonged the survival of DP thymocytes (Jones et al., 2000), making Akt/PKB an attractive candidate for mediating the death process.

Since Con A delivers signals which can block cell death and support positive selection, we hypothesised that distinct physiological effects induced by Con A and α-CD3ε in DP thymocyte populations could be the result of differential activation of the Akt/PKB related survival signalling pathway. In line with the above we show that apart from a decreased level of *bad* gene and protein expression, ligation of the TCR by Con A, as compared to α-CD3ε, results in differential phosphorylation of Bad and Bcl2, and that Con A phosphorylates the Bad kinase, Akt/PKB in a PI3K independent manner.

2. Materials and methods

2.1. Animals

DK, β2M −/− and major histocompatibility complex (MHC) I and II −/− (H-2^d), MHC double deficient mice embryos at day 15 of gestation, produced by timed matings, were used as sources of foetal material.

2.2. Purification of thymocyte subpopulations

Thymocyte sub-populations were purified by immunomagnetic selection from the thymi of newly born BALB/c (H-2^d) mice as described in details elsewhere (Moore et al., 1995).

2.3. Detection of DP thymocyte depletion

Thymus lobes (15 + 7 days DK mice) were treated in culture with 5 μg/ml of α-CD3ε antibody and 5 μg/ml of Con A for 18 h at 37 °C. DP thymocytes were immuno-labelled as described by Anderson et al. (1994). Briefly, thymus organ cultures were teased apart and the resulting cell suspensions were labelled with a mixture of PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 Abs, and fixed in 1% paraformaldehyde and analysed by flow cytometry.

2.4. Detection of apoptosis

Early signs of apoptosis were detected by Annexin staining since Annexin V has high affinity for the membrane phospholipid phosphatidyl serine, which is translocated from the inner membrane to the outer membrane in apoptotic cells earlier than DNA fragmentation would occur. Briefly, thymus lobes (15 + 7 days DK mice) were treated in culture with 5 μg/ml of α-CD3ε antibody and 5 μg/ml of Con A for 18 h at 37 °C then the thymus lobes were teased apart. The resulting cell suspensions were labelled with direct FITC-conjugate of Annexin V (final concentration: 1 μg/ml) (CLONTECH, Palo Alto) according to manufacturer's instructions and analysed by flow cytometry at 488 nm. Cells in later stages of apoptosis with increased membrane permeability were stained with propidium iodide and excluded from the analysis.

2.5. Western blotting

Thymocytes from embryonic organ cultures were teased out at t_0 and at 1, 4 and 12 h of treatments and lysed in lysis buffer (20 mM HEPES pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$ 137 mM NaCl, 50 mM β-glycerophosphate, 2 mM EGTA, 1% Triton X100 supplemented with 1 mM DTT, 2 mM PMSF, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatine, 2 mM Na_3VO_4, 0.1 M Na-pyrophosphate, 1 μM β-microcystin and 10 mM NaF) on ice for 20 min, then snap frozen in liquid nitrogen and stored at −70 °C until used. Just before loaded on 10% SDS-PAGE, the samples were boiled in 2× SDS sample buffer. Gels were blotted onto PVDF-membrane, blocked in 1% BSA and probed with anti-phospho-Akt (Upstate Biotechnology) or anti-phospho-Bad (Upstate Biotechnology) or mouse anti-Bad (Pharmingen) or anti-Bcl2 (Upstate Biotechnology) or β-actin (Sigma) antibodies.

Specific secondary antibodies were HRP-labelled and blots were visualised using the chemiluminescent Supersignal kit (Pierce) and densitometrically scanned for quantification (Alpha Imager, Flowgen).

2.6. Immunoprecipitation

Following treatment, thymocytes from embryonic organ cultures were teased out at t_0 and at 1 and 4 h of treatments, washed then lysed in lysis buffer (see in Western blotting) on ice for 20 min. The required antibody (anti-P-Serine, anti-Bcl2) plus protein A Sepharose were added to the lysates which were incubated O/N at 4 °C with constant rotation. Sepharose beads were collected by pulse centrifugation (5 min, 4 °C, 14,000 rpm) and washed in ice-cold TBS (10 mM Tris–HCl, pH 7.8). Sepharose beads were re-suspended in 50 μl TBS + 25 μl 2× SDS-sample buffer, then boiled for 3 min, spun down and the supernatant loaded on SDS-PAGE. Proteins separated on SDS-PAGE were blotted to Immobilon transfer membrane and either blocked in 1% BSA and probed with the relevant antibody or stained and placed for autoradiography to detect γ^{32}P-ATP (Amersham) incorporation.

2.7. Akt/PKB enzyme activity assay

Following 4 h treatment of foetal organ cultures with 5 μg/ml of α-CD3ε antibody and 5 μg/ml of Con A in DMEM, thymocytes were teased out and immunoprecipitated (see in detail above) for 2 h at 4 °C using anti-Akt/PKB antibody (Upstate Biotechnology). The sepharose beads were re-suspended in Akt/PKB assay buffer and enzyme

activity was measured by γ^{32}P-ATP incorporation into enzyme specific peptide substrates for 10 min at 30 °C, using a commercial Akt/PKB IP kinase assay kit (Upstate Biotechnology, TCS).

2.8. Inhibition of PI3K

Thymus lobes (15 + 7 days DK mice) were pre-incubated with the PI3K specific inhibitor LY294002 at the concentration of 1.5 μM for 60 min at 37 °C before adding treatment of 5 μg/ml of α-CD3ε antibody and 5 μg/ml of Con A in DMEM for 4 h at 37 °C. Thymocytes were then teased out and lysed in full lysis buffer (see in Western blotting) for immunoprecipitation or snap frozen for RNA extraction. For physiological studies thymocytes were incubated for 16 h at 37 °C then teased out and stained with CD4 and CD8 antibodies then analysed by FACS.

2.9. Inhibition of acidic sphingomyelinase and ceramide synthase

Thymus lobes (15 + 7 days DK mice) were pre-incubated with the acidic sphingomyelinase specific inhibitor SR33557 at the concentration of 30 μM and/or fumonisin at the concentration of 100 nM for 60 min at 37 °C before adding treatment of 5 μg/ml of α-CD3ε antibody and 5 μg/ml of Con A in DMEM for 4 or 16 h at 37 °C. Thymocytes were then teased out and lysed in full lysis buffer (see in Western blotting) for immunoprecipitation or snap frozen for RNA extraction or following 16 h incubation, thymocytes were immunostained for CD4 and CD8 expression.

2.10. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Reverse RT-PCR for the detection of all the gene mRNA investigated was conducted with β-actin as an internal standard, as described previously (Moore et al., 1993). *nur77* expression was used as positive control for gene expression in connection with T cell receptor ligation. Primer sequences used in PCR reactions: *β-actin*: (5′): 5′-GTT-ACC-AAC-TGG-GAC-GAC-A-3′; (3′): 5′-TGG-CCA-TCT-CCT-GC-T-CGA-A-3′; *nur77*: (5′): 5′-CCT-CGT-CGG-GTG-GAA-GAG-CTG-3′; (3′): 5′-GAG-GAG-GTA-CGT-CAG-TCT-TAG-3′; *bcl2*: (5′): 5′-CCT-GTG-CCA-CCA-TGT-GTC-CAT-C-3′; (3′); 5′-GCT-GAG-AAC-AGG-GTC-TTC-AG-A-GAC-3′; *tcf1*: (5′): 5′-AAA-GCA-CCA-AGA-ATC-CAC-AG-3′; (3′): 5′-ATG-CAT-TTC-TTT-TTC-CTC-CTG-CAC-3′; (3′); *bad*: (5′): 5′-GAG-TCG-CCA-CAG-TTC-GTA-C-3′; (3′): 5′-GAC-TCA-AGC-TGT-ACG-TCA-GC-3′.

3. Results

Since the thymic microenvironment has been shown to play an essential role in thymocyte development, the present study was performed in thymus organ cultures (Jenkinson and Anderson, 1994) to avoid disturbing the intra-thymic milieu, which perturbs thymocyte maturation (Marrack et al., 1988), and enhances thymocyte susceptibility to apoptosis (Porritt et al., 1998; van Ewijk, 1991). Furthermore, all the studies were carried out in MHC I/II deficient thymus lobes, which lack the necessary TCR ligands to trigger either positive or negative selection, so that thymocytes cannot progress beyond the DP developmental stage (Grusby et al., 1993). This system therefore represents a zero background signal level from the TCR and provides the ideal environment to investigate the effects of TCR ligation in developing thymocytes.

3.1. Physiological effects of α-CD3ε and Con A

To establish that both α-CD3ε Ab and Con A can penetrate intact thymus lobes and bind to DP thymocytes, we used direct FITC conjugates of α-CD3ε Ab and Con A to incubate intact thymus lobes in from MHC I/II deficient (DK) mice. Within 4 h 68% of DP thymocytes were labelled with α-CD3ε and 63% with Con A (Fig. 1a). To show that α-CD3ε and Con A did not just bind to DP thymocytes but also exerted physiological changes, a direct FITC-conjugate of Annexin V was used to detect early signs of apoptosis. While 18 h incubation with α-CD3ε resulted in a massive increase of Annexin positive thymocytes (42%) compared to controls (8%), Con A had no such effect (7%) (Fig. 1b) consistent with induction of death by α-CD3ε but not Con A driven signalling.

Having established that both α-CD3ε and Con A can penetrate the thymus lobes with the same efficiency, bind to DP thymocytes with different physiological consequences, it was also essential to examine the effects of α-CD3ε and Con A on changes in gene expression known to be related to TCR mediated signalling. As indicators, two genes, *nur77* and *tcf1* were selected. While *nur77* is a gene known to increase in response to TCR related apoptotic stimuli (Cheng et al., 1997; Winoto, 1997), the T cell restricted gene, *tcf1* (Verbeek et al., 1995), which is expressed very early in T cell development, has been reported to down-regulate during both TCR dependent positive (Verbeek et al., 1995) and negative selection (Jeon et al., 1998). Following 1 and 4 h incubation with α-CD3ε or Con A *nur77* mRNA expression was rapidly increased, while *tcf1* slowly decreased (Fig. 1c) indicating that TCR related signalling pathways were readily activated following both Con A and α-CD3ε stimulus, while physiological effects remained remarkably different.

3.2. Differential activation of signalling molecules

3.2.1. Akt/PKB activity

Akt/PKB is a key player in the survival of many cell types. To assess the role of Con A in activating the Akt/PKB dependent survival mechanism, we studied the level of Akt/PKB activation following Con A and α-CD3ε treatment.

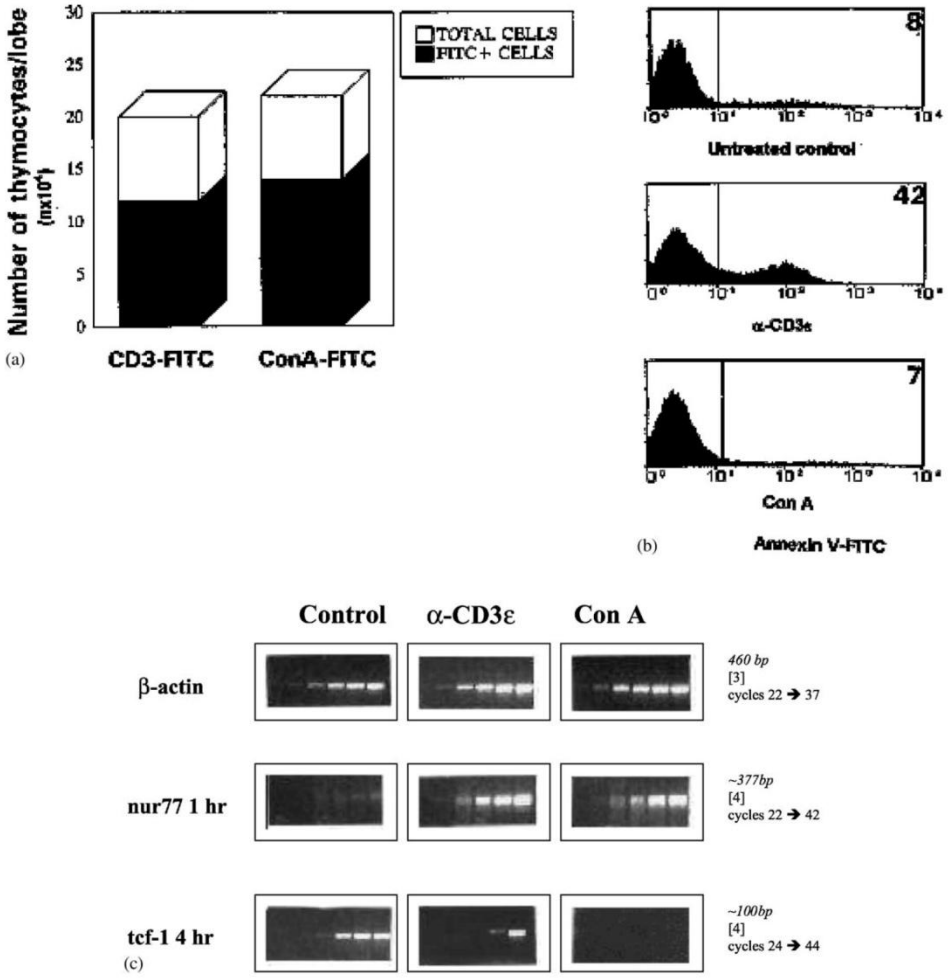

Fig. 1. Physiological effects of α-CD3ε (5 μg/ml) and Con A (5 μg/ml) on MHC DK thymocytes (15 + 7 days) in organ culture. (a) Number of thymocytes labelled with direct FITC conjugates of α-CD3ε and Con A following 4 h incubation shows an equal penetration rate to intact thymus lobes by both ligands. (b) Annexin V positive thymocytes following 18 h incubation in the presence or absence of α-CD3ε and Con A. The percentage of early apoptotic cells, 8 and 7% in control and Con A treated cultures, respectively, and 42% following α-CD3ε treatment, are indicated in the upper right corners of the figures. (The figures are representatives of five separate experiments). (c) Semiquantitative RT-PCR analysis of *nur77* and *tcf1* gene expression in MHC DK thymocytes (15 + 7 days) following 1 and 4 h incubation with α-CD3ε (5 μg/ml) and Con A (5 μg/ml) in organ cultures. Within 1 h of α-CD3ε and Con A treatment both ligand increased the message of *nur77*, *tcf1* mRNA began to decrease at the 4 h time point. (PCR data have β-actin as the inner standard. The size of PCR gene products in base pairs, the number of cycles and cycles were samples were taken for analysis (every three or four) are shown on the right hand side of the figures. The PCR data are representatives of two separate experiments).

It has been known that Akt/PKB is activated upon phosphorylation by upstream kinases (Franke et al., 1997), therefore the level of its phosphorylation is an indicator of the activation status of the enzyme. Following a 4 h incubation of thymocytes in thymic organ cultures with either α-CD3ε or Con A, a marked increase of Akt/PKB phosphorylation was detected by both Western blotting using anti-phospho-Akt antibody, and γ^{32}P-ATP incorporation into an Akt/PKB specific substrate (Fig. 2). Con A, however, increased Akt/PKB activity significantly above the level induced by α-CD3ε. To examine how their differences in Akt/PKB activation might be related to differential physiological outcomes, we investigated further elements of the Akt/PKB signalling pathway upstream and downstream of PKB.

3.2.2. PI3K

Since activation of Akt/PKB has been shown to be dependent on the presence of PI3K generated phospholipids, we theorised that inhibiting PI3K, and therefore the path to Akt/PKB activity, should render the effects of

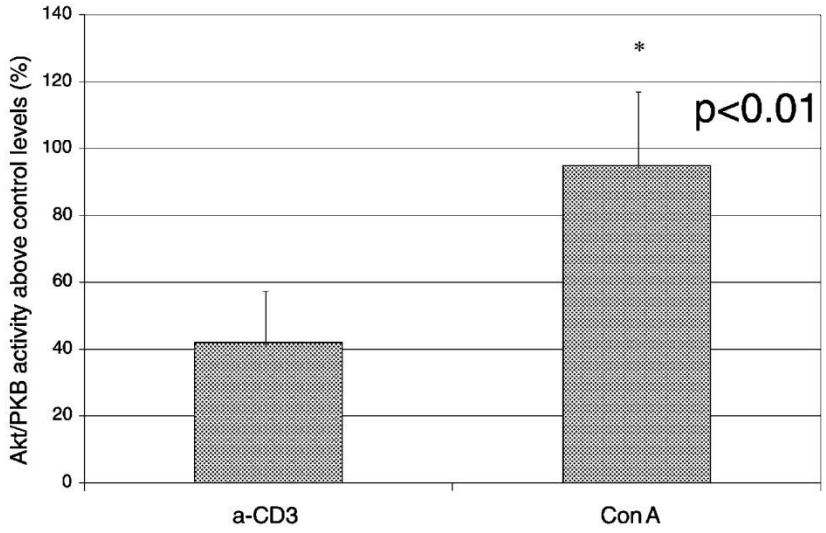

Fig. 2. Akt/PKB activity. MHC DK thymus lobes (15 + 7 days) were treated with α-CD3ε (5 μg/ml) and Con A (5 μg/ml) for 4 h in DMEM complemented with γ^{32}P-ATP. Thymocytes (10^6 per treatment) were lysed and Akt/PKB was immunoprecipitated. Kinase assay was performed according to manufacturers instructions and γ^{32}P-ATP incorporation was detected into the specific substrate.

TCR cross-linking by Con A similar to those of α-CD3ε. Firstly, we studied the effects of α-CD3ε and Con A on the phosphorylation of Akt/PKB in the presence of PI3K specific inhibitor, LY294002. Surprisingly, while α-CD3ε induced Akt/PKB phosphorylation was strongly inhibited by 1.5 μM LY294002, Con A induced Akt phosphorylation showed no reduction (Fig. 3), indicating that Akt/PKB phosphorylation induced by Con A can occur via a PI3K independent pathway. The above results were supported by experiments where 15 + 7 days DK thymus organ cultures were pre-incubated with 1.5 μM of LY294002, before adding α-CD3ε or Con A for a further 16 h incubation.

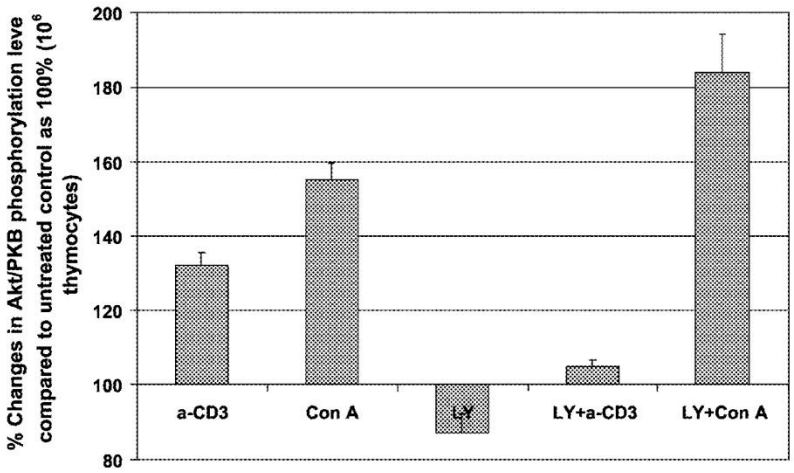

Fig. 3. Differential phosphorylation of Akt/PKB. MHC DK thymus lobes (15 + 7 days) following 4 h incubation with α-CD3ε (5 μg/ml) and Con A (5 μg/ml) in the continuos presence or absence of 1.5 μM LY294002 PI3 kinase inhibitor. Thymocytes (10^6 per treatment) were lysed and Akt/PKB protein immunoprecipitated using anti-Akt/PKB antibody. Proteins were separated on 10% SDS-PAGE and blotted onto Immobilon transfer membrane. The blots were probed with anti-phospho-Akt antibody then visualised using Chemiluminescent kit (Pierce) and densitometrically scanned for quantification (Alpha Imager, Flowgen) ($n = 5$). While the presence of PI3K inhibitor dramatically decreased Akt/PKB phosphorylation following α-CD3ε stimulus, it did not have a same effect following Con A treatment, suggesting that there is an independent mechanism for Akt/PKB phosphorylation used by Con A.

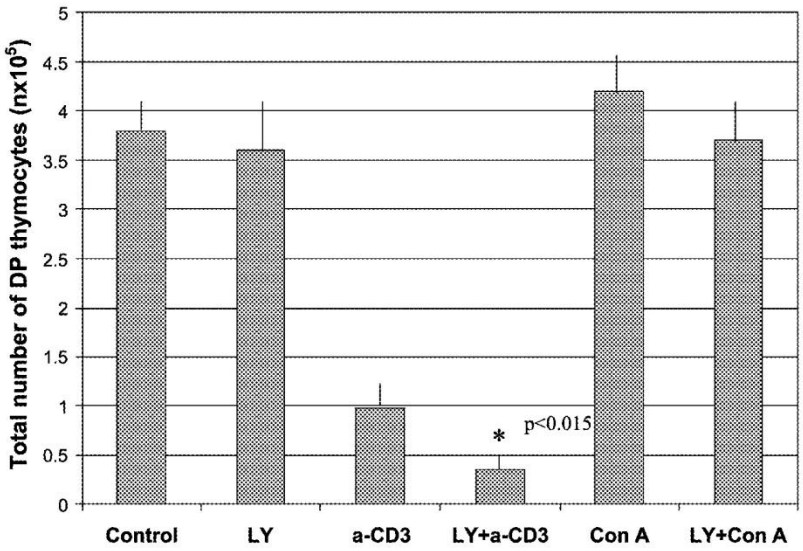

Fig. 4. Physiological effects of PI3K inhibition. Thymus lobes of (15 + 7 days) DK mice were pre-incubated with the PI3K specific inhibitor LY294002 at the concentration of 1.5 μM for 1 h at 37 °C before adding treatment of α-CD3ε (5 μg/ml) and Con A (5 μg/ml). Thymus lobe organ cultures were incubated for a further 16 h than stained for CD4 and CD8 and analysed by FACS. The presence of PI3K inhibitor did not affect thymocyte development in control cultures, but made them more sensitive to α-CD3ε induced apoptotic cell death, while had no effect on Con A treated organ cultures.

In agreement with studies using dominant negative PI3K, inhibition of PI3K did not affect thymocyte development (Sasaki et al., 2000). However, while decreased PI3K activity made thymocytes more sensitive to α-CD3ε induced apoptotic cell death, measured by an increase in DP thymocyte depletion (Fig. 4); while the effects of Con A were unchanged. The above results indicate that apart from a role for Akt/PKB phosphorylation in thymocyte survival, there is a pathway leading to Akt/PKB activation independently of PI3K, which is accessible to Con A.

3.2.3. *Bad phosphorylation*

In the context of cell death and survival, interactions between members of the Bcl2 family are known to play a crucial regulatory role and their activation is closely linked with the level of their phosphorylation. It has been shown in various cell systems, that cellular survival requires the phosphorylation of the pro-apoptotic protein Bad, which was identified as the first substrate of Akt/PKB (Datta et al., 1997; Franke et al., 1997; Gajewski and Thompson, 1996). When phosphorylated on serine residues, Bad dissociates from BclXL (Franke et al., 1997; Gajewski and Thompson, 1996), thus BclXL can block the release of cytochrome *c* from the mitochondria preventing subsequent caspase mediated apoptosis (Franke et al., 1997). We show that following treatment with Con A or α-CD3ε, Bad protein is rapidly phosphorylated (Fig. 5a), but while Con A treatment triggers a four-fold increase, α-CD3ε stimulation only increases Bad phosphorylation two-fold compared to untreated controls. At the 4 h time point using RT-PCR analysis a significant decrease was detected in *bad* mRNA expression following Con A but not α-CD3ε treatment (Fig. 5b). Compared with untreated controls, the resulting decrease at Bad protein levels was detectable in Con A treated thymocytes following a further 8 h incubation in the presence of Con A (Fig. 5c), while α-CD3ε Ab increased Bad protein levels (Fig. 5c).

3.2.4. *Bcl2 phosphorylation*

Another prominent member of the Bcl family is Bcl2, which has been shown to play an essential role in thymocyte survival (Siegel et al., 1992; Strasser et al., 1994). It is also known that Bcl2 to exert its anti-apoptotic effects depends on dimerisation with pro-apoptotic members of the Bcl2 family, such as Bax and Bid (Miller et al., 1997; Oltvai et al., 1993; Sato et al., 1994), and that its anti-apoptotic role is dependent on its level of phosphorylation (Ito et al., 1997; Srivastava et al., 1999). To examine whether differential activation of the anti-apoptotic Bcl2 is a factor in the differential effects of α-CD3ε and Con A to promote thymocyte death and survival, we studied the level of Bcl2 phosphorylation in thymocytes in the presence and absence of Con A or α-CD3ε. We have found that whilst Con A triggered a marked increase (Fig. 6), α-CD3ε decreased Bcl2 phosphorylation by approximately 30% (Fig. 6) as compared to untreated controls. Neither treatment seemed to have affected the level of Bcl2 protein expression at the time point studied, indicating that its activation status rather than the presence or absence

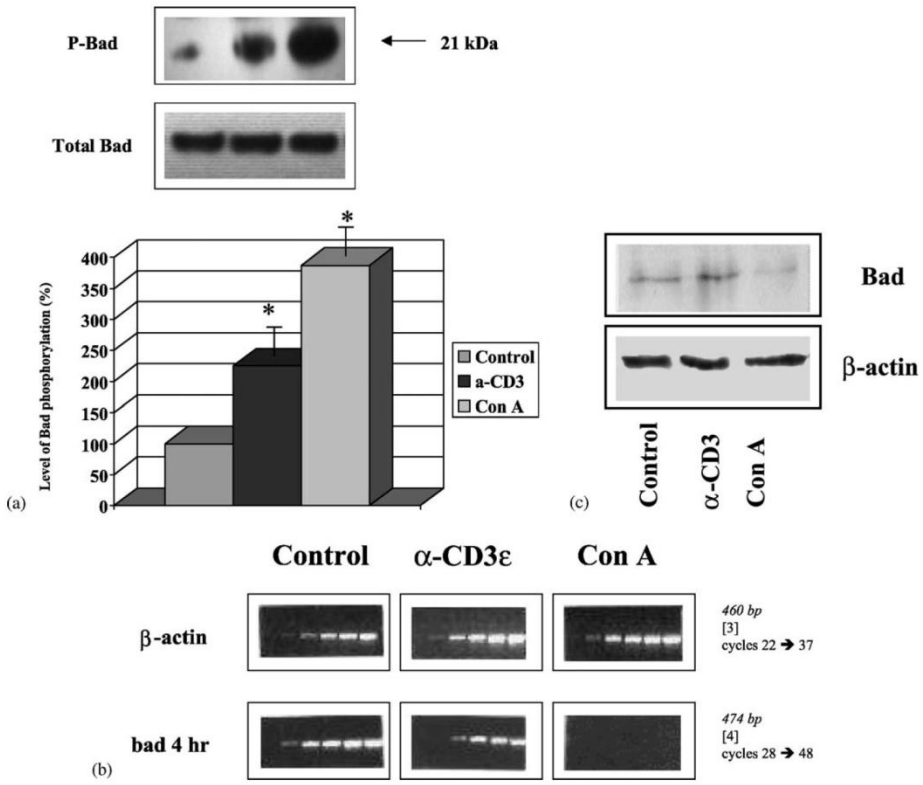

Fig. 5. Bad phosphorylation and mRNA levels. (a) MHC DK thymus lobes (15 + 7 days) were treated with α-CD3ε (5 μg/ml) and Con A (5 μg/ml) for 4 h in DMEM. Thymocytes (10^6 per treatment) were lysed and Bad was immunoprecipitated. Proteins were separated on 15% SDS-PAGE and blotted onto Immobilon transfer membrane. The blots were first probed with anti-phospho-Bad antibody and visualised using Chemiluminescent kit (Pierce). (b) To check equal protein loading, total Bad protein was revealed by probing the same blot with anti-Bad antibody. (c) MHC DK thymus lobes (15 + 7 days) were treated with α-CD3ε (5 μg/ml) and Con A (5 μg/ml) for 12 h in DMEM. Thymocytes (10^6 per treatment) were lysed and proteins were separated on 15% SDS-PAGE and blotted onto Immobilon transfer membrane. The blots were first probed with anti-Bad then anti-β-actin antibody. (The Western blots are representatives of four separate experiments).

of Bcl2 which is important to influence DP thymocyte survival.

3.2.5. *Inhibition of phosphatases*

Increased activity of two kinases, Akt/PKB and protein kinase C α (PKCα) and differential phosphorylation of their downstream substrates, Bad and Bcl2, raised the possibility of the involvement of phosphatases. Since it has been shown that PP2A regulates PKCα (Lee et al., 1996) activation and also that Bcl2 co-localises with a PP2A phosphatase (Deng et al., 1998) which dephosphorylates Bcl2 almost immediately following phosphorylation, okadaic acid, a potent PP2A inhibitor was introduced to inhibit the effects of α-CD3ε. Incubation of DP thymocytes in thymic organ cultures in the presence of okadaic acid had proved to be lethal for thymocytes, even at picomolar concentration (data not shown), therefore an alternative route was selected to try to inhibit phosphatase activity. PP2A phosphatases have been shown to be activated by ceramides (Chalfant et al., 1999; Ruvolo et al., 1999). Both acidic sphingomyelinase and ceramide synthase can catalyse biochemical processes leading to ceramide production. While acidic sphingomyelinase catalyses sphingomyelin break down into ceramide, ceramide synthase catalyses ceramide production from sphingosine. In order to inhibit ceramide production and therefore phosphatase activation in thymocytes, thymus organ cultures were pre-incubated for an hour with 30 μM of acidic sphingomyelinase inhibitor SR33557, or 100 nM of ceramide synthase inhibitor, fumonisin, before Con A and α-CD3ε stimulus. The cultures were incubated for a further 16 h and thymocyte depletion was determined by CD4 and CD8 down-regulation measured by FACS analysis. Neither fumonisin or the acidic sphingomyelinase inhibitor, SR33557, was able to inhibit α-CD3ε induced thymocyte depletion (data not shown and Fig. 7, respectively). Surprisingly, however, despite its ability to reduce the TCR

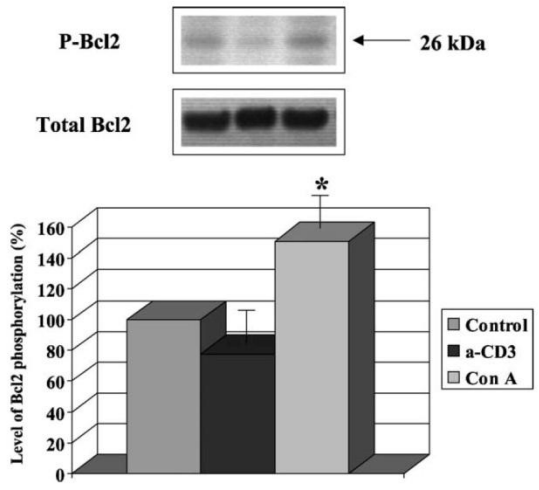

Fig. 6. Bcl2 phosphorylation. MHC DK thymus lobes (15 + 7 days) were treated with α-CD3ε (5 μg/ml) and Con A (5 μg/ml) for 4 h in DMEM complemented with γ^{32}P-ATP. Thymocytes (10^6 per treatment) were lysed and Bcl2 was immunoprecipitated. Proteins were separated on 15% SDS-PAGE and blotted onto Immobilon transfer membrane. To show that Bcl2 protein expression did not change at the time of the experiment, Bcl2 protein was revealed using differential protein staining (Pierce) "total Bcl2" while increased phosphorylation following Con A treatment was revealed by autoradiography (same membrane for 7 days at −70 °C) "p-Bcl2". (The blots are representatives of three separate experiments).

cross-linking mediated increase of *nur77* gene expression (data not shown), SR33557 was able to increase DP thymocyte depletion when co-cultured with Con A (Fig. 7) indicating, that Con A induced thymocyte survival might be a ceramide dependent process.

4. Discussion

Previously we have shown that whilst ligation of the TCR on immature thymocytes by either Con A or α-CD3ε can lead to phosphatidyl inositol phosphate (PIP2) hydrolysis and therefore the activation of downstream signalling pathways, only α-CD3ε and not Con A induced TCR cross-linking results in the induction of apoptosis (Anderson et al., 1996). In the present study we supported the above finding and investigated the molecular basis of this difference. We have theorised that since Con A has the ability to engage glycosylated surface molecules in addition to those of the TCR complex, activation of a well established survival mechanism might modulate the outcome of TCR cross-linking (Lu and Chen, 1994; Watanabe et al., 1996; Heinly et al., 2001).

The fact that Con A ligates LFA-1 (Watanabe et al., 1996) in addition to cross-linking the TCR and supports developmental events associated with cellular survival (Lovatt et al., 2000), made the Akt/PKB dependent signalling pathway an attractive candidate for two reasons. Firstly, that Akt/PKB is a key player in a signalling pathway which mediates cell survival in a variety of systems (Brunet et al., 1999; Cardone et al., 1998; Datta et al., 1997) and secondly, that LFA-1 has recently been reported to trigger a potent Akt/PKB activation (Perez et al., 2002).

Based on the above data we also hypothesised that the reason why high avidity TCR ligation does not always lead to apoptotic cell death in developing thymocytes, is that TCR induced apoptotic signals can be inhibited by the activation of the Akt/PKB survival signalling pathway.

We have shown that despite the strikingly different physiological outcome of Con A and α-CD3ε treatment, both Con A and α-CD3ε trigger an increase in *nur77* and a decrease in *tcf1* mRNA levels. Although *tcf1* down-regulation occurs during both TCR ligation dependent negative (death) (Jeon et al., 1998) and positive (survival) selection (Verbeek et al., 1995), increase in *nur77* gene expression is thought only to occur during TCR related, and therefore high avidity TCR ligation induced, apoptosis (Cheng et al., 1997; Winoto, 1997). Investigating possible gene expression changes associated with survival we discovered that while Con A triggered a marked decrease in *bad* mRNA and at a later time point Bad protein levels, α-CD3ε did not have the same effect. The pro-apoptotic Bad is part of the Bcl2 family of proteins, that are known to play an important role in regulating cell death and cell survival (Miller et al., 1997; Oltvai et al., 1993; Sato et al., 1994). It has also been discovered that for its de-activation and therefore the release of anti-apoptotic BclXL, Bad needs to be phosphorylated by Akt/PKB (Franke et al., 1997; Franke and Cantley, 1997; Gajewski and Thompson, 1996). Additionally, the anti-apoptotic role of Bcl2 (Ito et al., 1997; Srivastava et al., 1999), which molecule is one of the key regulators of thymocyte survival (Siegel et al., 1992; Strasser et al., 1994), is also dependent on its level of phosphorylation. Therefore, our finding that Con A, but not α-CD3ε, induces a marked increase in both Bad and Bcl2 phosphorylation as well as substantial down-regulation in *bad* expression, seem to prove the existence of a signalling mechanism, which once activated, can sufficiently protect thymocytes against apoptotic cell death induced by high avidity TCR ligation. Investigating the possible involvement of signalling pathways, we have discovered that both PKCα and Akt/PKB are differentially phosphorylated by Con A and α-CD3ε; and that Con A can activate the Bad kinase, Akt/PKB, in a PI3K independent manner. The known co-localisation of Bcl2 with a ceramide activated PP2A phosphatase (Ruvolo et al., 1999), which dephosphorylates both Bcl2 (Ruvolo et al., 1999) and its kinase PKCα (Lee et al., 1996; Ruvolo et al., 1998), raised the possibility that the substantial differences in Bcl2 protein phosphorylations are due to increased phosphatase activity induced by α-CD3ε and not by Con A. Although inhibiting ceramide production, and therefore phosphatase activity, did not significantly

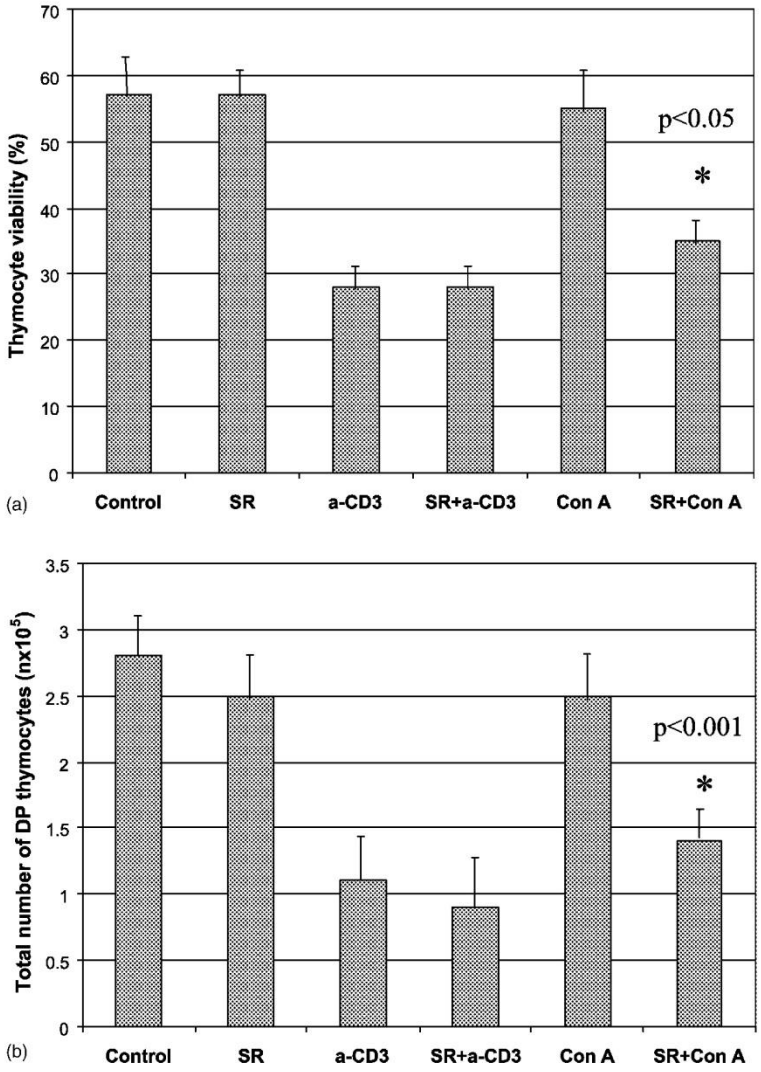

Fig. 7. The effect of acidic sphingomyelinase inhibitor, SR33557, on DP thymocyte depletion. Thymus lobes of (15 + 7 days) DK mice were pre-incubated with the acidic sphingomyelinase specific inhibitor SR33557 at the concentration of 30 μM for 1 h at 37 °C before adding treatment of α-CD3ε (5 μg/ml) and Con A (5 μg/ml) for a further 16 h. Thymocyte depletion was detected by CD4 and CD8 down-regulation using CD4-PE and CD8-FITC antibodies and analysed by FACS. Viability was detected by propidium iodide staining.

affect α-CD3ε induced DP thymocyte depletion; interestingly, SR3399 was able to significantly alter the effects of Con A. The above data revealed that without acidic sphingomyelinase enzyme activity, the physiological effects of Con A are remarkably similar to those triggered by α-CD3ε, indicating that ceramide, which normally associated with apoptotic cell death, might play an important role in regulating survival dependent developmental events in DP thymocytes. Based on experiments showing a role for ceramides in differentiation and proliferation (Kolesnik and Fuks, 1995) via the activation of PKCζ, a regulator of kB-dependent promoter activity and mitogenesis (Lozano et al., 1994) we can speculate, that a similar, ceramide dependent survival mechanism exist in thymocytes. A more detailed study of the role of acidic sphingomyelinase in TCR induced signal transduction, however, is awaiting further investigations.

In summary, we have shown that two different TCR ligands that both bind the TCR with sufficient avidity to trigger activation of mature T cells, nevertheless have a differential ability to activate apoptosis in immature thymocytes.

Differences in the pattern of TCR clustering between Con A and α-CD3ε and/or the ability of Con A to bind LFA-1 in addition to TCR that provide signals modulating the consequences of TCR ligation. Induction of survival during positive selection has been proposed to be regulated through LFA-1/ICAM-1 interaction in the thymic cortex (Kishimoto et al., 1996), which based on our signalling studies with LFA-1 binding Con A, is a highly likely mechanism. The above data have also highlighted the possibility that avidity of TCR ligation may not as a decisive factor in thymocyte selection as it was thought before and despite of previous beliefs, signal transduction initiated by high avidity TCR ligation could be modulated by activation of additional signalling pathways.

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Induction of thymocyte positive selection does not convey immediate resistance to negative selection

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SUMMARY

The acquisition of functional competence represents a critical phase during intrathymic development of T cells. Thymocytes reaching this stage represent cells which have been positively selected on the basis of major histocompatibility complex reactivity, but which have also been purged of potentially autoreactive T-cell receptor specificities by negative selection. While the developmental window in which thymocytes are subjected to positive selection is now well defined, the precise developmental timing of negative selection, in relation to positive selection events, is less clear. Moreover, the underlying mechanism allowing single-positive thymocytes to respond to T-cell receptor ligation by activation rather than death, remains controversial. Here we have analysed the developmental timing of negative selection in relation to positive selection, using measurement of thymocyte susceptibility to dendritic cell presentation of the superantigen staphylococcal enterotoxin B (SEB). We show that thymocytes which have received initial positive selection signals, namely $CD4^+$ $CD8^+$ $CD69^+$ thymocytes, like their $CD4^+$ $CD8^+$ $CD69^-$ precursors, are susceptible to negative selection, indicating that induction of positive selection does not convey immediate resistance to negative selection. In contrast, newly generated $CD4^+$ $CD8^-$ $CD69^+$ cells are not only resistant to deletion by SEB, but respond to SEB-mediated T-cell receptor-ligation by activation, indicating that the acquisition of functional competence occurs at the newly generated $CD4^+$ $CD8^-$ $CD69^+$ stage. Finally, by using direct retroviral infection of primary $CD4^+$ $CD8^+$ thymocytes, we also show that Notch-1 activation in $CD4^+$ $CD8^+$ thymocytes does not correlate with, nor convey resistance to superantigen-mediated negative selection. Thus, our data suggest that although Notch-1 has been implicated in resistance to thymocyte apoptosis, the acquisition of resistance to negative selection occurs independently of Notch-1 signalling.

INTRODUCTION

Specific interactions between the αβT-cell receptor (TCR) and self-peptide–major histocompatibility complex (MHC) complexes represent the basis of an intrathymic screening mechanism which governs the development of a non-autoreactive peripheral T-cell repertoire. This mechanism is dependent upon the operation of both positive and negative selection events at defined stages of thymocyte development. The former selects those thymocytes bearing a TCR of useful specificity, capable of recognizing foreign antigen-derived peptide in the context of self-MHC molecules, while the latter removes thymocytes bearing a TCR with potential for autoreactivity to self-peptide–self-MHC complexes.[1] While these two events are both dependent upon ligation of the TCR by peptide–MHC complexes, these interactions lead to very different outcomes. Positive selection of thymocytes, which requires medium to low avidity TCR ligation, triggers further differentiation and maturation,[2,3] eventually generating a population of phenotypically and functionally mature cells ready for export to the peripheral T-cell pool. In contrast, thymocytes making high avidity TCR–MHC interactions are negatively selected and triggered to undergo apoptosis, thus preventing generation of functional T cells with autoreactive specificities.

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The major population of thymocytes within the thymus is that of small cortical cells at the $CD4^+$ $CD8^+$ stage, and it is this population which is subjected to positive selection events. However, these cells are also targets of negative selection.[1,3] The developmental timing of negative selection in relation to positive selection is poorly defined, and it is not clear whether the window for negative selection encompasses a broad span of thymocyte development, or is more strictly limited to thymocytes at a specific stage of maturation.

An important change that is triggered by positive selection is the acquisition of increased resistance to apoptosis. Thus, TCR stimuli that lead to rapid apoptotic death in cortical thymocytes can result in activation in mature T cells.[4] However, the molecular basis for the increased resistance to apoptosis triggered by positive selection remains unknown. It has previously been demonstrated that Notch-1 signalling plays a role in mediating resistance to the induction of apoptosis in thymocyte cell lines,[5,6] although a recent paper suggests that Notch-1 does not play an essential role in thymocyte positive selection events.[7] With regard to negative selection, however, the role of Notch-1 remains unclear, since the latter report only studied the role of Notch-1 in resistance to dexamethasone-induced apoptosis, and so the relation of Notch-1 signalling to antigen-induced negative selection is still unknown. Thus, in this report we have studied the developmental timing of negative selection in relation to the induction of positive selection and activation of the Notch-1 signalling pathway. Using a system involving dendritic cell presentation of the superantigen staphylococcal enterotoxin B (SEB) we have investigated thymocyte susceptibility to negative selection at a number of phenotypic stages of development from the $CD4^+$ $CD8^+$ $CD69^-$ stage onwards. Our data indicate that while prepositive selection $CD4^+$ $CD8^+$ $CD69^-$ thymocytes, and also interestingly $CD4^+$ $CD8^+$ $CD69^+$ thymocytes, are still susceptible to negative selection events, resistance to negative selection is acquired at the $CD4^+$ $CD8^-$ $CD69^+$ stage. Moreover, this acquisition of resistance to deletion does not appear to correlate with activation of Notch-1 signalling, nor can constitutive activation of the Notch-1 signalling pathway overcome thymocyte susceptibility to negative selection. Collectively these data indicate that functional competence is achieved early in the $CD4^+$ $CD8^-$ phase, via a Notch-1-independent mechanism.

MATERIALS AND METHODS

Mice

BALB/c mice, Bcl-2 transgenic (tg) mice (under control of the p56lck promoter)[8] and MHC-deficient mice (Taconic) were bred and maintained at the Biomedical Sciences Unit, University of Birmingham, UK. Adult mice (4–6 weeks) were used as a source of $CD4^+$ $CD8^-$ $CD69^+$ HSA^+ (heat stable $antigen^+$) thymocytes, and mesenteric and inguinal lymph nodes, while thymocyte subpopulations were isolated from either BALB/c, Bcl-2 tg or MHC-deficient neonatal mice (0–2 days), or adult (4–6 weeks) BALB/c mice.

Antibodies and immunoconjugates

The following antibodies were coated onto anti-rat immunoglobulin G (IgG) or streptavidin-coated Dynabeads (Dynal, Wirral, UK) as appropriate: biotinylated anti-CD69 (clone H1.2f3, Pharmingen, San Diego, CA), anti-CD8 (clone YTS169.4, Seralab, Crawley Down, UK), anti-CD3 (clone KT-3, Serotec, Oxford, UK). Antibodies used for flow cytometric analysis were as follows: phycoerythrin (PE)-conjugated anti-CD4 (GK1.5, Pharmingen), fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (clone 53-6.7, Pharmingen), FITC-conjugated anti-BrdU (clone 3D4, Pharmingen), biotinylated anti-Vβ8 (clone F23.1, Pharmingen), biotinylated anti-CD25 (clone 7D4), biotinylated anti-CD24 (anti-HSA) (clone M1/69). Biotinylated antibodies were detected using a subsequent incubation in streptavidin allophycocyanin (APC) (Pharmingen).

Cell purification

Thymocytes. Methods to isolate thymocyte subsets have been described in detail elsewhere.[9,10] Briefly, pre-selection thymocytes were obtained from either MHC-deficient neonatal mice, which are halted at the $CD4^+$ $CD8^+$ stage and do not express CD69, due to a lack of exposure to positively selecting MHC molecules, or from Bcl-2 tg mice, as previously described.[11] Briefly, thymocytes from neonatal Bcl-2 tg mice were depleted of $CD3^+$ cells using anti-CD3-coated beads. This was followed by selection of $CD8^+$ cells using anti-CD8-coated beads, which were subsequently removed using Detachabead (Dynal). $CD69^+$ cells were prepared by immunomagnetic selection from neonatal BALB/c mice by positive selection using anti-CD69-coated beads, which were subsequently removed by Detachabead (Dynal). $CD4^+$ $CD8^+$ $CD69^+$ or $CD4^+$ $CD8^-$ $CD69^+$ cells were obtained by selection of $CD69^+$ cells, followed by either selection or depletion, respectively, of $CD8^+$ cells using anti-CD8-coated beads. $CD4^+$ $CD8^-$ $CD69^+$ HSA^+ thymocytes were purified from adult BALB/c mice by isolation of $CD4^+$ $CD8^-$ $CD69^+$ cells as described, and then selected for HSA^+ cells by labelling with biotinylated anti-HSA, followed by immunomagnetic selection using streptavidin microbeads (Miltenyi Biotec, Bisley, UK).

Dendritic cells. Cells from mesenteric and inguinal lymph nodes were pooled and selected for $CD11c^+$ cells, using anti-CD11c microbeads (Miltenyi Biotec). $CD11c^+$ cells were further purified by depletion of contaminating $B220^+$ cells, using precoated Mouse pan B (B220) Dynabeads (Dynal).

Deletion/stimulation assays

Isolated thymocyte populations were mixed together with isolated dendritic cells, at a ratio of 10 : 1, by centrifugation. The resultant cell pellet was transferred to the surface of a nucleopore filter in organ culture conditions, in the presence or absence of 10 μg/ml SEB (Toxin Technology, Sarasota, FL). Thymocyte proliferation in cultures was analysed simultaneously with expression of Vβ8. Thus cultures were pulsed with 5 μg/ml 5-bromo 2′-deoxyuridine

(BrdU, Sigma Chemical Co., Poole, UK) for the final 24 hr of a 2-day culture period. Cultures were harvested, and cells were labelled for surface expression of Vβ8, with BrdU incorporation being detected as described by Tough and Sprent.[12]

Real-time polymerase chain reaction

Multiplex polymerase chain reaction (PCR) was performed in ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Relative quantification of gene expression was achieved by using primer pairs to amplify the target genes in the presence of probes labelled with FAM reporter dyes and VIC reporter dye labelled probe for β-actin as endogenous control. Primer pairs and probes were designed using the *Taq* Man Probe and PRIMER DESIGN computer programme and data was analysed by the $\Delta\Delta C_T$ method. Primer sequences were as follows: deltex-1: F: CCGTGGTGTGGAACGAGATT, R: ACGTTGTCTAGGTAGCTGGCGT, Probe: CCTCACTGGTCACGGCTACCCCG; β-actin: F: CGTGAAAAGATGACCCAGATCA, R: TGGTACGACCAGAGGCATACAG, Probe: TCAACACCCCAGCCATGTACGTAGCC.

Semi-quantitative PCR

Total RNA was extracted from cells using TRIzol (Life Technologies, Paisley, UK) according to the manufacturer's instructions. RNA samples were treated with RNase-free Dnase1 (Pharmacia Biotech, Uppsala, Sweden) to remove any contaminating genomic DNA. This was followed by reverse transcription (RT). The RT-PCR was performed, with β-actin as a housekeeping gene. The sequences for β-actin and Deltex primers are as follows: β-actin: sense, 5′-GTTACCAACTGGGACGACA-3′; antisense, 5′-TGG CCATCTCCTGCTCGAA-3′: Deltex: sense, 5′-CACTGG CCCTGTCCACCCAGCCTTGGCAGG-3′; antisense, 5′-GAGGCATGTGCCAGGCTAGAGGCAAGGCAA-3′.

RT-PCR involved analysis of samples every three cycles from 18 to 33 (β-actin) or every four cycles from 24 to 44 (Deltex). PCR products were analysed by ethidium bromide agarose gel electrophoresis and identified by fragment size.

Retroviral infection of CD4$^+$ CD8$^+$ CD69$^-$ thymocytes

The cDNA encoding the intracellular domain of human Notch-1, ICN1, cloned into *Mig*RI, a vector that permits co-expression of cloned cDNA and green fluorescent protein (GFP) from single bicistronic message, was a generous gift from W.S. Pear (Department of Pathology and Laboratory Medicine, University of Pennsylvania Medical Center, Philadelphia, PA). Plasmid DNA was transfected into the ΦNX-A packaging cell line (American Type Culture Collection, Rockville, MD) by electroporation. Retrovirus-containing supernatant was collected following 8-hr incubation of the transfected packaging cells at 32°. Purified CD4$^+$ CD8$^+$ thymocytes from Bcl-2 tg mice were infected by re-suspending the thymocytes at 7×10^6/ml in retrovirus supernatant with 8 μg/ml polybrene followed by centrifugation for 60 min at 1000 ***g*** at room temperature and overnight incubation at 37°.

RESULTS

Positive selection does not convey immediate resistance to negative selection

Conflicting evidence exists regarding whether thymocytes which have initiated positive selection are still susceptible to deletion.[9,13–15] Thus it is still unclear whether the induction of positive selection conveys immediate resistance to negative selection, or whether a window exists following the initiation of positive selection where thymocytes are still susceptible to deletion. To address this controversy, in an initial set of experiments we compared the responses of a number of freshly isolated thymocyte populations to dendritic cell presentation of the bacterial superantigen SEB, a well-defined model of negative selection.[16] To study a range of thymocyte populations which encompass positive and negative selection events, we isolated preselection CD4$^+$ CD8$^+$ cells known to be at a prepositive selection stage by virtue of a CD69$^-$ phenotype. We also obtained purified CD69$^+$ thymocytes, in which CD69 expression is an indicator of the initiation of positive selection events,[17,18] and CD4$^+$ CD8$^-$ CD69$^+$ cells, representing a population of newly generated CD4$^+$ CD8$^-$ thymocytes.

MHC class II presentation of SEB is known to target specifically, amongst others, TCRs using the Vβ8 segment in their β-chain. Thus we have analysed here the SEB-reactive Vβ8$^+$ fraction of thymocytes. As expected, CD4$^+$ CD8$^+$ CD69$^-$ thymocytes cultured overnight with dendritic cells in the presence of 10 μg/ml SEB showed a specific reduction in numbers of Vβ8$^+$ CD4$^+$ CD8$^+$ cells (Fig. 1a,b). In fact, approximately 45% of Vβ8$^+$ CD4$^+$ CD8$^+$ thymocytes were deleted in comparison to the number of Vβ8$^+$CD4$^+$ CD8$^+$ cells recovered from culture in the absence of SEB (Fig. 1c). Interestingly, culture of CD69$^+$ thymocytes in the same culture system also showed evidence of deletion of Vβ8$^+$ CD4$^+$ CD8$^+$ cells, with approximately 55% of Vβ8$^+$ CD4$^+$ CD8$^+$ CD69$^+$ cells deleted by SEB (Fig. 1f). In contrast, however, CD4$^+$ CD8$^-$ CD69$^+$ thymocytes, representing newly generated T cells and the direct descendants of CD4$^+$ CD8$^+$ CD69$^+$ cells, were not deleted by SEB, with similar numbers of Vβ8$^+$ CD4$^+$ CD8$^-$ CD69$^+$ thymocytes recovered from cultures whether SEB was absent or present in the culture medium (Fig. 1d–f). It is important to note here that we have also looked at the response of a thymocyte population expressing a Vβ which does not interact with SEB. It has previously been demonstrated that Vβ2$^+$ thymocytes are not SEB-reactive,[19] and indeed, in these experiments, while culture with SEB led to deletion of CD4$^+$ CD8$^+$ thymocytes expressing Vβ8, CD4$^+$ CD8$^+$ Vβ2$^+$ thymocytes remained unaffected by culture in the presence of SEB (data not shown).

Since Vβ8$^+$ CD4$^+$ CD8$^-$ CD69$^+$ thymocytes showed no evidence of deletion following overnight exposure to dendritic cell presentation of SEB, we analysed Vβ8$^+$ CD4$^+$ CD8$^-$ CD69$^+$ thymocytes cultured in the absence or presence of SEB for evidence of activation. Interestingly, following a 24-hr culture period, we found that Vβ8$^+$ CD4$^+$ CD8$^-$ CD69$^+$ thymocytes recovered from cultures

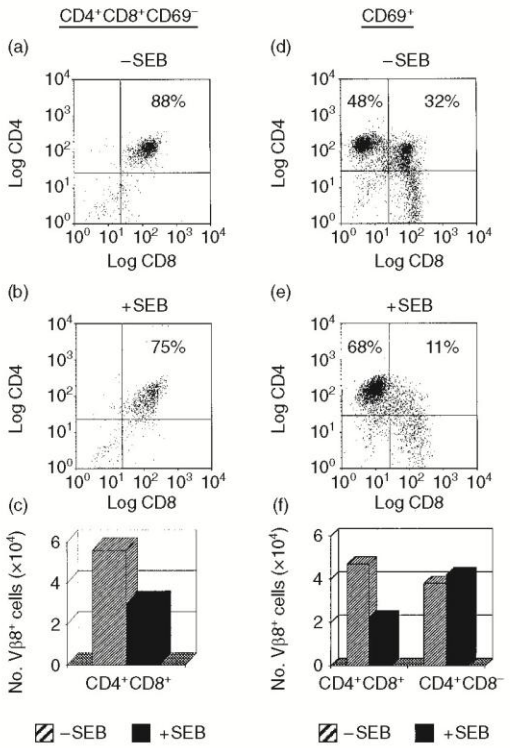

Figure 1. Induction of positive selection does not convey immediate resistance to negative selection. $CD4^+$ $CD8^+$ $CD69^-$ thymocytes (a, b) from MHC-deficient neonatal mice, or $CD69^+$ (d, e) thymocytes isolated from wild-type neonatal mice were placed in culture with freshly isolated dendritic cells at a ratio of 10 : 1, in the absence (a, d) or presence (b, e) of SEB at 10 μg/ml. Following a 24-hr culture period, thymocytes were harvested and analysed for expression of CD4, CD8 and Vβ8. In the experiment shown, 1×10^6 thymocytes were placed in each culture, with cell recoveries as follows: 9×10^5, 8×10^5, $4{\cdot}7 \times 10^5$ and $5{\cdot}5 \times 10^5$ for cultures (a), (b), (d) and (e), respectively. From this the total number of $V\beta8^+$ $CD4^+$ $CD8^+$ cells was calculated, as shown for the $CD4^+$ $CD8^+$ $CD69^-$ cultures in (c), with $1{\cdot}1 \times 10^5$ and 6×10^4 cells recovered from culture in the absence or presence of SEB, respectively. Similarly, from the $CD69^+$ cultures, $4{\cdot}6 \times 10^4$ and $2{\cdot}1 \times 10^4$ $V\beta8^+$ $CD4^+$ $CD8^+$ thymocytes were recovered from culture in the absence or presence of SEB (f). Similar results were obtained in three separate experiments.

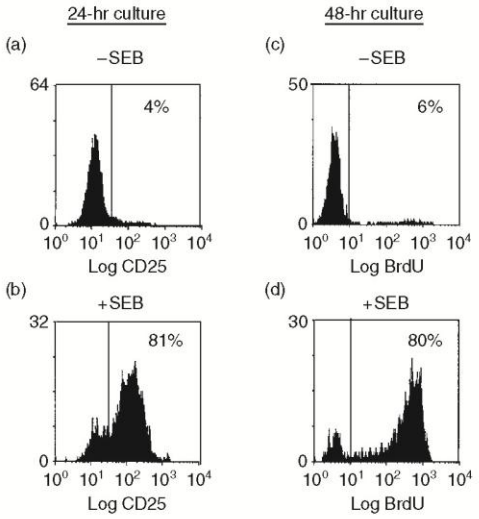

Figure 2. $CD4^+$ $CD8^-$ $CD69^+$ thymocytes show evidence of activation in response to dendritic cell presentation of SEB. $CD4^+$ $CD8^-$ $CD69^+$ thymocytes from wild-type neonatal mice were placed in culture with freshly isolated dendritic cells at a ratio of 10 : 1 in the absence (a, c) or presence (b, d) of SEB. Following a 24-hr culture period (a, b), thymocytes were harvested and analysed for expression of Vβ8 and CD25. In the experiment shown here, 1×10^6 thymocytes were placed in each culture, with recoveries of 4×10^5 and $3{\cdot}4 \times 10^5$ in the absence or presence of SEB, respectively. Of these, 1×10^4 (a) and $3{\cdot}3 \times 10^4$ (b) were of a $V\beta8^+$ $CD25^+$ phenotype. Similarly, the remaining cultures (c, d) were pulsed with BrdU after 24 hr, harvested after a further 24-hr culture period, and analysed for BrdU incorporation and Vβ8 expression. Total cell recoveries were $4{\cdot}3 \times 10^5$ (c) and $5{\cdot}8 \times 10^5$ (d), with the number of proliferating $V\beta8^+$ $CD4^+$ $CD8^-$ cells at $0{\cdot}4 \times 10^4$ and $9{\cdot}2 \times 10^4$ in the absence or presence of SEB, respectively. Similar results were obtained from three separate experiments.

in which SEB was present showed significant induction of CD25 expression, with 81% of cells having a $CD25^+$ phenotype (Fig. 2b), in contrast to the 4% of $V\beta8^+$ $CD4^+$ $CD8^-$ $CD69^+$ thymocytes found to express CD25 following culture in the absence of SEB (Fig. 2a). This evidence for activation induced by dendritic cell presentation of SEB was underlined further by assessing proliferation in $V\beta8^+$ $CD4^+$ $CD8^-$ $CD69^+$ thymocytes recovered from cultures after a 48-hr culture period. Thus, 80% of $V\beta8^+$ $CD4^+$ $CD8^-$ $CD69^+$ thymocytes cultured in the presence of SEB were found to be proliferating, as assessed by BrdU incorporation (Fig. 2d), while $V\beta8^+$ $CD4^+$ $CD8^-$ $CD69^+$ thymocytes recovered from culture in the absence of SEB were found to be out of cell cycle (Fig. 2c).

In the adult thymus, it has been postulated that newly generated $CD4^+$ $CD8^-$ HSA^{hi} medullary thymocytes are still sensitive to negative selection.[13] However, these results are based upon the induction of negative selection by injection of anti-TCR antibodies, and the system we use here, of dendritic cell presentation of SEB is perhaps more physiologically relevant. Therefore, we analysed HSA expression on our sorted $CD4^+$ $CD8^-$ $CD69^+$ neonatal thymocytes, and compared this with HSA expression on $CD4^+$ $CD8^-$ $CD69^+$ adult thymocytes. Interestingly, we found that $CD4^+$ $CD8^-$ $CD69^+$ neonatal thymocytes were uniform in their high expression of HSA (Fig. 3a). In contrast, $CD4^+$ $CD8^-$ $CD69^+$ adult thymocytes were heterogeneous for HSA, with 20% of cells being of an HSA^- phenotype (Fig. 3b). We therefore compared the response of neonatal and adult $CD4^+$ $CD8^-$ $CD69^+$ HSA^{hi} thymocytes to dendritic cell presentation of SEB. Interestingly, we found that $CD4^+$ $CD8^-$

$CD69^+$ HSA^{hi} thymocytes isolated from either neonatal or adult mice both responded to stimulation with SEB not by deletion but by activation. Indeed, the majority of both neonatal and adult $CD4^+$ $CD8^-$ $CD69^+$ HSA^{hi} thymocytes were found to express CD25 after a 24-hr culture period (Fig. 3c), and showed evidence of proliferation by BrdU incorporation after a 48-hr culture period (Fig. 3d).

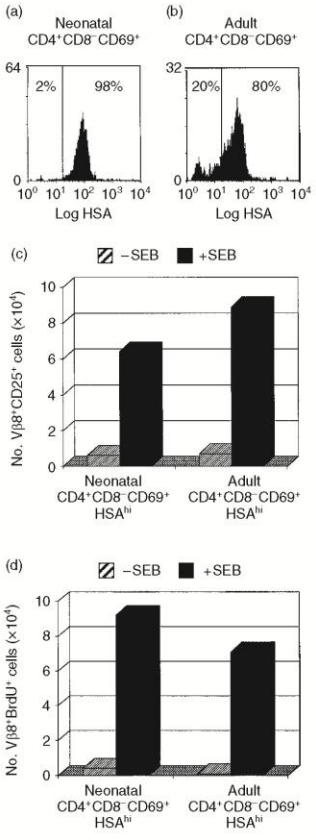

Figure 3. $CD4^+$ $CD8^-$ $CD69^+$ HSA^{hi} thymocytes from neonatal and adult mice show evidence of activation by SEB. $CD69^+$ neonatal (a) or adult (b) thymocytes were isolated from wild-type mice, and analysed for expression of CD4, CD8 and HSA. Gating on $CD4^+$ $CD8^-$ thymocytes revealed the HSA profile of $CD4^+$ $CD8^-$ $CD69^+$ cells from neonatal and adult thymus (a and b, respectively). Neonatal $CD4^+$ $CD8^-$ $CD69^+$ thymocytes (all HSA^{hi}) and adult $CD4^+$ $CD8^-$ $CD69^+$ thymocytes sorted for expression of HSA, were placed in culture with dendritic cells, at a ratio of 10 : 1 for 2 days, with BrdU added to cultures after 24 hr. Thymocytes were subsequently harvested and analysed for expression of Vβ8 and CD25 (c), or Vβ8 and BrdU incorporation (d). In the experiment shown, 1×10^6 thymocytes were placed into each culture, with recoveries of $4{\cdot}3 \times 10^5$, $5{\cdot}8 \times 10^5$ (neonatal, − or + SEB), $2{\cdot}8 \times 10^5$ and $4{\cdot}1 \times 10^5$ (Adult, − or + SEB). The number of $V\beta8^+$ $CD25^+$ cells (c) or $V\beta8^+$ $BrdU^+$ cells (d) were then calculated. This experiment is representative of three separate experiments.

Notch-1 signalling does not convey resistance to SEB induced negative selection

Mechanisms regulating negative selection and acquisition of resistance to deletion are poorly defined. It has been suggested that Notch-1 signalling plays a role in conveying resistance to apoptosis induced by dexamethasone, or by treatment with anti-TCR antibodies.[5,6] To see if Notch-1 activation correlates with phases of susceptibility to negative selection as defined above, we looked for evidence of Notch-1 activation in thymocyte subsets. As before, we isolated $CD4^+$ $CD8^+$ $CD69^-$, $CD4^+$ $CD8^+$ $CD69^+$, and $CD4^+$ $CD8^-$ $CD69^+$ thymocytes from neonatal mice, and used expression of Deltex, a downstream signalling molecule in the Notch-1 signalling pathway, as evidence of Notch-1 activation.[6] Deltex expression within these populations was analysed by real-time PCR to allow comparative analysis of mRNA (Fig. 4). While no evidence of Deltex mRNA was found in $CD4^+$ $CD8^+$ $CD69^-$ thymocytes, which is in agreement with a previous report,[6] we find that as positive selection progressed through the $CD4^+$ $CD8^+$ $CD69^+$ stage to a $CD4^+$ $CD8^-$ $CD69^+$ phenotype, an increase in Deltex expression was observed. Thus, these data indicate that the Notch-1 signalling pathway is activated upon the induction of positive selection, prior to the acquisition of resistance to negative selection, and persists into the post-positive selection $CD4^+$ $CD8^-$ $CD69^+$ stage.

To determine whether there is a causal relationship between Notch-1 signalling and resistance to negative selection, we isolated a population of preselection $CD4^+$ $CD8^+$

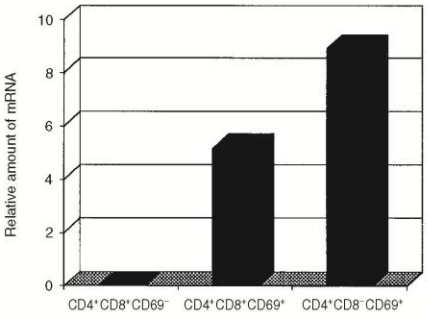

Figure 4. Measurement of Notch-1 activation in thymocyte subsets by analysis of Deltex expression. Pre-positive selection $CD4^+$ $CD8^+$ $CD69^-$ thymocytes were isolated from MHC-deficient neonatal mice, and populations of $CD4^+$ $CD8^+$ $CD69^+$ or $CD4^+$ $CD8^-$ $CD69^+$ cells were prepared from neonatal BALB/c mice, as described. Real-time PCR analysis was performed to allow relative quantitation of Deltex mRNA levels.

CD69$^-$ thymocytes and retrovirally infected them with a GFP-associated retroviral vector containing cDNA for the intracellular domain of human Notch-1,[20] resulting in a constitutively active form of Notch-1 (IC-Notch-1). Due to the lengthy manipulation procedure required prior to incorporation into deletion assays, CD4$^+$ CD8$^+$ CD69$^-$ thymocytes were, for these latter experiments, isolated from Bcl-2 tg mice, thymocytes from which have a longer lifespan than wild-type thymocytes. Thus, Bcl-2 tg CD4$^+$ CD8$^+$ CD69$^-$ thymocytes were infected with control (GFP only) or IC-Notch-1-containing vectors, and placed in culture with dendritic cells in the absence or presence of SEB, to see if constitutive activation of the Notch-1 signalling pathway affected their susceptibility to superantigen-mediated negative selection. Detection of GFP by flow cytometry enabled us to analyse specifically those thymocytes which had been successfully infected with the relevant virus (Fig. 5a,b), revealing an infection rate of 20–30%. Thus, thymocytes infected with the control GFP vector or IC-Notch-1 vector were cultured with dendritic cells in the absence or presence of SEB. Thymocytes used in these cultures required exposure to SEB for a longer culture period than wild-type thymocytes in order to see significant SEB-mediated deletion in the Vβ8$^+$ population, perhaps as a result of expression of the Bcl-2 transgene. However, following a 2-day culture period, subsequent analysis within the GFP$^+$ population revealed that Vβ8$^+$ thymocytes infected with the control GFP virus showed evidence of deletion in response to dendritic cell presentation of SEB (Fig. 5c). This level of deletion was similar to that observed in non-infected CD4$^+$ CD8$^+$ CD69$^-$ thymocytes in earlier experiments (Fig. 1c), measured following a 1-day culture period, thus indicating that retroviral infection, *per se*, did not influence this assay, although total cell recovery following a 2-day culture period (Fig. 5) was lower than the total cell recovery after 1 day in culture (Fig. 1). This is likely to be due to the limited lifespan of the CD4$^+$ CD8$^+$ thymocyte input population, but an increase in non-specific thymocyte death by an increased culture period is unlikely to mask any SEB-specific cell death, since background cell death does not affect the relative proportions of different Vβ subsets (data not shown).

Having established that retroviral infection of thymocytes did not influence this deletion assay, we looked at thymocytes which had been infected with the IC-Notch-1 vector. Surprisingly, we found that thymocytes infected with the GFP/IC-Notch-1 virus also showed considerable evidence of deletion following culture with SEB, with 58% of Vβ8$^+$ GFP/IC-Notch-1 infected cells being deleted as compared to the 42% of Vβ8$^+$ GFP control cells (Fig. 5c). From these results it therefore cannot be said that the protective effects of the Bcl-2 transgene in any way obscured protection conveyed by IC-Notch-1, since we saw specific deletion of Vβ8$^+$ thymocytes by SEB even following infection of thymocytes with IC-Notch-1. Importantly, using Deltex expression as a marker of Notch-1 signalling,[6] Fig. 5(d) shows that infection of primary CD4$^+$ CD8$^+$ thymocytes with the IC-Notch-1 construct leads to up-regulation of Deltex expression, indicative of activation of the Notch-1 signalling pathway by IC-Notch-1. Thus the lack of resistance to deletion observed in IC-Notch-1 infected cells is not merely attributable to the lack of activation of Notch-1 signalling. Collectively then, these data suggest that introduction of IC-Notch-1 into primary CD4$^+$ CD8$^+$ thymocytes is capable of activating the Notch-1 signalling cascade, but fails to convey resistance to superantigen-mediated negative selection.

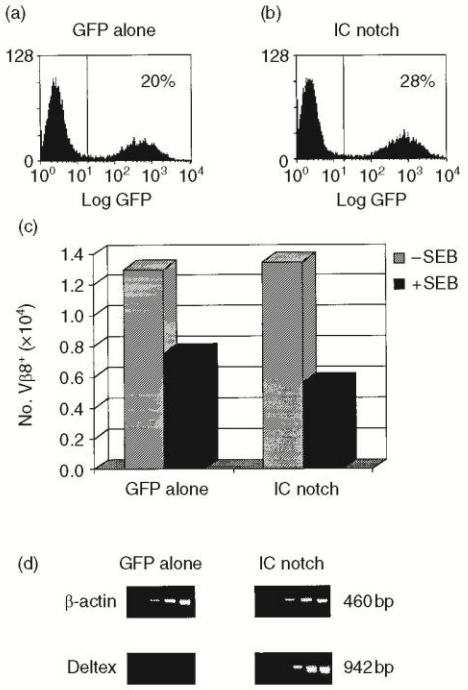

Figure 5. Expression of a constitutively active form of Notch-1 does not protect CD4$^+$ CD8$^+$ thymocytes from superantigen-mediated cell death. CD4$^+$ CD8$^+$ CD69$^-$ thymocytes from Bcl-2 tg neonatal mice, giving them an extended lifespan to allow for complex and lengthy manipulation procedures, were retrovirally transfected with a GFP-associated vector containing cDNA for IC-Notch-1 (b) or a control GFP-associated vector (a). After overnight incubation with the retrovirus, control infected and IC-Notch-1 infected CD4$^+$ CD8$^+$ cells were analysed for GFP expression by flow cytometry (a, b) and for expression of Deltex by semiquantitative PCR (d), and placed in culture with dendritic cells, at a ratio of 10 : 1, in the absence or presence of SEB at 10 μg/ml. Following a 48-hr culture period, thymocytes were harvested and analysed for expression of GFP and Vβ8. 1×10^6 CD4$^+$ CD8$^+$ thymocytes were placed in each culture, with recoveries of $3{\cdot}1 \times 10^5$, $1{\cdot}9 \times 10^5$ cells (control virus, without and with SEB, respectively), $2{\cdot}2 \times 10^5$ and $1{\cdot}5 \times 10^5$ (IC-Notch virus without and with SEB, respectively). Thus, this enabled calculation of numbers of transfected (GFP$^+$) Vβ8$^+$ cells for each culture (c).

DISCUSSION

In this study we have addressed mechanisms regulating the transition from a functionally immature thymocyte to a

functionally mature T cell. We have shown firstly that induction of positive selection does not convey immediate resistance to negative selection. Moreover, thymocytes which have initiated positive selection as identified by a $CD69^+$ phenotype, are also known to have up-regulated levels of TCR expression. Therefore the continued susceptibility of $CD4^+$ $CD8^+$ $CD69^+$ thymocytes to deletion at a stage where they have increased avidity for TCR–MHC interactions represents a biologically important point in development for the screening and removal of potentially autoreactive T cells. Interestingly, our results also indicate that, once thymocytes reach the $CD4^+$ $CD8^-$ $CD69^+$ stage, not only are they resistant to deletion, but they are capable of responding to the same stimulus by activation, as shown by CD25 expression and by BrdU incorporation. This is in contrast to previous observations by Kishimoto and Sprent,[13] who found that medullary $CD4^+$ $CD8^-$ thymocytes, from adult mice, expressing a semi-mature HSA^{hi} phenotype are still susceptible to apoptosis induced by anti-TCR antibody. However, the discrepancy between these and our data cannot be attributed to differences in the adult and neonatal thymocyte populations, since we show here that $CD4^+$ $CD8^-$ HSA^{hi} thymocytes from both adult and neonatal mice respond to dendritic cell presentation of SEB by activation. Thus, it may be that variation in TCR stimuli, such as between superantigen and anti-TCR antibody, causes activation of different responses.

By analysing activation of Notch-1, a molecule purported to play a role in resistance to negative selection,[6] we have also shown that activation of Notch-1 in $CD4^+$ $CD8^+$ thymocytes does not correlate with, nor convey resistance to, negative selection. Thus, our results suggest that although Notch-1 has been shown to convey resistance to a variety of triggers of apoptosis, including non-TCR-mediated apoptosis induced by steroid treatment,[6] it is not the sole factor in regulating the change in susceptibility of thymocytes to TCR-mediated negative selection signals. On a similar point, the differences between our results using primary thymocytes and superantigen, and those reported by Jehn *et al.*[5] using thymocyte lines with anti-TCR antibody may additionally reflect intrinsic differences between cell lines and primary thymocytes and methods of TCR stimulation. Indeed, these discrepancies may indicate that whilst Notch-1 signalling can confer resistance to apoptosis induced by anti-TCR antibodies alone, it is insufficient to overcome the more physiological stimulus of TCR ligation in association with co-stimulatory signals provided by professional antigen-presenting cells, as studied here. In summary, our results show that there is a developmental overlap between positive and negative selection events, with resistance to negative selection being acquired at the $CD4^+$ $CD8^-$ $CD69^+$ stage but not immediately following the initiation of positive selection. Moreover, the acquisition of this resistance to deletion appears to occur independently of Notch-1 signalling, suggesting that additional, and as yet undefined, cell interactions may be required for this process.

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Notch ligand-bearing thymic epithelial cells initiate and sustain Notch signaling in thymocytes independently of T cell receptor signaling

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Thymic epithelial cells are specialized to play essential roles at multiple stages of T cell development in the thymus, yet the molecular basis of this specialization is largely unknown. Recently, the Notch family of transmembrane proteins has been implicated in thymocyte development. Such proteins interact with cell surface proteins of the Delta-like and Jagged families. It is known that Notch ligands are expressed intrathymically, and that Notch signaling is regulated by Notch ligands expressed on either the same or third-party cells. However, functional analysis of Notch ligand expression, and elucidation of the mechanism of Notch ligand signaling in thymocyte development, are unclear. Here, we find that Notch ligand expression in the thymus is compartmentalized, with MHC class II$^+$ thymic epithelium, but not thymocytes nor dendritic cells, expressing Jagged-1, Jagged-2 and Delta-like-1. We also provide evidence that contact with Notch ligands on thymic epithelium is necessary to activate and sustain Notch signaling in thymocytes, and that this can occur independently of positive selection induction. Our data suggest that Notch ligand expression by thymic epithelium may partly explain the specialization of these cells in supporting thymocyte development, by regulating Notch activation via an inductive signaling mechanism independently of signaling leading to positive selection.

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Key words: Thymus / Stromal cell / Cell-cell interaction

1 Introduction

The development of immature thymocytes is tightly regulated by interactions with thymic stromal cells. Thymic epithelial cells are key regulators of a variety of events during thymocyte development, such proliferation, initiation of TCR gene rearrangement, and thymocyte positive selection. How thymic epithelial cells regulate these processes is largely unclear, and few molecules expressed by these cells have been shown to play functionally important roles in thymocyte development [1].

Recently, considerable attention has focussed on interactions between Notch receptors and their ligands in T cell development. Such molecules are attractive candidates as mediators of intrathymic differentiation since they influence cell fate decisions in various other developmental systems [2]. In the thymus, Notch-1 has been implicated in T cell commitment [3, 4], and in thymic selection events [5], while Notch-3 may play a role in pre-TCR selection events [6]. Recently, use of conditional knockout mice has ruled out an essential role for Notch-1 in later stages of thymocyte development [7]. However, whether this reflects functional redundancy between Notch family members, which can interact with the same Jagged and Delta-like family members, is not clear. In addition, while Notch-1 may be nonessential for positive selection, the possibility of subtle effects of Notch signaling on the selected repertoire cannot be excluded [8].

Consistent with the involvement of Notch molecules in T cell development, expression of certain Notch ligands has been demonstrated in the thymus [9, 10]. However, analysis of Notch ligand expression has been largely confined to either thymic epithelial cell lines [9], whose phenotypic and functional relevance to normal thymic epithelium is uncertain, or involved the use of in situ hybridization, where localization of expression to individual cell types in thymic sections can be difficult [10]. In addition, there is conflicting evidence relating to the expression of Notch ligands by thymocytes [9, 11]. Thus, clarifying the pattern of expression of Notch ligands in the thymus is essential to allow discrimination between

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Abbreviations: WT: Wild type **RT:** Reverse transcription

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Notch activation by lateral inhibition (where Notch and Notch ligands are expressed on the same cells) and inductive signaling (where Notch and Notch ligands are expressed on different cell types). Moreover, assessing the functional ability of Notch ligands to activate Notch signaling may provide a clearer understanding of the possible functions of Notch and Notch ligand family members in thymus development.

Here, we have mapped the expression of Notch ligands on different cellular components of the developing thymus and have used reaggregate organ cultures to investigate the functional ability of ligand expressing cells to activate the Notch pathway in pre-selection $CD4^+8^+$ thymocytes. Our data provide direct evidence that thymic epithelial cells are responsible for regulating Notch activation during thymocyte development via an inductive signaling mechanism. Furthermore, separation of Notch signaling from TCR-MHC interactions leading to positive selection induction may be indicative of a general mechanism by which thymic epithelial cells influence the survival and maturation of thymocytes at multiple stages.

2 Results

2.1 Analysis of Notch ligand expression in the thymus

Due to the current paucity of suitable antibodies, we analyzed expression of Notch ligands in the thymus by reverse transcription (RT)-PCR. cDNA were obtained from purified thymocyte subsets, purified MHC class II^+ thymic epithelial cells, which we have previously shown to be unique mediators of positive selection [12], and from thymic dendritic cells. Fig. 1a shows that the thymocyte subsets analyzed failed to show expression of the Notch ligands Jagged-1, Jagged-2 and Delta-like-1. In marked contrast, freshly purified MHC class II^+ thymic epithelial cells were found to contain readily detectable mRNA for Notch ligands (Fig. 1b). Interestingly, MHC class II^+ thymic dendritic cells lack Jagged-1 and Delta-like-1 expression, and express only a trace of Jagged-2 mRNA (Fig. 1b).

2.2 Exposure to Notch ligand-bearing thymic epithelium is necessary and sufficient for Notch-1 activation in $CD4^+8^+$ thymocytes

We next sought to determine the functional activity of Notch ligands expressed by thymic epithelial cells, and analyzed the requirements for Notch activation in immature $CD4^+8^+$ thymocytes. To assay Notch signaling, we analyzed expression of Hes-1 and Deltex, downstream indicators of the CBF-1-dependent and -independent pathways of Notch signaling [5]. Thus, we prepared $CD4^+8^+\alpha\beta TCR^-$ thymocytes from bcl-2 transgenic mice, and cultured these cells either alone or in reaggregate organ culture with thymic epithelium. Use of thymocytes expressing a bcl-2 transgene allows analysis of Notch activity in the absence of thymic stroma, since unlike wild-type (WT) thymocytes, bcl-2 transgenic cells have a prolonged life-span in the absence of stromal cell sup-

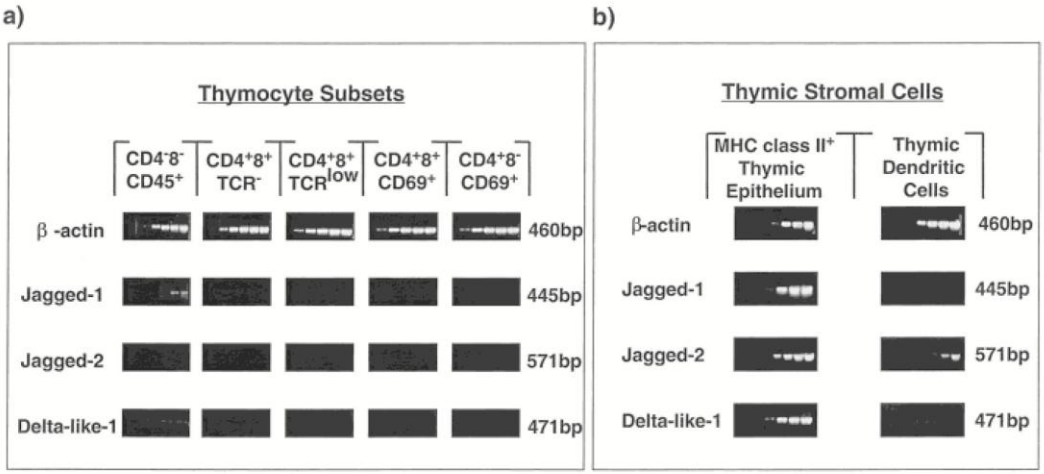

Fig. 1. Thymic Notch ligand expression is limited to MHC class II^+ epithelium. cDNA from indicated cells were subjected to RT-PCR for Jagged-1, Jagged-2 and Delta-like-1, with equal cDNA loadings monitored by β-actin expression. PCR product sizes are in base pairs (bp). Similar data came from three separate experiments.

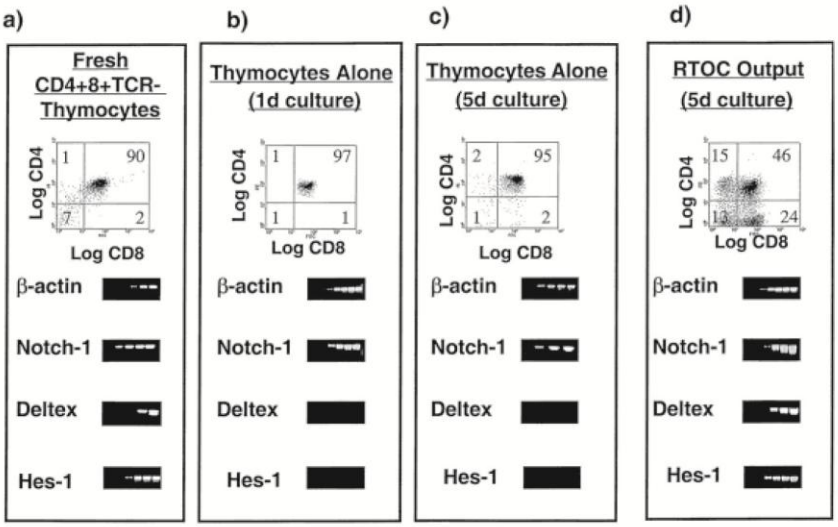

Fig. 2. Exposure to thymic epithelial cells regulates, and is necessary for, Notch signaling in $CD4^+8^+$ thymocytes. Bcl-2 transgenic $CD4^+8^+TCR^-$ thymocytes (a) were cultured alone overnight (b), and then for a further 5 days in the absence (c) or presence (d) of thymic stroma, the latter as a reaggregate culture. Cells were analyzed for CD4, CD8 expression and Notch-1, Deltex and Hes-1 mRNA expression. In this experiment, 1×10^6 thymocytes were used for overnight cultures, yielding 8×10^5 viable cells. From this, 5-day cultures of either 8×10^5 thymocytes alone, or 8×10^5 thymocytes reaggregated with 8×10^5 WT thymic stroma were initiated. Yields obtained: thymocytes alone, 6×10^5; thymocytes from reaggregate culture, 4×10^5. Similar data came from three separate experiments.

port [12]. As shown in Fig. 2a, $CD4^+8^+\alpha\beta TCR^-$ thymocytes freshly isolated from bcl-2 transgenic mice express Deltex and Hes-1, indicating these cells have received Notch-activating signals, perhaps reflecting events at earlier stages of development [6]. Importantly, Deltex and Hes-1 are also expressed in WT BALB/c $CD4^+8^+\alpha\beta TCR^-$ thymocytes (not shown), ruling out the possibility that expression is related to bcl-2 transgene expression.

When bcl-2 transgenic $CD4^+8^+\alpha\beta TCR^-$ thymocytes cells are maintained in culture overnight, although they continue to survive, expression of both Deltex and Hes-1 is lost (Fig. 2b), indicating that thymocyte-thymocyte cell contact is insufficient to activate Notch signaling, in agreement with our PCR data indicating the absence of Notch ligand expression by these cells (Fig. 1a). We then exploited this overnight culture period in which Deltex and Hes-1 expression in $CD4^+8^+$ thymocytes is lost to analyze the requirements for Notch activation in thymocytes purged of Notch-activating signals generated by exposure to Notch ligands *in vivo*. Interestingly, while bcl-2 tg $CD4^+8^+$ thymocytes maintained alone in culture for 5 days still survived and maintained Notch-1 mRNA expression together with a $CD4^+8^+$ phenotype, they failed to re-express either Deltex or Hes-1 (Fig. 2c), indicating that prolonged thymocyte-thymocyte contact does not induce Notch signaling. However, purified $CD4^+8^+$ thymocytes purged of *in vivo* Notch signals by overnight culture, when reassociated with Notch ligand bearing thymic epithelium in reaggregate cultures, were found to have undergone induction of Deltex and Hes-1 mRNA expression and generated $CD4^+$ and $CD8^+$ cells (Fig. 2d).

2.3 Thymic epithelial cells can activate Notch signaling in $CD4^+8^+$ thymocytes independently of positive selection induction

Given the correlation between positive selection and the induction of Notch signaling [6], we were interested in determining whether Notch signaling is causally dependent upon induction of positive selection. We adopted two strategies to eliminate $\alpha\beta$TCR-peptide/MHC interactions, while still allowing the possibility of Notch-Notch ligand interactions between pre-selection $CD4^+8^+$ thymocytes and thymic epithelium. We first re-associated bcl-2 transgenic $CD4^+8^+$ thymocytes, purged of *in vivo* generated Notch signals by overnight culture, with either WT or MHC class I/class II double-deficient ($MHC^{-/-}$) thymic stromal cells in reaggregate thymus organ culture, which therefore still express Notch ligands but differ only in their provision of $\alpha\beta$TCR ligands. Fig. 3 shows once again that 'purged' $CD4^+8^+$ thymocytes

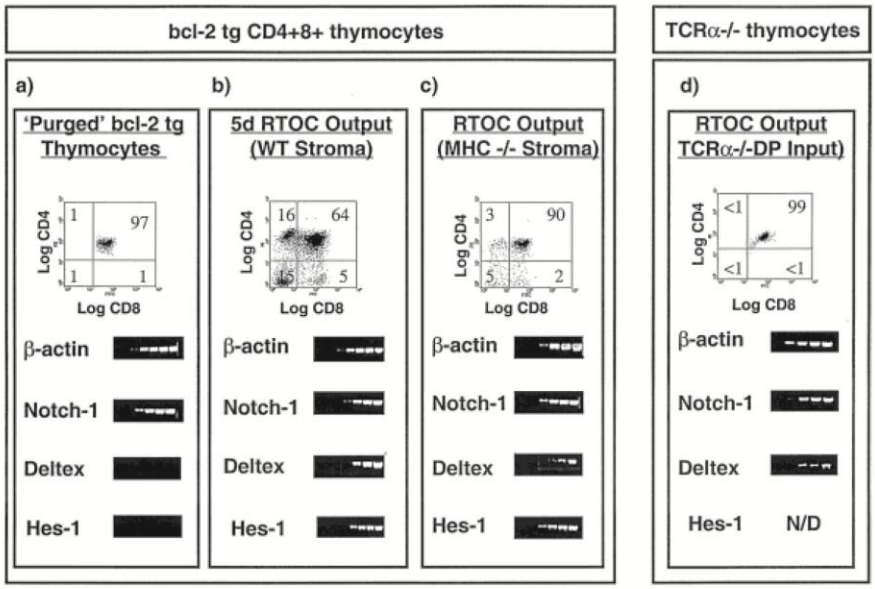

Fig. 3. Thymic epithelial cells can activate Notch signaling in $CD4^+8^+$ thymocytes in the absence of TCR-MHC interactions. $CD4^+8^+TCR^-$ thymocytes from bcl-2 transgenic neonates were cultured overnight (a), and then reaggregated with either WT (b) or $MHC^{-/-}$ thymic stroma (c), with reaggregate cultures harvested at day 5. Thymocytes were analyzed for CD4, CD8 expression and Notch-1, Deltex and Hes-1 mRNA expression. Yields were as follows: 1×10^6 overnight cultured $CD4^+8^+$ thymocytes gave 5×10^5 from WT stroma cultures and 4×10^5 from $MHC^{-/-}$ stroma cultures. To study Notch signaling in the absence of αβTCR expression, $CD4^+8^+$ thymocytes from $TCR\alpha^{-/-}$ neonates were cultured overnight and then reaggregated with WT thymic stroma. At day 5, cultures were analyzed as above. In this experiment, 1×10^6 $TCR\alpha^{-/-}$ thymocytes were reaggregated with 1×10^6 WT stromal cells, yielding 4×10^5 viable cells. Similar results came from three separate experiments.

(Fig. 3a), when reassociated with WT epithelial cells, undergo positive selection and are induced to express Deltex and Hes-1 (Fig. 3b). In contrast, the same thymocytes re-associated with $MHC^{-/-}$ thymic stroma over a similar culture period remain largely at a $CD4^+8^+$ stage, but importantly show evidence of Notch activation, as indicated by re-expression of Deltex and Hes-1 (Fig. 3c). Thus, eliminating TCR-MHC interactions by removal of MHC molecules on thymic epithelium prevents positive selection induction, but not Notch activation.

Another approach to investigate the relationship between TCR-MHC interactions and Notch signaling is to disrupt TCR-MHC interactions by preventing expression of a complete αβTCR. Thus, we next analyzed Notch activation in $CD4^+8^+$ thymocytes isolated from $TCR\alpha^{-/-}$ neonates, where development is blocked at the $CD4^+8^+$ stage [13]. In agreement with the above, when associated with MHC bearing WT thymic stroma, $TCR\alpha^{-/-}$ thymocytes as expected remain at the $CD4^+8^+$ stage, but show evidence of Notch activation, as indicated by Deltex expression (Fig. 3d).

3 Discussion

Accumulating evidence has implicated Notch signaling at various stages of T cell development [3–5]. As Notch signaling depends upon interaction with cell membrane ligands of the Jagged and Delta-like families, defining the distribution and functional competence of these ligands in the thymus is fundamental to understanding the control of Notch-mediated events in thymocyte maturation and selection.

Here we show that Notch ligands Jagged-1, Jagged-2 and Delta-like-1 are expressed by MHC class II^+ epithelium, but not by thymocyte subsets or thymic dendritic cells. This distribution, together with our finding that interaction with thymic epithelium in reaggregate cultures induces Notch signaling in thymocytes, provides evidence for an inductive model of Notch signaling in thymocytes, where Notch activation is mediated by interaction with stromal cells, rather than a lateral inhibition model involving interaction between thymocytes themselves.

Recently, an essential requirement for the Notch-1 molecule in later stages of thymocyte development has been ruled out by Wolfer et al. [7], who show that generation and positive selection of $CD4^+8^+$ thymocytes occurs normally in thymocytes rendered Notch-1-deficient by Cre-lox technology. However, the Notch ligands we show here to be expressed by thymic epithelium allow for possible signaling through other Notch family members such as Notch-2, Notch-3 and Notch-4, all of which are expressed in the thymus [11]. Thus, expression of Notch ligands by thymic epithelium may be an important component of their poorly understood yet specialized ability to regulate thymocyte maturation. Indeed, bone marrow precursors lacking Notch-1 are blocked at the earliest stage of T cell development [4], while constitutively active Notch-3 disrupts pre-TCR-mediated development [6]. Collectively, these observations indicate that one aspect of the importance of epithelium in early T cell development [14] may involve provision of Notch ligands. Current efforts are aimed at delivery of antisense retroviral constructs to thymic epithelium [15] to analyze the role of Notch ligands in T cell development.

Finally our data shows that Notch signaling can be triggered by contact between thymic epithelium and thymocytes in the absence of TCR-MHC interactions, suggesting that, although Notch signaling is active during positive selection [5], Notch activation is not dependent upon an initial step involving TCR signaling. Thus, we would suggest that the ability of thymic epithelial cells to provide Notch ligands to activate signaling via Notch family members may be a general feature of the specialized ability of thymic epithelial cells to support and regulate multiple stages of thymocyte development.

4 Materials and methods

4.1 Mice

The following mice were used: BALB/c, MHC-deficient ($MHC^{-/-}$, Taconic [16]), bcl-2 transgenic (a gift of Dr. S. J. Korsmeyer, Dana-Farber Cancer Institute, Boston), RAG1 deficient ($RAG1^{-/-}$, Jackson Laboratories), and TCRα deficient ($TCR\alpha^{-/-}$ [13]). Day of vaginal plug detection was designated day 0.

4.2 Antibodies and immunoconjugates

Antibodies used to coat onto magnetic beads were as described [16]. Antibodies used for flow cytometry (PharMingen) were: anti-CD4 PE (GK1.5), anti-CD8 FITC (53-6.7).

4.3 Purification of thymocyte subsets

Thymocyte subsets used for RT-PCR analysis were obtained from BALB/c mice as described [16]. $CD4^+8^+$ thymocytes purged of *in vivo* Notch signals were prepared from bcl-2 transgenic $CD4^+8^+TCR^-$ thymocytes as described [12], cultured on 0.8-μm Nucleopore filters overnight and harvested. $CD4^+8^+$ thymocytes were enriched from $TCR\alpha^{-/-}$ thymuses, using anti-CD4 FITC and anti-FITC MicroBeads (Miltenyi Biotech).

4.4 Purification of thymic epithelial cells and thymic dendritic cells

Thymic stroma was prepared from BALB/c (WT) and $MHC^{-/-}$ 15-day embryo thymuses by culture in 1.35 mM 2-deoxyguanosine (Sigma) [16]. Dendritic cells were prepared from 4–6-week-old $RAG1^{-/-}$ thymuses as described [17], using CD11c MicroBeads and a MiniMacs Separation System, giving 90–95% purity (not shown).

4.5 RT-PCR

RT-PCR was performed as before [18]. cDNA normalized to β-actin were amplified for all target genes over 27–44 cycles, apart from Deltex, which was amplified over 27–47 cycles at 4-cycle intervals. Annealing temperatures were 55°C except for Deltex (75°C). Primer sequences used were: Notch-1: (forward) 5'-CCCAGCAGGTGCAGCCACAG-3', (reverse) 5'-GGTGATCTGGGACGGCATGG-3'; Deltex: (forward) 5'CACTGGCCCTGTCCACCCAGCCTTGGCAGG-3', (reverse) 5'-GAGGCATGTGCCAGGCTAGAGGCAAGGCAA-3'; Hes 1: (forward) 5'-GCCAGTGTCAACACGACACCGG3', (reverse) 5'-TCACCTCGTTCATGCACTCG-3'; Jagged-1: (forward) 5'-CATTACGTGTTGCCTGTAAGCC-3', (reverse) 5'-GTGGTTCAGCATTACATACG-3'; Jagged-2: (forward) 5'-GTCCTTCCCACATGGGAGTT-3', (reverse) 5'-GTTTCCACCTTGACCTCGGT-3'; Delta-like-1: (forward) 5'-GTCACAGAGCTCTGCAGGAG-3', (reverse) 5'-TGTGGGCAGTGCGTGCTTCC-3'.

4.6 Preparation of reaggregate thymus organ cultures

Reaggregate cultures were made from 1:1 mixtures of thymic stromal cells and thymocytes as described [16].

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Spontaneous Neutrophil Apoptosis Involves Caspase 3-mediated Activation of Protein Kinase C-δ*

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Neutrophils are short-lived leukocytes that die by apoptosis. Whereas stress-induced apoptosis is mediated by the p38 mitogen-activated protein (MAP) kinase pathway (Frasch, S. C., Nick, J. A., Fadok, V. A., Bratton, D. L., Worthen, G. S., and Henson, P. M. (1998) *J. Biol. Chem.* 273, 8389–8397), signals regulating spontaneous neutrophil apoptosis have not been fully determined. In this study we found increased activation of protein kinase C (PKC)-β and -δ in neutrophils undergoing spontaneous apoptosis, but we show that only activation of PKC-δ was directly involved in the induction of apoptosis. PKC-δ can be proteolytically activated by caspase 3. We detected the 40-kDa caspase-generated fragment of PKC-δ in apoptotic neutrophils and showed that the caspase 3 inhibitor Asp-Glu-Val-Asp-fluoromethylketone prevented generation of the 40-kDa PKC-δ fragment and delayed neutrophil apoptosis. In a cell-free system, removal of PKC-δ by immunoprecipitation reduced DNA fragmentation, whereas loss of PKC-α, -β, or -ζ had no significant effect. Rottlerin and LY379196 inhibit PKC-δ and PKC-β, respectively. Only Rottlerin was able to delay neutrophil apoptosis. Inhibitors of MAP-ERK kinase 1 (PD98059) or p38 MAP kinase (SB202190) had no effect on neutrophil apoptosis, and activation of p42/44 and p38 MAP kinase did not increase in apoptotic neutrophils. We conclude that spontaneous neutrophil apoptosis involves activation of PKC-δ but is MAP kinase-independent.

Neutrophils are short-lived terminally differentiated blood cells that play a vital role in inflammatory responses. Chemotactic factors generated at sites of infection induce the movement of neutrophils from the blood into the affected tissues. A variety of molecules, including cytokines and bacterial products, then activate the defense systems of the neutrophil, which include phagocytosis, degranulation, and activation of NADPH oxidase. Although neutrophils are crucial in the defense against infection, they have also been implicated in the pathogenesis of tissue injury seen in inflammatory diseases of the lung, kidney, joints, and other organs (1–3). During the resolution of inflammation, effete neutrophils are removed from inflammatory sites by apoptosis, leading to their recognition and phagocytosis by macrophages (4). Any significant delay to neutrophil apoptosis can lead to excessive neutrophil accumulation and damage to healthy tissue (5). Although apoptosis is an intrinsic cell process, a variety of cytokines, primarily those that prime neutrophils (6), are able to delay apoptosis. How these effects are achieved is not known, although the modulation of signaling pathways regulating apoptosis is an obvious target in such a short-lived cell.

Members of the MAP[1] kinase family of signaling enzymes, specifically, p38 MAP kinase, have been shown to be involved in accelerating neutrophil apoptosis in response to stress but do not appear to be involved in spontaneous neutrophil apoptosis (7). An alternative signaling pathway that has been implicated in the regulation of apoptosis in a wide variety of cells (8) is protein kinase C (PKC). PKC is a family of 11 isoenzymes that are well conserved across species, suggesting they have specific functions within cells (9). Neutrophils have been shown to express several PKC isoenzymes including PKC-α, -β, -δ (10, 11), and -ζ (12). Selective involvement of PKC isoenzymes in the regulation of apoptosis has been indicated in recent studies in a variety of cells, including those of myeloid (13–15) and lymphoid (16, 17) origin. In Molt 4 cells, Lee *et al.* (17) have shown that ceramide induced apoptosis via the inhibition of PKC-α, and Whelan and Parker (18) have shown that down-regulation of PKC-α induces apoptosis in COS cells, suggesting an anti-apoptotic role for this isoenzyme. In contrast, MacFarlane *et al.* (13) showed that PKC-β expression was required for promyeloid HL60 cells to undergo differentiation and apoptosis in response to phorbol ester treatment, and the expression of PKC-β was differentially regulated during apoptosis in myelomonocytic U937 cells (14). Also in U937 cells, PKC-δ has been shown to be activated by caspase 3, leading to the generation of a 40-kDa catalytic fragment after the induction of apoptosis by a variety of agents, including Fas ligation (19). Thus, PKC isoenzymes appear to be differentially involved in the regulation of the apoptotic program.

Although PKC has been implicated in signaling pathways regulating spontaneous apoptosis in promyeloid cells (20), the involvement of PKC isoenzymes in neutrophil apoptosis has not been established. However, the Fas/Fas ligand system has been reported to be involved in mediating spontaneous neutrophil apoptosis (21), although the signaling pathways downstream of Fas ligation in the neutrophil have not been established. Because PKC-δ has been shown to be activated after Fas ligation in promyeloid cells (19), a role for the activation of

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[1] The abbreviations used are: MAP, mitogen-activated protein; DEVD-fmk, Asp-Glu-Val-Asp-fluoromethylketone; PKC, protein kinase C; ERK, extracellular signal-regulated kinase.

specific PKC isoenzymes in spontaneous neutrophil apoptosis was investigated. In this report, we show that both PKC-β and -δ were activated during apoptosis, although only the inhibition of PKC-δ delayed spontaneous neutrophil apoptosis. We also confirm that p42/44 and p38 MAP kinases are involved in stress-induced apoptosis but are not involved in the regulation of spontaneous neutrophil apoptosis.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Human Peripheral Blood Neutrophils—Venous blood (20–100 ml) was taken from healthy volunteers, and neutrophils were isolated on Percoll gradients as described previously (22). Neutrophil preparations contained >95% neutrophils. Neutrophils were resuspended in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma) and used immediately as healthy control cells, or they were cultured in a humidified 5% CO_2 atmosphere to provide apoptotic cells. Cytospin preparations (3 min, 10 × *g*; Cytospin 2, Shandon) were made after 6 and 24 h of incubation, differentially stained using a commercial May-Grunwald Giemsa stain (Diff-Quick, Baxter Healthcare Products), and assessed for apoptotic morphology (22).

Treatment of Neutrophils with Inhibitors—PKC inhibitors, Go6976 and Rottlerin were purchased from Calbiochem and used at the concentrations shown. LY379196 is a novel PKC-β inhibitor (23) and was kindly provided by Eli Lilly and used at 10 nM. The caspase 3 inhibitor DEVD-fmk (Calbiochem) was used at 20 μM. Inhibition of caspase 3 was confirmed by assaying caspase 3 activity in 100 μl of neutrophil cell lysates, using a commercial kit and according to manufacturer's instructions (CaspAce; Promega). The MAP-ERK kinase and p38 MAP kinase inhibitors, PD98059 and SB202190 (Calbiochem), were used at 30 and 10 μM, respectively. All inhibitors were included in the incubation medium, and apoptosis was assessed by morphology after 6 h or overnight treatment.

Assay of PKC Isoenzyme Activation—PKC activation was assessed by determining the translocation of PKC from the cytosol to the particulate fraction of neutrophils (24). Briefly, neutrophils were resuspended in Buffer A (20 mM Tris/HCl buffer, pH 7.5, and 1.5 mM EGTA supplemented with 1.5 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin) and lysed by freezing and thawing in liquid nitrogen. The cell lysate was spun (100,000 × *g*, 4 °C, 30 min), and the supernatant was removed and treated as the cytosol extract. Particulate proteins were then extracted from the resulting pellet by incubation for 10 min on ice in Buffer A containing 0.5% (v/v) Triton X-100. Degradation of PKC during the fractionation of neutrophils was monitored by Western blotting and was not greater than 5% for any of the isoenzymes tested. Protein concentrations were determined in cytosol and particulate preparations, and the samples were boiled in SDS sample buffer. Equivalent amounts of protein were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore UK, Ltd). PKC isoenzyme immunoreactivity was detected with rabbit polyclonal antibodies to PKC-α, -β, and -δ (Santa Cruz Biotechnology). The anti-PKC-ζ antibody (25) was an anti-peptide antibody raised in rabbits and was a kind gift from Dr. J. Ransom (Syntex Research Ltd., Palo Alto, CA). Horseradish peroxidase-conjugated sheep anti-rabbit IgG antibody (Amersham International) was used as a secondary antibody. Enhanced chemiluminescence (ECL; Amersham International) was used to reveal immunoreactive bands. 10 ng of human recombinant PKC for each isoenzyme (Calbiochem) was applied to the outer lane in each gel to confirm antibody specificity.

Immunostaining and Confocal Microscopy—The subcellular distribution of PKC isoenzymes was assessed using indirect immunofluorescence staining and confocal microscopy. Cytospin preparations were made of apoptotic and non-apoptotic neutrophils and air dried for at least 1 h before fixing in 4% paraformaldehyde for 10 min. Cells were incubated with antibody to PKC-δ (Santa Cruz Biotechnology) for 30 min at room temperature in a humidified atmosphere and incubated with fluorescein isothiocyanate-conjugated secondary antibody for an additional 30 min. Nuclei were counterstained with propidium iodide. Fading of fluorescence was retarded by treatment of stained cells with 2.5% (w/v) 1,4-diazobicyclo(2,2,2)-octane (DABCO; BDH Ltd.) in 80% glycerol. Immunofluorescence was analyzed by laser scanning confocal microscopy using a MRC 500 confocal microscope (Bio-Rad).

Assay for Active p42/44 and p38 MAP Kinase—Protein extracts from freshly isolated neutrophils and neutrophils cultured overnight were separated on 10% SDS-polyacrylamide gel electrophoresis gels and analyzed by Western blotting using antibodies specific for the phosphorylated, active forms of p42/44 and p38 MAP kinase (Promega). Immunoreactive bands were revealed by enhanced chemiluminescence.

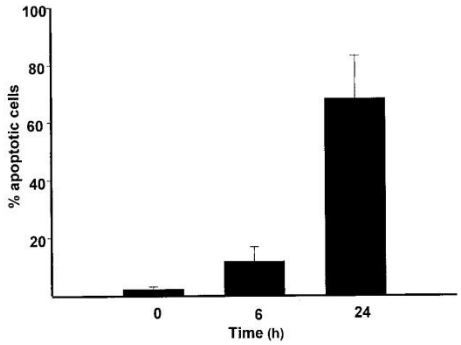

FIG. 1. **Neutrophils die by apoptosis when cultured *in vitro*.** Neutrophils were isolated from human peripheral blood and cultured at 37 °C for the times shown. Apoptosis was detected by morphological analysis of neutrophils stained with a commercial May-Grunwald Giemsa stain. Results are the mean ± S.D. of five separate experiments.

Cell-free Apoptosis System—Nuclei were isolated from neutrophils using a rapid procedure developed for hemopoietic cells (26). Cytosol was prepared from neutrophils incubated for 24 h to give >75% apoptotic cells. Neutrophils were lysed in Buffer A and spun at 100,000 × *g* to recover the cytosol (supernatant) fraction. Healthy nuclei were combined with apoptotic cytosol, and DNA fragmentation was measured after 30 min using an enzyme-linked immunosorbent assay-based commercial kit (Roche Molecular Biochemicals). To deplete PKC isoenzymes, the apoptotic cytosol was incubated with PKC isoenzyme antibodies (Santa Cruz Biotechnology) at 10 μg/ml extract for 30 min at 4 °C, followed by a mouse anti-rabbit IgG antibody and protein A coupled to agarose beads (Upstate Biotechnology). Immunocomplexes were removed by centrifugation, and the remaining cytosol was combined with the healthy nuclei. Depletion of PKC was confirmed by Western blotting. Mouse immunoglobulin G was used as control.

Statistics—Data presented here represent a minimum of three experiments, and, where appropriate, data are expressed as mean ± SD. Statistical significance was assessed by Student's *t* test, and $p < 0.05$ was taken as a significantly different value.

RESULTS

PKC Isoenzymes and Neutrophil Apoptosis—The level of apoptosis in freshly isolated neutrophils (Fig. 1) was low (2.2 ± 0.7%; $n = 5$) and increased significantly as cells were aged in culture to 11.6 ± 5.1% after 6 h ($p < 0.05$; $n = 5$) and 68.0 ± 15% after 24 h ($p < 0.001$; $n = 5$).

PKC resides in the cytosol in the inactive state and is translocated to the membrane fraction upon activation. Assessment of particulate (membrane) and cytosolic PKC can thus give an indication of enzyme activation. Immunoblotting of neutrophil particulate and cytosolic protein extracts revealed an increase in PKC-β and -δ isoenzymes associated with the particulate fraction during apoptosis (Fig. 2*A*). Translocation of PKC-α was not detected, and the majority of PKC-α was located in the cytosol. PKC-ζ was also detected only in the cytosol fraction of neutrophils, but its subcellular localization did not alter as neutrophils were aged in culture (Fig. 2*A*).

PKC-δ can also be activated by caspase 3. A 40-kDa fragment of PKC-δ was not detected in freshly isolated neutrophils, and only the full-length 78-kDa form was seen. However, as cells were aged overnight in culture and entered apoptosis, a 40-kDa fragment was detected, and the level of the 78-kDa PKC-δ was reduced concomitantly (Fig. 2*B*). The appearance of the 40-kDa PKC-δ fragment was reduced when DEVD-fmk was included in the medium (Fig. 2*B*), confirming that generation of the active

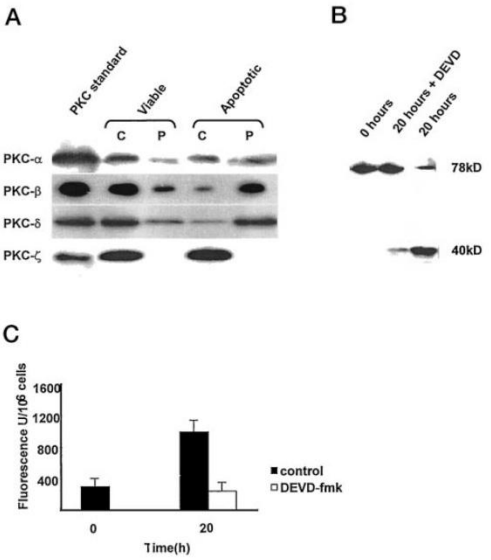

FIG. 2. **PKC isoenzyme activation during neutrophil apoptosis.** *A*, PKC isoenzyme activation was detected by translocation of PKC from the cytosol (*C*) to the particulate (*P*) fraction in extracts from freshly isolated, viable neutrophils and neutrophils cultured for 20 h to become apoptotic. PKC isoenzymes were detected by immunoblotting, and the blot shown is representative of three separate experiments. 10 ng of recombinant human PKC-α, -β, -δ, or -ζ was run on adjacent lanes as standards. *B*, whole cell extracts were prepared from freshly isolated neutrophils and neutrophils cultured for 20 h in medium in the absence or presence of 20 μM DEVD-fmk by the addition of SDS-polyacrylamide gel electrophoresis sample buffer to the cell pellets. The full-length 78-kDa and caspase-generated 40-kDa fragments were detected by immunoblotting. The blot shown is representative of three experiments. *C*, the inhibition of caspase 3 by DEVD-fmk was confirmed by assaying parallel extracts from neutrophils cultured for 20 h in the absence or presence of 20 μM DEVD-fmk and freshly isolated neutrophils. Activity was measured using a fluorescence-tagged caspase 3 substrate according to manufacturer's instructions and is expressed as fluorescence units/10^6 cells. Data are the mean ± SD of three separate experiments.

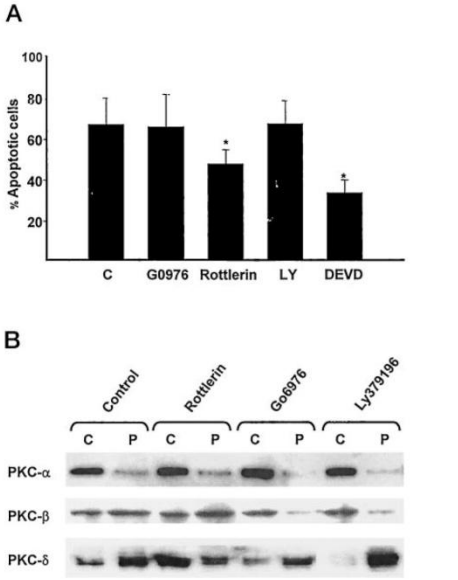

FIG. 3. **Effect of inhibitors of PKC-δ and caspase 3 on neutrophil apoptosis.** Neutrophils were cultured for 20 h in the absence or presence of PKC inhibitors Go6976 (20 nM) or LY379196 (10 nM) or the caspase 3 inhibitor DEVD-fmk (20 μM). *A*, apoptosis was assessed by morphological analysis of cytospins of neutrophils stained with May-Grunwald Giemsa stain. Results are the mean ± S.D. of three separate experiments, and * denotes $p < 0.05$. *B*, inhibition of PKC-β and -δ by the PKC inhibitors used was confirmed by immunoblotting of cytosol and particulate extracts from apoptotic neutrophils treated with the various inhibitors.

fragment of PKC-δ was mediated by caspase 3. Inhibition of caspase 3 activity by DEVD-fmk was confirmed in the same extracts (Fig. 2*C*). Although PKC-ζ can also be cleaved by caspase 3, we did not detect the cleaved form of this isoenzyme in neutrophils (data not shown).

Effect of PKC-specific Inhibitors on Neutrophil Apoptosis—To determine whether the activation of PKC-β and -δ seen in apoptotic cells was involved in the apoptotic process, three PKC isoenzyme-selective inhibitors were used. Go6976 inhibits the classical PKC isoenzymes (PKC-α, -β, and -γ), LY379196 is a PKC-β-specific inhibitor (23), and Rottlerin inhibits PKC-δ (27). Because PKC-δ can be activated by caspase 3, the effect of the caspase 3 inhibitor (DEVD-fmk) on neutrophil apoptosis was also determined. Go6976 and LY379196 did not decrease apoptosis in neutrophil cultures after an overnight incubation (Fig. 3*A*). However, Rottlerin and DEVD-fmk inhibited neutrophil apoptosis significantly (Fig. 3*A*). The inhibition of PKC isoenzymes by the inhibitors used was confirmed by Western blotting of neutrophil cytosol and particulate extracts (Fig. 3*B*).

Effect of Removal of PKC Isoenzymes on DNA Fragmentation in a Cell-free System—To attempt to confirm the data gained with pharmacological inhibitors of PKC isoenzymes, a cell-free system was used. Nuclei were isolated from non-apoptotic neutrophils and combined with cytosol from apoptotic neutrophils after the removal of individual PKC isoenzymes by immunoprecipitation (Fig. 4*A*). Apoptosis was then assessed using an enzyme-linked immunosorbent assay method to detect DNA fragmentation. Cytosol from apoptotic neutrophils (A_+) induced a significant increase in DNA fragmentation in non-apoptotic nuclei, compared with the addition of cytosol from healthy neutrophils (A_0, Fig. 4*B*). The removal of PKC-δ by immunoprecipitation significantly decreased DNA fragmentation induced by the apoptotic cytosol. Loss of PKC-β gave a slight but not significant reduction in DNA fragmentation, and removal of PKC-α or -ζ or the addition of a mouse IgG had no effect (Fig. 4*B*).

Involvement of p42/44 MAP Kinase and p38 MAP Kinase in Spontaneous Neutrophil Apoptosis—Inhibitors of MAP-ERK kinase 1 (PD98059), which lies upstream of p42/44 MAP kinase, and p38 MAP kinase (SB202190) were used to determine whether members of the MAP kinase family were involved in the regulation of spontaneous neutrophil apoptosis. Neither of these agents affected the level of neutrophil apoptosis when they were included in the culture medium for 6 h (data not shown) or overnight (Fig. 5*A*). In addition, activated p42/44 MAP kinase and p38 MAP kinase detected by antibodies specific for the active forms of these kinases were barely detectable in freshly isolated or spontaneously apoptotic neutrophils (Fig. 5*B*). In contrast, activated p38 MAP kinase was present at a high level in neutrophils induced to die by UV irradiation (Fig. 5*B*), and p42/44 MAP kinase was activated by treatment of neutrophils with phorbol myristic acid (Fig. 5*B*).

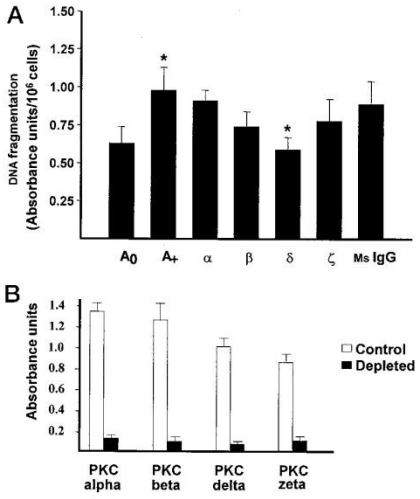

FIG. 4. **Effect of PKC isoenzyme depletion on nuclear DNA fragmentation in a cell-free system.** Cytosol and nuclei were isolated from apoptotic and non-apoptotic neutrophils, respectively, and combined (A_+), or they were isolated after the depletion of PKC isoenzymes by immunoprecipitation. DNA fragmentation was then measured using a commercial enzyme-linked immunosorbent assay-based kit (*A*). Healthy nuclei were combined with non-apoptotic cytosol (A_o) as a control for the fractionation procedure, and a mouse anti-rabbit IgG antibody (*Ms IgG*) was a control for the depletion procedure. The removal of PKC isoenzymes was confirmed by immunoblotting of the depleted cytosol and analysis of band intensity determined by scanning densitometry and expressed as absorbance units per mm² (*B*). Results are the mean ± S.D. of three separate experiments, and * denotes a value of $p < 0.05$.

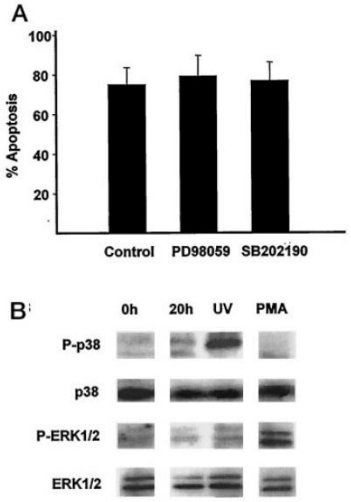

FIG. 5. **p42/44 and p38 MAP kinase activity during spontaneous neutrophil apoptosis.** *A*, inhibitors of MAP-ERK kinase 1 (30 μM PD98059) or p38 MAP kinase (10 μM S202190) were added to cultures of freshly isolated neutrophils, and the level of apoptosis was determined after 20 h by examination of cell morphology. The results are the mean ± S.D. of four separate experiments. *B*, extracts were prepared from freshly isolated neutrophils (*0 h*), neutrophils cultured for 10 min with 10 nM phorbol myristic acid, neutrophils cultured for 20 h in medium alone (*20 h*), and neutrophils induced to apoptosis by UV irradiation for 3 min at 254 nm (*UV*). Active phosphorylated (P-p38 and P-ERK 1/2) and total (p38 and ERK 1/2) p38 MAP kinase and p42/44 MAP kinase were detected by immunoblotting. The blot shown is representative of three separate experiments.

Nuclear Localization of PKC-δ during Neutrophil Apoptosis—The Western blotting data indicating PKC-δ translocation to the particulate fraction were investigated further by immunohistochemistry. Comparison of PKC-δ immunostaining in freshly isolated neutrophils (Fig. 6*A*) and neutrophils cultured for 8 h (Fig. 6*B*) revealed an increase in PKC-δ associated with the nucleus in many cells. The increase in nuclear PKC-δ was quantitated by determining the percentage of total PKC-δ fluorescence that was coincident with the red fluorescence of the propidium iodide nuclear counterstain. In each study, 100 cells were examined, and the mean value for nuclear PKC-δ increased from 7.7 ± 0.4% in healthy neutrophils to 21.4 ± 1.5% in neutrophils cultured for 8 h. Staining of neutrophils aged for a longer time period in culture was not possible because these cells were more fragile and were not suitable for immunostaining.

DISCUSSION

Much research has focused on the apoptotic program in proliferating cells, but little is known about the regulation of apoptosis in fully differentiated cells, such as neutrophils. However, the pathological consequences of an altered neutrophil life span are considerable (1–3). In this study, the involvement of PKC isoenzymes and MAP kinases in spontaneous neutrophil apoptosis has been investigated. We confirmed previous reports that p38 MAP kinase is involved in inducing signals for neutrophil death in response to stress (7). However, the p38 MAP kinase pathway is not involved in spontaneous apoptosis, suggesting that this pathway in the neutrophil may be activated solely in response to stress-induced apoptosis. PKC is known to be involved in neutrophil activation and in the regulation of apoptosis in a variety of cells. The 11 PKC isoenzymes appear to play various roles in the regulation of apoptosis (8), but PKC-δ is consistently a pro-apoptotic PKC isoenzyme. PKC-δ can be cleaved by caspase 3, and transfection of cells with the caspase-generated catalytic fragment of PKC-δ is sufficient to induce apoptosis (28). The data reported here show that PKC-δ is activated during spontaneous neutrophil apoptosis. Furthermore, inhibition of PKC-δ activation by either use of a selective inhibitor or inhibition of caspase 3 delayed neutrophil apoptosis significantly. The caspase 3 inhibitor produced a greater delay in apoptosis than the PKC-δ inhibitor. This is not surprising, because PKC-δ is one of the many targets of this protease. Other substrates of caspase 3 include ICAD, the inhibitor of the apoptosis-specific endonuclease caspase-activated DNase (29), and cytoskeletal proteins such as actin (30) and fodrin (31). Thus, inhibition of PKC-δ may remove one of the elements of the apoptotic program but would not be able to prevent it altogether.

Until recently, PKC-δ and the very closely related isoform PKC-θ were the only PKC isoenzymes reported to undergo proteolytic cleavage and activation by caspase 3. It has now been shown that PKC-ζ can also be cleaved by caspase 3, but in this case, apoptosis was associated with inhibition of PKC-ζ enzyme activity rather than activation (32). Thus, activation of caspase 3 during apoptosis can result in the cleavage of both PKC-δ and -ζ, simultaneously effecting the activation of a pro-apoptotic PKC and the inhibition of an anti-apoptotic PKC isoenzyme. Although PKC-ζ was expressed in neutrophils, we did not detect proteolytic cleavage in spontaneously apoptotic neutrophils. Therefore, this pathway may either operate only

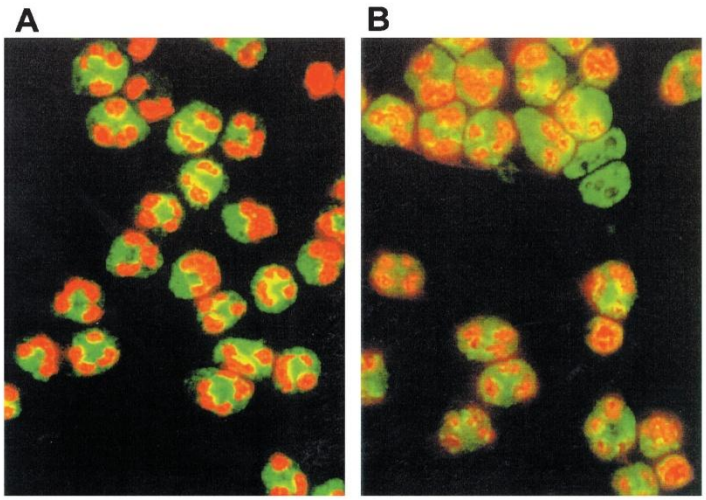

FIG. 6. **Subcellular location of PKC-δ in healthy and apoptotic neutrophils.** Freshly isolated neutrophils (*A*) and neutrophils cultured for 8 h (*B*) were indirectly immunostained for PKC-δ (*green fluorescence*). Nuclei were counterstained with propidium iodide, and coincident fluorescence is shown as *yellow pseudo-color*. Immunofluorescence was visualized using confocal microscopy. The image shown is representative of three separate experiments.

in certain cells or be activated only during stress-induced apoptosis.

We detected translocation of PKC-β in apoptotic neutrophils but did not detect proteolytic fragmentation of this isoenzyme. In a cell-free system, depletion of PKC-β from apoptotic cytosol did not significantly reduce DNA fragmentation in healthy nuclei, and the PKC-β inhibitor LY379196 did not delay neutrophil apoptosis. These data do not support a primary role for PKC-β in spontaneous apoptosis. Moreover, PKC-β has been proposed to play a role in neutrophil activation rather than apoptosis and may be involved in NADPH complex assembly and superoxide generation (33, 34). We excluded the possibility that PKC-β translocation was associated with activation of neutrophils in these studies by measuring superoxide generation. None was detected, suggesting that the translocation of PKC-β is an apoptosis-associated event.

The data reported here suggest that PKC isoenzymes play several roles in the regulation of spontaneous neutrophil apoptosis, with PKC-δ playing a key role. Because p38 MAP kinase was not activated during spontaneous neutrophil apoptosis, we propose that PKC-δ represents a primary signaling pathway, maintaining a basal rate of spontaneous apoptosis. Additional pathways can be recruited to increase the rate of neutrophil death, including the MAP kinase pathway, in situations requiring accelerated apoptosis. The upstream mechanisms leading to activation of caspase 3 and PKC-δ in spontaneous neutrophil apoptosis are still unclear. Ligation of Fas has been suggested (21) because neutrophils express both Fas and its ligand. However, this is unlikely because Fas-blocking antibodies are ineffective (35). An alternative explanation is that spontaneous apoptosis is a result of cytokine deprivation; several pro-inflammatory cytokines are able to prevent neutrophil apoptosis (6, 36). Furthermore, we have shown recently that cytokine deprivation-induced apoptosis of T cells also involves activation of PKC-δ (37).

The association of PKC-δ with the nucleus during apoptosis and its subsequent activation by caspase 3 have recently been reported in U937 cells after ionizing irradiation (38) and during cytokine deprivation and Fas-induced apoptosis in T cells (37). Nuclear translocation of PKC-δ may therefore be an early event in the apoptotic program, preceding the common pathway that follows caspase 3 activation. The tyrosine kinase c-abl can be activated by ionizing irradiation, resulting in phosphorylation of PKC-δ and translocation to the nucleus. Tyrosine phosphorylation may be a general mechanism for nuclear translocation of PKC-δ.

In summary, PKC isoenzymes play several distinct roles in the regulation of spontaneous neutrophil apoptosis. The translocation of PKC-δ to the nucleus and its activation by caspase 3 appear to be crucial events. PKC-δ may therefore represent a useful therapeutic target in conditions of disregulated neutrophil apoptosis.

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The Lipoxygenase Product 13-Hydroxyoctadecadienoic Acid (13-HODE) Is a Selective Inhibitor of Classical PKC Isoenzymes

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13-Hydroxyoctadecadienoic acid (13-HODE), hydroxylinoleic acid, is a major lipoxygenase metabolite which is produced by myeloid inflammatory cells and modifies inflammatory cell activity. The biological effects of 13-HODE in non-haemopoietic cells (HUVEC) have been attributed to the incorporation of 13-HODE into 13-HODE containing diacylglycerol and the selective inhibition of protein kinase C. Our studies, using whole promyeloid cells (HL60) and recombinant PKC isoenzymes in an *in vitro* assay, showed that 13-HODE inhibited PKC-α, β1, and PKC-βII, but did not affect the activity of PKC-δ. These data suggest that the actions of hydroxylinoleic acid on myeloid cells include the selective inhibition of classical PKC isoenzymes.

Linoleic acid is a major component of membrane fatty acids with potential as a signalling molecule (1). Recently, oxidized derivatives of linoleic acid have been identified in a variety of cells and these metabolites have been shown to exhibit a number of biological functions, including modulation of cell proliferation, apoptosis and inflammation (2–4). 13-hydroxyoctadecadienoic acid (13-HODE) is a 15-lipoxygenase metabolite produced in significant amounts by myeloid inflammatory cells (5). 13-HODE has a variety of effects on myeloid cell function, acting as a chemoattractant for neutrophils (6), inhibiting the effects of superoxide produced by macrophages (7) and modulating the degranulation of human neutrophils and expression of surface adhesion molecules (CD11b) required for extravasation (8). The mode of action of hydroxylinoleic acid on myeloid cells has not been established, though Cho and Ziboh (9) reported that 13-HODE was a selective inhibitor of PKC-β in hyperproliferating HUVEC cells. As PKC is involved in the regulation of several aspects of inflammatory cell function, its modulation by 13-HODE could explain the range of effects of linoleic acid derivatives on immune responses.

PKC is a multigene family consisting of 11 isoenzymes that are selectively expressed in different tissues and have been proposed to play specific roles in cell regulation (10). The cells used by Cho and Ziboh, human umbilical vein endothelial cells, expressed PKC β and α as the main PKC isoenzymes (9). Caution is therefore required in interpreting these data, as the effect of 13-HODE on the modulation of a range of PKC isoenzymes, including the novel (δ,ϵ,η,θ) and atypical (ζ,λ/ι,μ) PKC isoenzymes, was not investigated in these studies. To improve understanding of the effect of 13-HODE on intracellular signalling, the effect of 13-HODE was investigated on the activity of PKC isoenzymes in a promyeloid cell line, HL60, which expresses a wide range of PKCs (α, β1, βII, δ and ζ). Inhibition of PKC was also determined using recombinant PKC isoenzymes in an *in vitro* assay system.

MATERIALS AND METHODS

Cell Culture

HL60 cells were cultured in RPMI 1640 medium (GIBCO-BRL), supplemented with 10% fetal calf serum (FCS, GIBCO-BRL) and containing 1mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Cells were treated for 30 min with 13-HODE (97% pure, confirmed by HPLC, Sigma), at 3 ng/ml, with an equivalent concentration of ethanol as a solvent control, for 10 min and 1 h. To assess inhibition of PKC activity, control and 13-HODE-treated cells were incubated for 10 min with 10nM 12-tetradecanoyl 13-phorbol acetate (TPA), a potent activator of classical and novel PKC isoenzymes (10).

PKC Activation Assays

In vitro PKC assay. PKC activity was assessed in whole cells using an *in vitro* kinase assay for PKC immunoprecipitated from cells (11). Briefly, 2×10^6 control or 13-HODE-treated cells were lysed in 200 μl of lysis buffer (Tris-HCl, pH7.4 containing 100 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM phenylmethylsulfonylfluoride (PMSF) and 0.1% Nonidet-P 40). 1 μg of a polyclonal anti-PKC

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antibody, raised against peptides specific to PKC isoenzymes α, $\beta 1$, $\beta 2$ or δ (Santa Cruz Biotechnology) was added to the lysate and incubated for 2 h at 4°C. The immunoprecipitate was then isolated by incubation with protein A-Sepharose (Sigma) for 1 h at 4°C, spun down (13,000 rpm, 2 min, MSE Microcentaur) and washed in PKC assay buffer (20 mM Hepes, pH 7.2 containing 137 mM NaCl, 5.4 mM KCl, 0.3 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 25 mM β-glycerophosphate, 10 mM $MgCl_2$, 5 mM EGTA and 2.5 mM $CaCl_2$). The sepharose beads were resuspended in 100 μl of PKC assay buffer and 20 μl of each sample was used in a PKC activity assay. Protein kinase activity was measured by incorporation of γ-^{32}P-ATP (ICN-Flow, High Wycombe, UK) into a PKC substrate peptide (QKRPSQRSKYL) for 10 min at 30°C, using a commercial PKC assay kit (Upstate Biotechnology).

In addition to using immunoprecipitated PKC in the activity assay, the effect of 13-HODE on recombinant human PKC isoenzymes (Pan Vera Corporation, USA) was also evaluated. 1 unit of recombinant PKC α, $\beta 1$, $\beta 2$, δ or ζ were incubated with 10 nM TPA and 3 ng/ml 13-HODE and their activity measured using the same commercial kit.

PKC translocation. PKC activation in whole cells can also be assessed by determining the relative amounts of PKC in the membrane and particulate fractions of cells (12). Proteins were extracted from cells by lysis in 20 mM Tris-HCl buffer, pH 7.4 containing 100 μg/ml leupeptin, 10 μg/ml pepstatin A and 1 mM PMSF. Lysates were spun at 100,000 × g for 30 min at 4°C. A 6 μl aliquot of supernatant was assayed for protein using a commercial kit (Bio-Rad). SDS sample buffer was added to the remainder of the sample and boiled for 5 min (cytosol protein extract). Membrane proteins were extracted from the pellet using the same lysis buffer containing 2% CHAPS (Sigma). Pellets were resuspended in buffer, incubated on ice for 10 min, then spun at 30,000 × g for 10 min at 4°C. The supernatant was again assayed for protein and boiled in SDS sample buffer (membrane protein extract). Equivalent amounts of protein were separated on 10% SDS-PAGE gels and transferred to Immobilon PVDF transfer membrane (Millipore, Harrow UK) using a wet-blotting apparatus (BioRad). PKC isoenzymes were detected with affinity-purified rabbit anti-peptide antibodies specific to PKC α, $\beta 1$, $\beta 2$, δ and ζ (Santa-Cruz). The second antibody was a horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG antibody (Amersham International, UK). Immunoreactivity was detected using an enhanced chemiluminescence (ECL) kit (Amersham International).

Statistics

Results presented here represent a minimum of three experiments and where appropriate, data are expressed as mean ± SD. Statistical significance was assessed by Student's t test and $p < 0.05$ was taken as significant.

RESULTS

Inhibition of PKC isoenzymes by 13-HODE. Figure 1 shows that addition of 10 nM TPA to HL60 cells produced a rapid translocation of PKC-α, $\beta 1$ and βII and δ from the cytosol (C) to the membrane (M) fraction, which is indicative of PKC activation. TPA did not induce translocation of PKC-ζ, (data not shown) which is in agreement with its reported unresponsiveness to phorbol esters and diacylglycerols. Pre-incubation of HL60 cells for 30 min with 13-HODE, prior to addition of TPA, inhibited the translocation of PKC-$\beta 1$ from cytosol to membrane (Figure 1). 13-HODE also inhibited the TPA-induced translocation of PKC-$\beta 2$ and α, but to a lesser degree. 13-HODE did not affect the translocation of PKC-δ, suggesting that its inhibitory actions may be restricted to the classical PKC isoenzymes. As TPA did not activate PKC-ζ and this isoenzyme was detected only in the cytosol of proliferating HL60 cells (data not shown), we could not determine whether 13-HODE was able to inhibit this atypical PKC. When HL60 cells were not pre-incubated with 13-HODE prior to addition of TPA, 13-HODE did not inhibit PKC translocation (Figure 1).

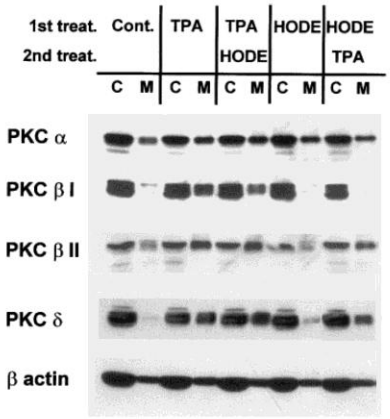

FIG. 1. Effect of 13-HODE on PKC isoenzyme translocation in HL60 cells. Extracts of cytosolic (C) and membrane (M) fractions of HL60 cells treated with 10 nM TPA alone, 10 nM TPA and 3 ng/ml 13-HODE simultaneously, 3 ng/ml 13-HODE alone, or 3 ng/ml 13-HODE 30 min prior to addition of 10 nM TPA were separated by SDS-PAGE and analysed by Western blotting. Blots were probed with PKC isoenzyme-specific antibodies (α, βI, βII, and δ) and revealed by ECL. Anti-β-actin antibody was used to confirm equal loading of protein. The blots shown are representative of three separate experiments.

To confirm that the translocation of PKC isoenzymes seen in whole cells reflected altered enzyme activity, the 13-HODE treated HL60 cells were lysed and PKC isoenzymes were isolated by immunoprecipitation. The activation status of the isoenzymes was then tested using an in vitro PKC activity assay. These assays revealed that 13-HODE significantly inhibited the activation of PKC α, $\beta 1$ and βII. 13-HODE had no effect on PKC δ enzyme activity (Figure 2). Interestingly, 13-HODE alone was also able to inhibit the activity of PKC-β isoenzymes that was ongoing in proliferating HL60 cells, in the absence of further stimulation by TPA (Figure 2).

PKC activity assays were also performed using recombinant human PKC isoenzymes and the data supported the results obtained with immunoprecipitated PKC proteins (Figure 3). 13-HODE was able to inhibit the activation of PKC α, $\beta 1$ and $\beta 2$ induced by TPA, but had no effect on TPA-induced activation of PKC δ.

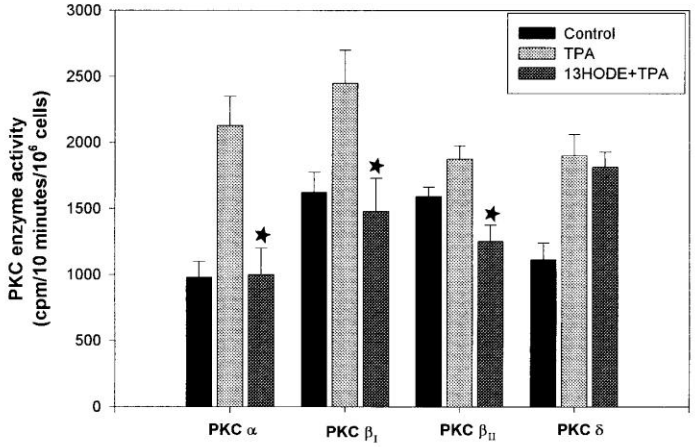

FIG. 2. Effect of 13-HODE on PKC isoenzyme activity in HL60 cells. Cells were treated with 10 nM TPA and/or 3 ng/ml 13-HODE and then lysed after 30 min. PKC α, βI, βII, and δ isoenzymes were immunoprecipitated from the lysates and PKC enzyme activity was determined using an *in vitro* PKC activity assay to detect [γ-^{32}P]ATP incorporation into a PKC specific peptide. The data are mean ± SD of 3 separate experiments. A star denotes $p < 0.05$.

DISCUSSION

In this study hydroxylinoleic acid, 13-HODE, has been shown to be a potent inhibitor of PKC-β, in partial agreement with published data in HUVEC (9). In addition, a pre-incubation period was required for inhibition of PKC activation in whole cells. These data support the findings of Cho and Ziboh, who showed that 13-HODE was incorporated first into membrane phospholipids and subsequently into 13-HODE containing diacylglycerols released following receptor mediated lipid hydrolysis (9). Furthermore, studies of the activation status of PKC isoenzymes precipitated form HL60 cells (Figure 1), showed that a 30 min incubation of cells with 13-HODE alone, could inhibit the normal activity of PKC-β in proliferating HL60 cells. These data further support the proposal that 13-HODE containing diacylglycerols can compete with physiological activators of PKC (including agonistic diacylglycerols) *in vivo*. In contrast to the reports of Cho and Ziboh

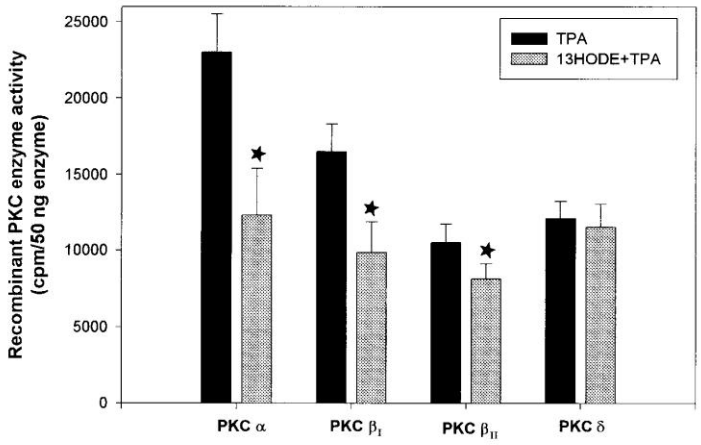

FIG. 3. Effect of 13-HODE on recombinant PKC isoenzyme activity. Recombinant human PKC isoenzymes (α, βI, βII, and δ) were activated by TPA (10 nM) and the effect of 3 ng/ml 13-HODE on enzyme activity was determined by measuring [γ^{32}P]ATP incorporation into a PKC specific peptide. Data are mean ± SD of 3 separate experiments. A star denotes $p < 0.05$.

concerning HUVEC cells (9), we showed that the effects of 13-HODE were not limited to PKC β in HL60 cells. 13-HODE was able to inhibit PKC-α, both *in vitro* and in whole cell assays. The apparent discrepancy between our data and those of Cho and Ziboh (9), is likely to be technical in origin. These authors relied upon the membrane translocation assay and also measured PKC activity in epidermal extracts following purification by DE52 and hydroxylapatite. In our studies, PKC activity was assessed by translocation in whole cells, by assay of immunoprecipitated PKC and using recombinant PKC protein. These approaches are rapid and direct and do not involve lengthy chromatographic purification of a labile enzyme activity.

We have also shown that 13-HODE did not inhibit PKC-δ. The novel PKC isoenzymes were not considered by Cho and Ziboh. These data indicate that the effects of 13-HODE are limited to the classical PKC isoenzymes and in this context it may be a useful research and therapeutic agent. 13-HODE thus represents a physiological lipid inhibitor of PKC with a degree of isoenzyme selectivity. Such a role has been demonstrated previously for ceramide and PKC α, though the inhibitory effect of this lipid was indirect (13). Our studies with purified recombinant PKC, showed that 13-HODE was able to inhibit PKC activation directly and it may therefore be able to function as a signalling molecule in its own right. The importance of a selective and naturally occurring PKC isoenzyme inhibitor is clear. PKC isoenzymes are differentially regulated during cell events such as proliferation and apoptosis, but it has not been demonstrated how this is achieved at the molecular level *in vivo,* though differential modulation by lipid species varying in fatty acid composition is one possibility (14). 13-HODE is a lipid species with isoenzyme selective activity and represents a route to differential regulation of PKC isoenzyme activity *in vivo*. To determine exactly how the functions of inflammatory cells are modified by 13-HODE will necessitate determining the role of the classical PKC isoenzymes in the regulation of the response being studied.

ACKNOWLEDGMENTS

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Superoxide Production in Human Neutrophils: Evidence for Signal Redundancy and the Involvement of More Than One PKC Isoenzyme Class

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Selective protein kinase C (PKC) activators and inhibitors and a physiological agonist, fMLP, were used to study superoxide production and PKC isoenzyme activation in human neutrophils. The data show that the classical PKC isoenzymes, α and β, were activated by TPA and at a time prior to NADPH oxidase complex assembly. fMLP induced activation of PKC-β over a similar time course. Inhibition of c-PKCs reduced, but did not block, TPA-induced superoxide production completely, suggesting additional PKC isoenzymes were involved beyond NADPH oxidase assembly. PKC inhibitors were unable to inhibit fMLP-induced superoxide generation, indicative of signal redundancy in the induction of superoxide generation in human neutrophils.

Neutrophils are short lived, terminally differentiated blood cells, which play a vital role in inflammatory responses. A variety of molecules, including cytokines and bacterial products, then activate the defence systems of the neutrophil, which include phagocytosis, degranulation and activation of NADPH oxidase (1). NADPH oxidase is a multi-component enzyme that catalyses the reduction of oxygen, at the expense of NADPH, to produce a range of microbicidal reactive oxygen species. In resting cells the various components of the NADPH oxidase complex are dissociated within the neutrophil, activation results in their association into a functional enzyme complex at the cell membrane (2,3). Assembly of the complex is rapid and phosphorylation is thought to play a key role in mediating formation of the active oxidase complex (4,5). Two major components of NADPH oxidase, $p47^{phox}$ and $p67^{phox}$ are rapidly phosphorylated after activation of neutrophils (6-8) and a key role for protein kinase C (PKC) has been demonstrated (9-11). Interestingly, studies with potent inhibitors of PKC suggest that not all of the events associated with neutrophil activation require PKC. For example, Ro 31-8425 inhibited superoxide generation induced by TPA, but had little effect on adhesion or response to the chemoattractant C5a (12).

PKC is a family of 11 isoenzymes, which are well conserved across several species, suggesting they have specific functions within cells (13). The PKC isoenzyme family has been divided into three groups, according to their co-factor requirements and structure (14); classical PKC's (α,βI,βII,γ); novel PKC's (δ,ϵ,η,θ) and atypical PKC's (ζ,λ/ι,μ). Stimulation of neutrophils with receptor agonists, including fMet-Leu-Phe (fMLP), results in activation of phospholipase C with the production of inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (15). IP3 releases calcium from intracellular stores and both Ca^{2+} and diacyglycerol activate PKC.

Although PKC is clearly involved in the regulation of superoxide production, little is known of the role of the individual PKC isoenzymes. Neutrophils express several PKC isoenzymes including PKC-α, βI, βII, δ (16,17) and ζ (18) and purified PKC can stimulate superoxide production by solubilised NADPH oxidase (19). Sergeant and McPhail have shown very recently that the three predominant isoenzymes, PKC-βII, δ and ζ were all activated during stimulation of neutrophils with opsonized zymosan. The activation of these PKC isoenzymes was rapid, occurring within 1 minute, and preceded the formation of the NADPH enzyme complex (20). Moreover, the activation of PKC isoenzymes was transient, suggesting that PKC activation was required for the assembly of the NADPH complex, but may not be required for the maintenance of the active enzyme complex (20). Although all three isoenzymes investigated in the latter study were activated by zymosan treatment, the precise involvement of the isoenzymes was not established and it is possible that not all were required for superoxide production.

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The short life-span and differentiated state of the neutrophil, restrict the methods that can be used to manipulate PKC isoenzymes. In the studies described here, we have used a variety of pharmacological agents to activate and inhibit PKC isoenzymes and have correlated this with their ability to affect superoxide generation. PKC activation was assessed after two minutes of treatment to determine which isoenzymes were activated prior to NADPH complex assembly. As both PKC-β and δ have been implicated in previous studies (10,20,21), we have specifically used agents with reported selectivity for these two isoenzymes. The deoxyphorbol ester Doppa, activates PKC-β selectively *in vitro* (22), though reports of its selectivity *in vivo* are inconsistent (23). 13-HODE, a pro-inflammatory molecule, has been shown to inhibit PKC-β in HUVEC (24). BisA activates PKC-δ both *in vitro* and in whole cells (25) and Rottlerin, which has its highest potency for inhibition of PKC-δ (IC_{50} 3-6μM) and is less effective against the classical (IC_{50} 30-42μM) and atypical (IC_{50} 80-100μM) PKC isoenzymes (26).

MATERIALS AND METHODS

Isolation and culture of human peripheral blood neutrophils. Venous blood was taken from healthy volunteers and neutrophils were isolated on Percoll gradients as described previously (27). Neutrophil preparations contained greater than 95% neutrophils. Neutrophils were resuspended in RPMI 1640 medium (Gibco-BRL), supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma).

Treatment of neutrophils with PKC modulating compounds. To determine the ability of PKC activating agents to stimulate superoxide production, neutrophils were treated for 1h with the various compounds at the concentrations shown. SA was kindly provided by Professor F.J.Evans (London School of Pharmacy) and BisA was provided by Dr D Watters (Queensland Institute for Medical Research). The remaining PKC modulating compounds were purchased from Calbiochem-Novabiochem. fMLP (Sigma) was used at 100 nM, as a physiological receptor agonist. Following treatment, neutrophils were assayed for superoxide production (see below). To test the effect of PKC inhibitors on superoxide production, neutrophils were treated with 500mM Bisindolylmaleimide 1, 20nM Go6976, 5μM Rottlerin or 300nM 13-HODE for 30 min prior to activation of neutrophils with 100 nM fMLP or 10 nM TPA. 13-HODE was purchased from Sigma and was >97% pure as established by HPLC. Superoxide production was then measured after a 1h incubation. All of the agents used were prepared as stocks in DMSO or ethanol and kept at −70°C, the appropriate amount of solvent was added to control neutrophil cultures.

Assay for superoxide production. Superoxide production was determined by measuring the superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C (28). The assay was performed in fibronectin-coated 24 well linbro plates, for 1 h, at 37°C. The plates were then spun at 400 × g for 5 min and the absorbance of the supernatant was determined at 550 nm (PYE Unicam PU 8600 UV/Vis spectrophotometer, Philips).

Measurement of PKC isoenzyme activation. PKC resides in the cytosol in the inactive state and associates with cell membranes and the cytoskeleton upon activation. PKC activation can therefore be assessed by determining translocation of PKC from the cytosol to the membrane fraction of cells (25). Translocation of PKC isoenzymes was determined after a 2 min incubation with 100 nM fMLP, 10 nM TPA, or 50 nM concentrations of Dopp, Doppa, ThyA or BisA. Briefly, neutrophils were resuspended in buffer A [20 mM Tris/HCl buffer, pH 7.5; 1 mM PMSF, 100 μg/ml leupeptin and 10 μg/ml pepstatin] and lysed by freezing and thawing in liquid nitrogen. The cell lysate was spun (100,000 × g, 4°C, 30 min) and the supernatant removed and treated as the cytosol extract. Membrane proteins were then extracted from the resulting pellet in buffer A containing 0.5% (v/v) Triton X-100. Samples were boiled in SDS sample buffer and equivalent amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membrane (Immobilon-P, Millipore UK Ltd). PKC isoenzyme immunoreactivity was then detected by incubating the blot with primary antibodies to the individual PKC isoenzymes, followed by a HRP-conjugated sheep anti-rabbit (Amersham Imternational) or donkey anti-sheep IgG antibody (The Binding Site Ltd), as appropriate. Enhanced chemiluminescence (ECL, Amersham International) was used to reveal immunoreactive bands. The PKC antibodies used were affinity purified, polyclonal antipeptide antibodies and were obtained from more than one source. The anti-PKC-ϵ antibody was raised in rabbits (Gibco-BRL), the anti-PKC-ζ antibody was also raised in rabbits and was a kind gift from Dr J Ransom (Syntex Research Ltd, Palo Alto, USA). The antibodies to PKC-α, βI, βII and δ were raised in sheep (The Binding Site Ltd). The specificity of the rabbit antibodies has already been reported (29). The sheep antibodies detected single bands in extracts from Jurkat T cells (Santa Cruz) of appropriate relative molecular weight (Mr 76-80 kDa) on Western blots and inclusion of the immunogenic peptide removed these bands on Western blots and reduced immunostaining to the background level seen with second antibody alone (data not shown).

Statistics. Results presented here represent a minimum of three experiments and where appropriate, data are expressed as mean ±

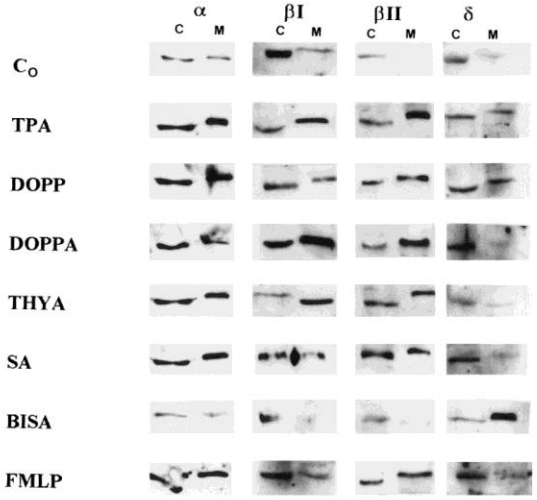

FIG. 1. Activation of PKC isoenzymes prior to superoxide generation. PKC isoenzyme translocation was detected in cell extracts from control (Co) neutrophils and neutrophils treated with 10 nM TPA, 25 nM DOPP, 25 nM DOPPA, 25 nM THYA, 25 nM SA, 25 nM BISA, or 100 nM fMLP for 2 min. Protein was extracted from cytosol (C) and membrane (M) fractions; and individual PKC isoenzymes (α, βI, βII, δ) were detected by Western blotting. Data shown are from a single experiment, representative of three identical studies performed.

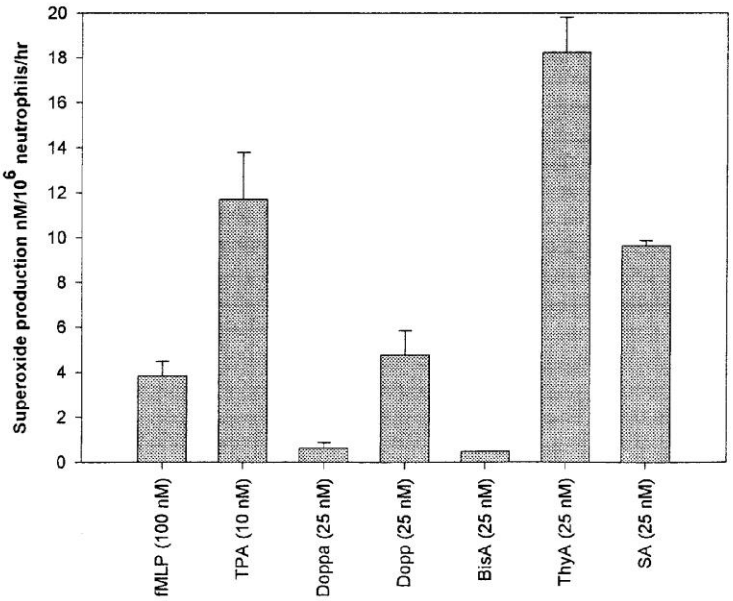

FIG. 2. Superoxide production by human neutrophils treated with PKC activators or fMLP. Neutrophils were incubated with 100 nM fMLP, 10 nM TPA, or 25 nM DOPP, SA, ThyA, BISA, or DOPPA for 1 h at 37°C. Superoxide generation was measured as described under Materials and Methods and data are presented as mean ± SD of three separate experiments.

SD. Statistical significance was assessed by Student's t test and a p value of <0.05 was taken as a significantly different value.

RESULTS

PKC Isoenzyme Activation prior to Superoxide Production

Activation of PKC isoenzymes was assessed after a 2 min treatment with pharmacological activators of PKC and also after treatment with fMLP. The short time point was selected to determine PKC isoenzyme activation which coincided with NADPH complex formation (20). Western blotting of freshly isolated neutrophils (Co, Fig.1) showed that these cells expressed PKC-α, βI, βII and δ. In resting neutrophils PKC isoenzymes were located predominantly in the cytosol, indicative of an inactive state, with the exception of PKC-α which was distributed evenly between the cytosol and membrane. The atypical isoenzyme, PKC-ζ, was also detected, but was not affected by any of the PKC activators used here (data not shown). All four isoenzymes showed evidence of translocation to the membrane (M) fraction, indicative of activation, within 2 min of the addition of TPA or Dopp, though the effect on PKC-α was less marked than for PKC-βI, βII or δ (Fig.1). ThyA and SA caused a significant translocation of PKC-βI and βII, with no effect on PKC-δ, again the translocation of PKC-α was apparent, though less marked than for PKC-β. Doppa activated only PKC-βI and βII over the time course tested and BisA translocated only PKC-δ. fMLP has been reported to induce translocation of PKC (30) and Fig. 1 shows that in our studies the only isoenzyme affected after 2 min was PKC-βII.

PKC Isoenzymes and Neutrophil Superoxide Production

PKC activators. Both fMLP and TPA induced significant superoxide production (Fig.2). Dopp, which does not show isoenzyme selectivity (22), was as effective as TPA in the induction of superoxide generation (Fig.2). SA and ThyA (Fig.2) also produced a superoxide burst equivalent to that of TPA. BisA, a specific activator of PKC-δ (25), did not induce superoxide production (Fig.2), suggesting that if PKC-δ is involved in superoxide generation, it is acting in conjunction with other PKC isoenzymes or other signalling pathways. The deoxyphorbol ester Doppa, which activated only PKC-β within 2 minutes, failed to increase production of superoxide (Fig.2), suggesting that activation of PKC-β alone was not sufficient to effect formation of the NADPH oxidase complex and superoxide generation.

PKC inhibitors. The broad range PKC inhibitor bisindolylmaleimide 1 and the c-PKC specific inhibitor Go6976, significantly reduced TPA-induced superoxide

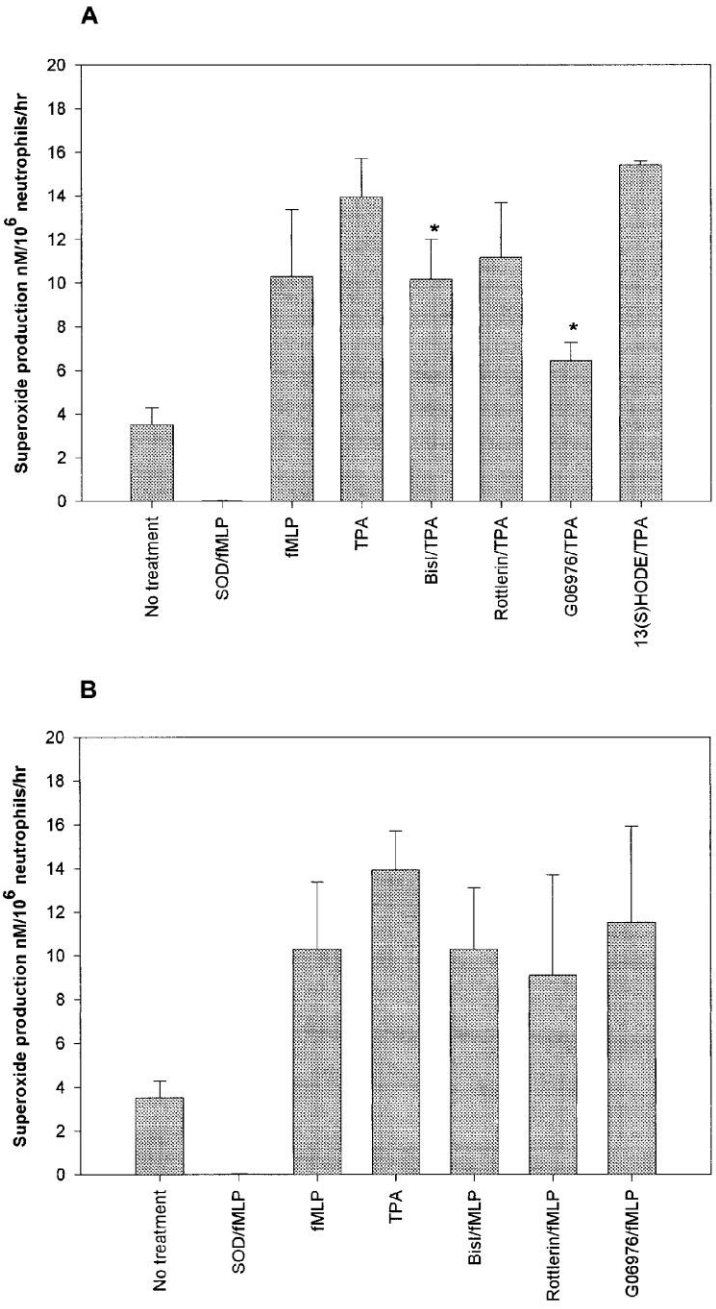

FIG. 3. Effect of PKC inhibitors on superoxide production induced by fMLP or TPA. Neutrophils were incubated with 500 nM bisindolylmaleimide 1, 20 nM Go6976, 300 nM 13-HODE, or 5 μM Rottlerin for 30 min prior to the addition of (A) 10 nM TPA or (B) 100 nM fMLP. Superoxide production was measured as described under Materials and Methods and was completely inhibited by the addition of superoxide dismutase (SOD). Data are shown as mean ± SD for three separate experiments. * Denotes $p < 0.05$.

generation (Figure 3A). In contrast, inhibitors with a more restricted effect on PKC isoenzymes, 13-HODE and Rottlerin, which have been reported to inhibit PKC-β and δ respectively, showed no inhibition of superoxide production by induced by TPA (Fig.3A). None of the inhibitors used were able to affect superoxide production induced by fMLP (Fig.3B).

DISCUSSION

In this study the involvement of PKC isoenzymes in superoxide generation in human neutrophils has been investigated. More specifically, we have determined which PKC isoenzymes were activated within two minutes of the addition of a range of agonists, to identify isoenzymes of PKC that may be specifically involved in the assembly of the NADPH oxidase enzyme complex (20). Superoxide production was induced by the PKC activator TPA and also by the physiological receptor agonist fMLP. Both SA and ThyA, which activated only PKC-α and β isoenzymes in neutrophils within two minutes, were equipotent with TPA, inferring a role for activation of PKC-α and/or β in the formation of the NADPH oxidase complex prior to superoxide generation. Doppa, which activated only PKC-β within two minutes, did not induce superoxide production in neutrophils. Merritt et al (31) have previously reported the ability of SA to induce superoxide generation in neutrophils and they also showed that Doppa was unable to induce superoxide production, even at concentrations as high as 1μM. However, these authors did not determine PKC isoenzyme translocation in their studies. Thus, although PKC-β has been proposed by several authors (10,21) to mediate superoxide production, our data suggest that activation of both PKC-α and β are required. Interestingly, both PKC-α and βII have been identified as major components of neutrophil granule cytoplasts which contain components of the NADPH oxidase complex (32). From the studies employing selective inhibitors of PKC isoenzymes, it is not possible to conclude that activation of the c-PKC isoenzymes was sufficient to effect maximal superoxide generation induced by TPA. We have shown that the activation of specific isoenzymes, PKC-α and β, was rapid and correlates with the time previously recorded for the assembly of the NADPH oxidase complex (20). However, to assume that signalling cascades initiated beyond two minutes by the various PKC activators continued to involve only PKC-α and β, would not be justified. Firstly, ThyA (23), has been shown to activate a range of PKC isoenzymes with prolonged treatment (20min-1h). Secondly, Go6976, which is a potent inhibitor of the c-PKC isoenzymes, was able to reduce superoxide production induced by TPA, but only by 50%. Broad range inhibitors of PKC isoenzymes, such as Ro 31-8425 (12), have been shown to abolish superoxide production. Not surprisingly, the inhibitors showing the greatest isoenzyme selectivity, 13-HODE and Rottlerin, had no effect on TPA-induced superoxide production.

Although PKC is clearly able to promote superoxide generation in neutrophils, there is mounting evidence of redundancy in this signal transduction pathway, which is confirmed in this report. For example, induction of superoxide generation by the chemoattractant C5a was 12-fold less sensitive to inhibition by Ro 31-8425 than was induction by TPA (12). Previous studies have reported the translocation of several PKC isoenzymes following treatment of neutrophils with fMLP, including PKC-βII and δ (31) and PKC-βI, βII and ζ (18). However, these studies did not determine whether inhibition of these isoenzymes would affect fMLP induction of superoxide generation. fMLP induced the rapid translocation of PKC-βII in the studies reported here. However, the inability of Go6976 to inhibit fMLP-induced superoxide generation would suggest that a PKC-independent signalling pathway is able to mediate the actions of fMLP with regard to superoxide generation. El Benna et al (33) have shown recently that the phosphorylation of $p67^{phox}$ induced by fMLP was not inhibited bisindolylmaleimide 1, whereas TPA-induced phosphorylation was inhibited. Thus formation of the NADPH oxidase complex and subsequent generation of superoxide, can be achieved via PKC-dependent or PKC-independent signalling pathways.

Finally, as TPA is not a physiological activator of neutrophils, there has been some suggestion that the activation of PKC may not normally contribute to neutrophil activation. This may be the case for certain receptor agonists, including fMLP, but there is also evidence that PKC signal transduction pathways are employed under physiological conditions. For example, in monocytes inhibition of superoxide production by Legionella pneumophila was shown to be associated with depletion of PKC α and β (34) and the effects of a variety of physiological agonists are affected by inhibitors such as Ro 31-8425 (12). If PKC is to be a target for the design of novel therapeutics for diseases involving abnormal neutrophil function, it is clearly necessary to establish which PKC isoenzymes are to be targeted and more importantly, whether PKC activation is a redundant signalling pathway for that function.

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DOPPA INDUCES CELL DEATH BUT NOT DIFFERENTIATION OF U937 CELLS: EVIDENCE FOR THE INVOLVEMENT OF PKC-βI IN THE REGULATION OF APOPTOSIS

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Abstract—Recent reports have claimed that activation of protein kinase C (PKC)-β is sufficient for both differentiation and apoptosis in promyeloid HL60 cells. Phorbol esters which differentially activate PKC isoenzymes *in vitro* were used to induce differentiation and apoptosis in U937 cells; TPA and Dopp activate all U937 PKC isoenzymes, except PKC-ζ and Doppa activate only PKC-βI. At concentrations of Doppa below 50 nM, only PKC-βI was activated by 2 min and apoptosis was induced, but there was no differentiation of cells towards monocytes. TPA (1–25 nM) and Dopp (5–100 nM) activated PKC-α,-βI and-δ within 2 min and induced differentiation, but only increased apoptosis at the highest concentrations used. Thus, initial activation of PKC-βI is insufficient for differentiation of U937 cells, but may lead to the induction of apoptosis. Copyright © 1996 Elsevier Science Ltd.

Key words: Protein kinase C (PKC), apoptosis, differentiation, Doppa, U937, PKC translocation.

Introduction

Protein kinase C (PKC) is a family of at least 11 isoenzymes, which are differentially regulated by physiological ligands, have distinct co-factor requirements and show tissue specific expression [1]. These facts suggest that the different PKC isoenzymes have specific functions within cells, very few of which have been determined. The compound 12-tetradecanoyl-13-phorbol acetate (TPA) is a potent activator of PKC; its ability to induce differentiation and modulate apoptosis in promyeloid cells suggests a role for PKC in the regulation of these processes [2, 3]. There are many reports of the selective involvement of PKC isoenzymes in differentiation (reviewed in [2]) and it is clear that PKC-β is a key regulator of this process. In contrast, the literature regarding PKC and apoptosis appears contradictory, with reports that TPA prevents apoptosis in some cells [4] and induces it in others [5]. These studies did not determine the isoenzyme content of the treated cells or take into consideration the ability of TPA to both activate and down-regulate the majority of PKC isoenzymes.

Recently, mutant promyeloid HL60 cells (HL60 PET), deficient in PKC-β, were shown to be resistant to both differentiation and apoptosis [6] induced by phorbol esters. Increasing expression of PKC-β in these cells restored their ability to differentiate and apoptose in response to the phorbol esters TPA and Doppa [7]. Whilst TPA is relatively non-selective in its activation of PKC isoenzymes *in vitro* and in whole cells (hereafter referred to as *in vivo*), 12-deoxyphorbol-13-phenylacetate-20-acetate (Doppa) activates only PKC-βI *in vitro* [8]. The authors therefore concluded that the activation of PKC-β was necessary and sufficient for both HL60 cell differentiation and apoptosis [7, 9]. This conclusion may not be entirely correct.

Whilst Doppa is specific for activation of PKC-βI *in vitro* [8], recent *in vivo* studies show that concentrations of Doppa of 100 nM and above can activate PKC isoenzymes other than PKC-β [10, 11]. Macfarlane and co-workers did not show data confirming the PKC-β specificity of Doppa *in vivo*, which was used at

Abbreviations: TPA, 12-tetradecanoyl-13-phorbol acetate; *Dopp*, 12-deoxyphorbol-13-phenylacetate; *Doppa*, 12-deoxyphorbol-13-phenylacetate-20-acetate.

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concentrations of up to 200 nM in their studies. In addition, hydrolysis of Doppa to 12-deoxyphorbol-13-phenylacetate (Dopp) can occur within 6 h *in vivo* [11]. Dopp is not PKC isoenzyme specific [8]. This possibility was not considered in the HL60 PET studies and substantial Doppa hydrolysis may have occurred over the long treatment periods required for differentiation of HL60 cells.

In this study, the promonocytic cell line U937 was used, as we have recently shown that the expression of PKC isoenzymes is differentially modulated in spontaneously apoptotic U937 cells [12]. Most notably, there was a dramatic increase in the expression of PKC-β. Here we have used a range of concentrations of Doppa, in addition to Dopp and TPA, to investigate more directly the role of PKC isoenzyme activation in differentiation and apoptosis. We have also assessed the activation of PKC isoenzymes by these phorbol esters in U937 cells. The data suggest that rapid activation of PKC-βI can lead to apoptosis, but that the modulation of several PKC isoenzymes is required for differentiation.

Materials and Methods

Cell culture

Myelomonocytic human leukaemia cells (U937), from the Cell Culture Collection at Porton Down, U.K., were grown in RPMI 1640 (Gibco, Paisley, U.K.) containing 10% fetal calf serum (Gibco) and antibiotics, as previously described [13]. Cells in exponential growth were incubated for up to 72 h with a range of concentrations of phorbol esters (Calbiochem Novabiochem, U.K.): 1–25 nM TPA, 5–100 nM Dopp or 5–100 nM Doppa.

Assessment of differentiation and apoptosis

To assess differentiation of U937 cells towards monocytes, cells were harvested and cell lysates prepared to determine expression of the monocyte specific enzyme Cathepsin B, as previously described in detail [14]. Apoptosis was assessed by three different methods. In 48 h treated cultures, DNA was extracted from control and treated cells and electrophoresed, using a standard protocol to detect oligonucleosomal DNA fragmentation associated with apoptosis [13]. In cultures treated for shorter time periods (2 h), more sensitive methods of detecting apoptotic cells were required. The ability to 3′-end label fragmented DNA has been used as a sensitive measure of the endonuclease activity associated with apoptosis [15]. Cells treated for 2 h with 10 nM TPA or 25 nM Doppa were lysed in LB buffer (10 mM Tris–HCl, pH 8.0, containing 100 mM EDTA and 0.5% Triton X-100) and spun at 35,000 × *g* for 20 min. The DNA was ethanol-precipitated from the supernatant, re-dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, containing 1 mM EDTA) and 3′-end labelled with ^{32}P-ddATP using a commercial kit (Amersham International, U.K.). Specific incorporation of radioactivity was determined by scintillation counting and expressed as a percentage of counts incorporated into DNA of untreated cells. In the third method, apoptosis was detected by FACS analysis, identifying apoptotic cells at an early stage by their reduced size and increased granularity [16]. Control cells and cells treated for 2 h with phorbol esters were fixed in PBS containing 5% fetal calf serum and 1% formaldehyde. Forward and side light scatter (fsc and ssc) were analysed using a Becton–Dickinson FACScan analyser, with apoptotic cells separated from healthy cells on the basis of their reduced fsc and increased ssc [16]. The percentage of cells in the apoptotic and non-apoptotic populations was then determined.

Isoenzyme translocation

Protein kinase C isoenzyme translocation was assessed to determine activation of PKC isoenzymes. Briefly, PKC was extracted and partially purified by anion exchange chromatography (DE52, Whatman Ltd), from cytosol and membrane fractions of control and phorbol ester-treated cells [17]. The PKC isoenzyme content of extracts was determined by Western blotting, using affinity purified PKC isoenzyme-specific antibodies. The antibodies were raised against peptides specific to each of the PKC isoenzymes-α,-βI,-δ, -ϵ (Gibco–BRL, U.K.) and -ζ (kindly provided by Dr J. Ransom, Syntex Research, Palo Alto, U.S.A.). These antibodies are suitable for Western blotting, detecting a band of appropriate molecular weight that was removed by incubation with immunogen [18]. Second antibodies were peroxidase-conjugated sheep anti-rabbit IgG (The Binding Site Ltd, U.K.) and immunoreactivity was detected by enhanced chemiluminescence, using a commercial kit (ECL, Amersham International, U.K.) and pre-flashed X-ray film (X-Omat, MAS Stirling). Immunoreactive bands were scanned densitometrically (LKB Ultroscan) to provide a semi-quantitative determination of PKC isoenzyme down-regulation. The density of immunoreactive bands in treated cells was determined for each isoenzyme and expressed as a percentage of the control band density.

Confocal microscopy

The U937 cell cytospin preparations (Shandon II cytocentrifuge) were fixed in acetone and indirectly immunostained with a PKC-βI specific antibody (Gibco–BRL). The suitability of this antibody for immunohistochemistry has already been reported [19, 20]. The second antibody was a fluorescein-conjugated sheep anti-rabbit IgG (The Binding Site

Ltd, U.K.). The sub-cellular distribution of immunofluorescence was determined using a laser scanning confocal microscope (Bio–Rad, model MRC 500).

Statistical analysis

Where appropriate, data are expressed as mean ±S.D. and the Students *t*-test was used to compare data. A significant difference between means was represented by $P \leqslant 0.05$.

Results

Induction of differentiation by phorbol esters

Figure 1 shows that TPA was a potent inducer of monocyte differentiation, with maximal Cathepsin B activity reached by 48 h with 5 nM TPA. Dopp was less potent, with a maximal effect at 100 nM (Fig. 1). These figures are in broad agreement with values published for the induction of differentiation by these agents in the promyelocytic cell line HL60 [21]. In contrast, Cathepsin B activity was only induced by Doppa at the highest concentration used (100 nM) and did not reach the levels seen with TPA or Dopp. Other authors have also reported less than maximal induction of monocyte differentiation of promyeloid HL60 cells by Doppa, even using concentrations up to 1 μM [21]. There is, therefore, good agreement between the effects of phorbol esters on induction of differentiation in both U937 cells and HL60 cells.

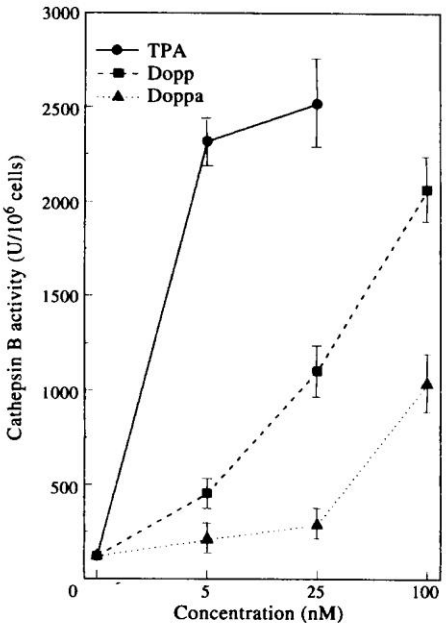

Fig. 1. Induction of differentiation by TPA, Dopp or Doppa. The U937 cells were cultured for 72 h with TPA (1–25 nM), Dopp (5–100 nM) or Doppa (5–100 nM) and assessed for Cathepsin B activity to determine monocyte differentiation. Data shown are for enzyme activity (Units per 10^6 cells) and are mean ±S.D. of three determinations.

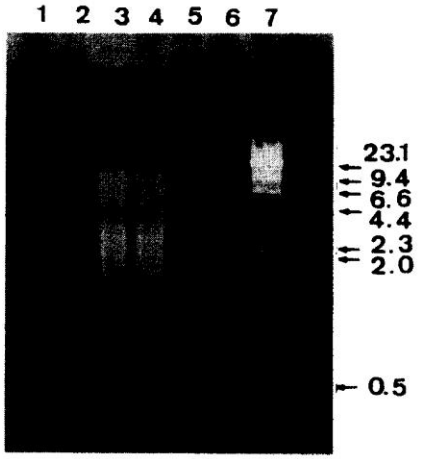

Fig. 2. Fragmentation of DNA in the presence of TPA or Doppa. The U937 cells were incubated for 72 h with medium alone (lane 1), Doppa at 5 nM (lane 2), 25 nM (lane 3), 50 nM (lane 4), or TPA at 10 nM (lane 5), or 25 nM (lane 6). The DNA was extracted and electrophoresed (12) to detect oligonucleosomal fragments typical of apoptosis. Lane 7 = DNA standards.

Induction of apoptosis by Doppa, Dopp and TPA

Doppa treatment increased DNA fragmentation in U937 cells at all concentrations tested (Fig. 2), with 25 nM producing a maximal effect. There was no detectable increase in DNA fragmentation by 48 h with 10 nM TPA, although increasing TPA to 25 nM did cause DNA fragmentation (Fig. 2). Similarly, only 100 nM Dopp caused an increase in DNA fragmentation (data not shown). The detection of gross DNA fragmentation by this method is one of the standard diagnostic tests for apoptotic cell death. However, accumulation of sufficient DNA fragments can take up to 24 h, as cells do not enter apoptosis synchronously [22]. In addition, the breakdown of DNA into 180 kb fragments represents a relatively late stage in apoptosis. In order to detect apoptosis in a shorter time frame, we decided to measure apoptosis by two more sensitive methods. Fragmentation of DNA assessed by 3′-end labelling [14] was detected within 2 h of Doppa treatment. In three separate experiments, the amount of radioactivity incorporated (kcpm/10^6 cell equivalents) into the DNA of 2 h Doppa treated cells (31.61 ± 1.51) was significantly more than in control cells (18.7 ± 1.47, $P \leqslant 0.01$). The 3′-end labelling of DNA

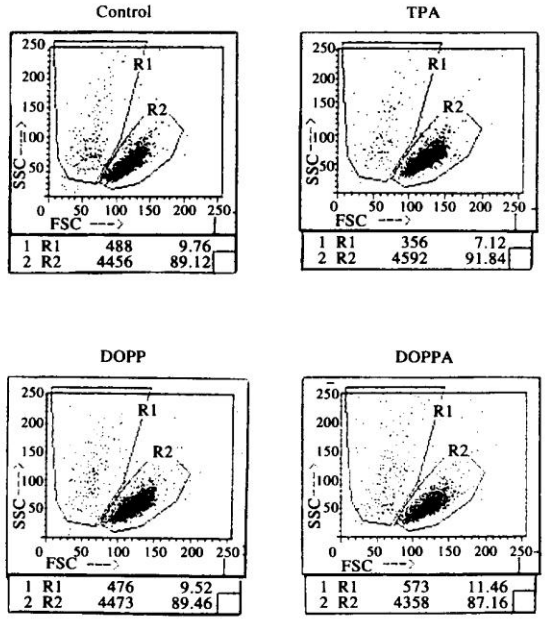

Fig. 3. The FACS analysis of apoptotic cells. The U937 cells were treated for 2 h with medium alone (control), 25 nM Doppa, 10 nM TPA or 100 nM Dopp. Cells were fixed and stained with propidium iodide prior to FACS analysis. Data are plotted for forward (fsc) and side (ssc) light scatter. Apoptotic cells were identified by their reduced fsc and ssc and enumerated. The regions of the plot identifying apoptotic cells and non-apoptotic cells are marked as R1 and R2, respectively. The plots shown are from a single experiment, representative of three performed.

from 2 h TPA treated cells (21.22 ± 2.84) was not significantly different from control cells. The FACS analysis of formaldehyde-fixed cells showed that the percentage of cells with an early apoptotic morphology in control cultures was 9.54 ± 0.46%. Following a 2 h treatment with 10 nM TPA, 25 nM Dopp or 25 nM Doppa, FACS analysis confirmed that only Doppa caused an early increase in apoptosis. In three separate experiments, the percentage of cells in the apoptotic region (R1) of the FACS plot was 8.25 ± 0.74% for TPA, 9.65 ± 0.7% for Dopp and 11.24 ± 0.35% ($P < 0.05$) for Doppa. Although the actual increase in the number of apoptotic cells is small (Fig. 3) with Doppa treatment at this early time point, Fig. 1 clearly shows that substantial apoptosis develops in Doppa treated cultures. Taken together, these data show that TPA and Dopp can induce differentiation in U937 cells, but they do not initially increase apoptosis. Apoptosis, seen with these agents in longer term treated cultures, is likely to be a consequence of differentiation [23]. In contrast, Doppa was able to induce significant DNA fragmentation without differentiation.

Modulation of PKC isoenzymes by Doppa, Dopp and TPA

The major isoenzymes present in U937 cells were PKC-α, -βI, -δ and -ζ. Figure 4 shows a single Western blot which is representative of three performed. Within 2 min, 25 nM Doppa caused a translocation of PKC-βI to the membrane fraction, which was maintained after 1 h. No other isoenzymes were activated after 2 min but, by 1 h, PKC-α and -δ were also translocated with Doppa treatment. There was no significant down-regulation of PKC immunoreactivity after 2 min treatments with any of the phorbol esters (data not shown) and no down-regulation of PKC-βI by 1 h of Doppa treatment (Fig. 5). Total PKC-α was decreased to 65.3 ± 4.5% ($P \leq 0.05$) of control after 1 h of Doppa treatment. Although PKC-δ was reduced after 1 h of Doppa treatment, the change was not significant (Fig. 4). In contrast, 10 nM TPA and 25 nM Dopp caused translocations of PKC-α, -βI and -δ (Fig. 4) by 2 min of treatment. Significant down-regulation of -α, -βI and -δ isoenzymes was detected by 1 h with TPA and down-regulation by Dopp was significant for PKC-α and -δ by 1 h (Fig. 5). None of the

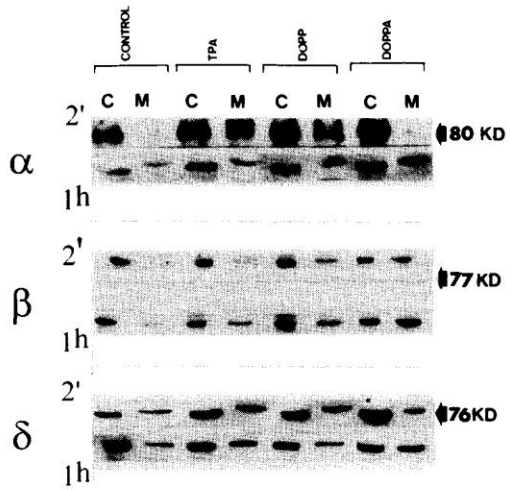

Fig. 4. Activation of PKC isoenzymes by TPA, Dopp and Doppa. The U937 cells were treated with medium alone (control), 10 nM TPA, 25 nM Dopp or 25 nM Doppa for 2 min and 1 h prior to extraction of PKC from cytosol (C) and membrane (M) fractions. Extracts were separated on SDS gels and PKC isoenzymes were detected by Western blotting with isoenzyme specific antibodies. Immunoreactivity was visualized by enhanced chemiluminescence and the autoradiographs shown are from a single experiment, representative of three performed.

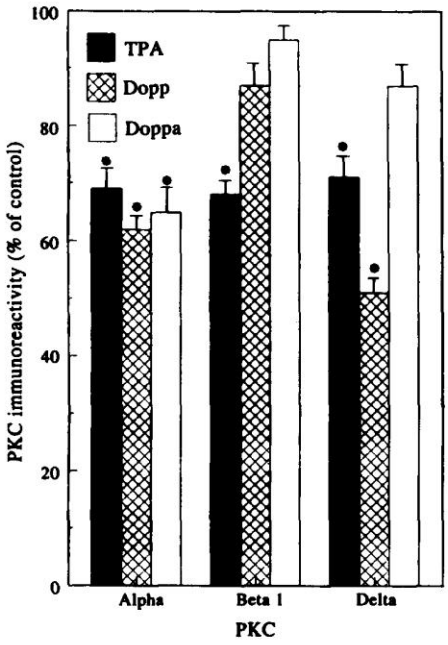

Fig. 5. Down-regulation of PKC isoenzymes by TPA, Dopp and Doppa. The U937 cells were incubated with 10 nM TPA, 25 nM Dopp or 25 nM Doppa for 60 min. The PKC isoenzyme content of cell extracts was determined by Western blotting using PKC isoenzyme specific antibodies. Immunoreactivity was visualized by enhanced chemiluminescence, quantitated by scanning densitometry and expressed as a percentage of the control immunoreactivity. Data shown are mean ±S.D. and significant differences between means ($P \leq 0.05$) are marked by an asterisk (*).

phorbol esters effected a translocation of PKC-ζ over the time course tested (data not shown). This observation would be expected, as this isoenzyme does not contain a phorbol ester binding domain [24].

Sub-cellular distribution of PKC-βI

Confocal microscopy revealed that the sub-cellular disposition of PKC-βI was different in cells treated with 25 nM Doppa or 10 nM TPA. Immunoreactivity was distributed throughout the cell in untreated U937 cells (Fig. 6, panel A) but was predominantly nuclear following 2 min Doppa treatment (Fig. 6, panel C). After 1 h of Doppa treatment PKC-βI was located predominantly in the cytosol (Fig. 6, panel D). In contrast, TPA caused a rapid evacuation of nuclear PKC-βI, which was detected after 2 min (data not shown) and was maximal by 10 min (Fig. 6, panel B). Confocal microscopy also confirmed that down-regulation of PKC-βI was a feature of treatment with TPA rather than Doppa (Fig. 6, panels B and D).

Discussion

In this study we have examined the role of PKC isoenzymes in the induction of differentiation and apoptosis in the promonocytic cell line U937, using phorbol esters with differing abilities to activate PKC isoenzymes *in vitro*. The compound TPA activates all isoenzymes except for the atypical PKCs (ζ, λ, μ) and Doppa is PKC-βI specific *in vitro* [8]. Doppa can be converted within cells to Dopp, by hydrolysis of the C-20 acetate group. Dopp is not PKC-βI specific and we have used this phorbol ester to assess the effect of any possible Doppa hydrolysis.

Several authors have clearly demonstrated that the expression of PKC-β is required for the differentiation of HL60 cells into macrophage-like cells induced by TPA [7, 25], with PKC-βI or -βII being equally effective [25]. However, a recent report has further suggested that the activation of PKC-β is sufficient for both the induction of differentiation and apoptosis in promyeloid HL60 cells [9]. Our data would indicate that activation of this isoenzyme is not sufficient for both of these events in U937 cells. Although caution is required when comparing results from two different cell lines, many of our data indicate that such comparisons may be valid between U937 and HL60 cells. Whilst U937 and HL60 are distinct cell lines, they are both induced along the macrophage lineage by TPA and express similar PKC isoenzymes [26]. We show here that the ability of phorbol esters TPA, Dopp and Doppa to induce differentiation of U937 cells is similar to that reported for HL60 cells [21].

In these studies, only TPA and Dopp were effective inducers of differentiation in U937 cells; TPA and Dopp also induced some apoptosis at the higher concentrations used. However, the apoptosis induced by TPA and Dopp appeared to be subsequent to differentiation, as apoptosis could not be detected at earlier time points with these agents. In effect, these agents may delay apoptosis by first inducing differentiation towards monocytes. In contrast, Doppa induced a sub-maximal expression of Cathepsin B activity, but only at a high concentration, i.e. 100 nM. However, Doppa effectively induced DNA fragmentation at concentrations well below 100 nM and in the absence of differentiation.

The proposal that activation of PKC-β was sufficient for differentiation of promyeloid HL60 cells was based upon the ability of Doppa (50–200 nM) to induce monocyte differentiation and apoptosis in promyeloid HL60 cells [7, 9]. The authors used Doppa as a PKC-β specific agonist *in vivo*, based upon its selectivity for PKC-βI *in vitro* [8]. There is now evidence that Doppa may not be PKC-β specific *in vivo* [10, 11] at the concentrations used by Macfarlane and Manzel. Moreover, the differentiation induced by Doppa in these

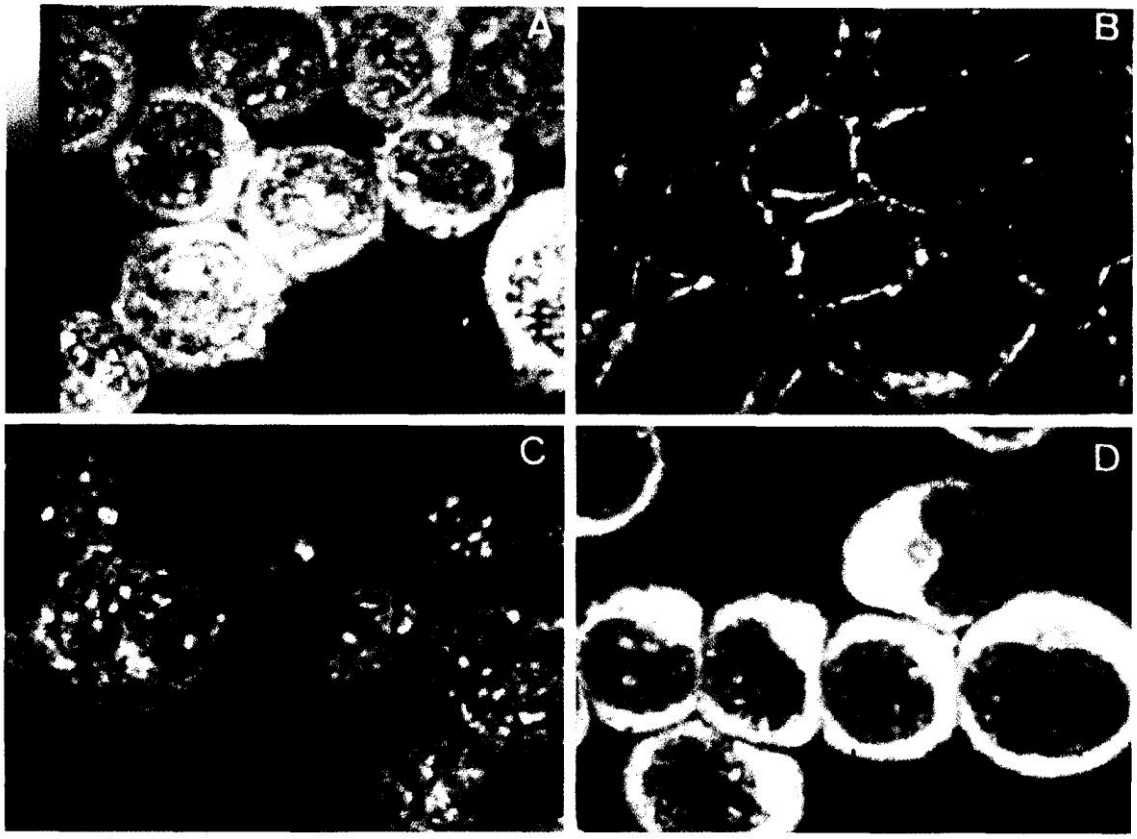

Fig. 6. Sub-cellular translocation of PKC-βI with TPA or Doppa. The U937 cells were treated with medium alone (A), 10 nM TPA for 10 min (B), or 25 nM Doppa for 2 min (C) and 1 h (D). Cells were indirectly immunostained with an antibody to PKC-βI and fluorescence staining imaged by laser scanning confocal microscopy. Fluorescence intensity is depicted using pseudo-colour, with a non-linear scale (shown top left), where white represents the most intense fluorescence and dark brown the least intense.

studies may be due to its conversion to Dopp, during the long treatment period required for differentiation. We have used a range of concentrations of Doppa and show that at higher concentrations, i.e. 100 nM, at which Doppa is not PKC-β specific *in vivo* [10], Doppa was able to induce partial U937 cell differentiation. We propose that conversion to Dopp also could account for this effect, as lower doses of Dopp were found to be less effective for induction of differentiation. In contrast, at low concentrations, Doppa was most effective for induction of apoptosis and could not induce monocyte differentiation. At these lower concentrations of Doppa, we found that PKC-βI was the only PKC isoenzyme translocated within 2 min. Although activation of other isoenzymes, i.e. PKC-α and -δ, was detected after 1 h, this result is not surprising and could be expected 1 h after the initiation of a signalling cascade. Thus, we feel that it may be more correct to say that PKC-βI shows the highest affinity for Doppa *in vivo*, with the other isoenzymes requiring higher concentrations for activation. Such differential sensitivity of PKC isoenzymes to lipid co-factors has already been demonstrated (reviewed in [26]). Thus, the initial and rapid activation of PKC-βI may be sufficient for apoptosis but not for differentiation of U937 cells. This conclusion is in accordance with other studies that have shown the involvement of several PKC isoenzymes in cell differentiation [2, 27, 28].

The data reported here also suggest that the down-regulation of PKC is associated with differentiation rather than apoptosis. Other authors have already shown that loss of specific PKC isoenzymes is as important as their differential activation [29]. Clemens *et al.* [2], have reviewed the extensive literature concerning PKC, proliferation and differentiation. They concluded that stimulation of PKC leading to down-regulation was associated with growth arrest and/or differentiation and that stimulation of PKC without extensive down-regulation promoted a mitogenic response. In our studies, TPA was a potent inducer of differentiation and caused a rapid loss of PKC-α, -βI and -δ. Doppa (< 100 nM) was a poor inducer of differentiation and

had not down-regulated PKC-β1 or -δ significantly by 1 h of treatment. Interestingly, Dopp was a less potent inducer of differentiation than TPA and a notable difference in their modulation of PKC isoenzymes was the reduced ability of Dopp to down-regulate PKC-β1 (a 13% reduction with 25 nM Dopp, compared with 37% for 10 nM TPA after 1 h). These data therefore also imply that maintenance of PKC-β1 and -δ expression, following activation, may be required for apoptosis.

Finally, the sub-cellular location of PKC-β1 was differentially modified in cells induced to differentiate with TPA, or apoptose with Doppa. It has already been suggested that decreased levels of nuclear PKC are associated with differentiation [2]. The rapid loss of nuclear PKC-β1 with TPA in this study would support this hypothesis. Whether the rapid nuclear translocation of PKC-β1 upon Doppa treatment results in the modulation of nuclear proteins involved in apoptosis is one possibility. Fields and co-workers [30] have recently shown that PKC-βII is a mitotic lamin kinase, causing phosphorylation of lamin B and disassembly of the nuclear lamina prior to mitosis. These studies did not determine the ability of other PKC isoenzymes to phosphorylate lamin B. As PKC-β1 and -βII are alternate splice forms of the PKC-β gene, it is possible that lamin B is also a substrate for PKC-β1. We are now investigating this possibility as nuclear lamina disassembly is also a pre-requisite for apoptosis [31]. The nuclear lamins may therefore represent early targets for PKC-β1 activated by Doppa. Identification of substrates for PKC-β1 in U937 cells induced to apoptose is a priority for future research.

Finally, the role of other PKC isoenzymes must also be considered, notably PKC-δ which was activated following 1 h of Doppa treatment and was also poorly down-regulated by this agent. Clearly, PKC is only one element in a signalling cascade regulating entry into apoptosis and the PKC isoenzymes involved may be cell type specific. Iwata *et al.* have recently shown that the activation of PKC-ε is involved in glucocorticoid-induced apoptosis in immature thymocytes [32]. Thus, identification of the PKC isoenzymes involved in apoptosis and their substrates will increase our understanding of apoptosis and may help to target sites for therapeutic intervention.

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The Polyether Bistratene A Activates Protein Kinase C–δ and Induces Growth Arrest in HL60 Cells

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Bistratene A (BisA) induced growth arrest in G2/M in HL60 cells. In addition, BisA-treated cells (50nM for 48 h) became adherent and expressed the adhesion molecule CD11c, but did not express the monocyte enzyme α-napthyl acetate esterase or phagocytose complement coated yeasts. BisA activated protein kinase C (PKC)-δ and induced translocation of PKC-δ to the nucleus. This suggests that activation of PKC-δ can induce growth arrest and cell adhesion, but is insufficient to mediate full differentiation of HL60 cells. BisA has potential as a new probe for determining the function of PKC isoenzymes, specifically PKC-δ.

Protein kinase C (PKC) is a serine/threonine kinase which play a role in the regulation of cell proliferation and differentiation (1) and cell morphology (2). The 12 known isoenzymes of PKC differ in their tissue and sub-cellular distribution, requirements for co-factors and substrate specificity (3). They are thought to play specific roles in the regulation of cell function (4). To date, few of the functions of individual PKC isoenzymes have been determined, in part due to a lack of suitable molecular or pharmacological probes. Compounds that can selectively activate (5) or inhibit (6, 7) PKC isoenzymes are now being identified.

Bistratene A (Bis A) is a polyether isolated from the ascidian *Lissoclinum bistratum* (8). Bis A induces cytostasis and incomplete differentiation of promyeloid HL60 cells towards monocytes/macrophages (9). Cells are arrested at the G2/M phase of the cell cycle (10) by Bis A treatment. Bis A enhanced the activity of type 11 PKC fraction from bovine spleen (9), this crude fraction of PKC is likely to have contained more than one PKC isoenzyme (11). These studies were performed to determine which PKC isoenzymes are activated by Bis A and may be mediating its effects on cell proliferation and differentiation.

MATERIALS AND METHODS

Bis A was isolated from *L. bistratum* collected at Heron Island Reef, Australia, as described previously (8). Antisera to PKC isoenzymes α, β1, β11, δ, and η, were raised in sheep, against peptides unique to the individual PKC isoenzymes (The Binding Site Ltd, Birmingham, U.K.). The anti-sera to PKC-ε and ζ were also raised against isoenzyme specific peptides, in rabbits (Dr J Ransom, Syntex Research, USA). All antibodies were affinity purified and precipitated or immunoblotted a band of appropriate molecular size, which could be ablated by co-incubation with the immunogenic peptide. The same antisera were used for indirect immunostaining. Staining could be titred out to an end point and was removed by co-incubation with the immunogenic peptide.

Assays for Monocyte Differentiation

Promyeloid HL60 cells were treated with 10–100 nM Bis A, after 48h cell adherence was determined after gently shaking culture flasks and removing non-adherent cells. The expression of CD11c was determined by indirect immunostaining (antibody provided by DR N Ling, Birmingham University). Expression of α-napthyl acetate esterase (ANAE) was determined on cytospin preparations of HL60 cells (12) and phagocytosis was assessed by the ability of cells to ingest complement-coated yeasts (13). FACS analysis was used to determine cell cycle status in HL60 cells treated with 50 nM Bis A.

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PKC Isoenzyme Activation Studies

Activation of PKC isoenzymes in vitro. PKC was extracted from 2×10^8 HL60 cells and fractionated as described (14). Alternate 1ml fractions eluted from the hap column were assayed for PKC activity by incorporation of [^{32}P]-ATP (ICN) into histone 111-S or a synthetic PKC peptide substrate MBP_{4-14} (Amersham), using a micro-titre plate method (15). To assess activation of PKC by Bis A, DAG was replaced by 50 nM Bis A. To determine the isoenzyme content of each hap fraction, HL60 cells were cultured for 3 days in medium containing 5μCi/ml ^{3}H-Lysine (Amersham). PKC was extracted and purified as described above. An aliquot of each fraction from the hap column was immunoprecipitated using PKC isoenzyme specific antisera and radioactivity of immunocomplexes determined by liquid scintillation counting. Radioactivity precipitated non-specifically, in the presence of antibody and immunogenic peptide, was subtracted to give values for specific isoenzyme precipitation.

Translocation of PKC isoenzymes. HL60 cells were treated for up to 10 minutes with 50 nM Bis A. Cytospin preparations were fixed in acetone and stained with primary antibodies to PKC-α, β1, β11, δ, η, ϵ, and ζ (1:15) and appropriate fluorescein-conjugated second antibodies (1:40, The Binding Site Ltd). Nuclei were counterstained with propidium iodide and fading of fluorescence was retarded by 2.4% DABCO (1,4 diazobicyclo (2, 2, 2) octane) in 80% glycerol (16). In Western blotting studies, HL60 cells were lysed and cytosol and membrane fractions extracted directly into SDS-sample buffer. Protein extracts were electrophoresed on 10% SDS-PAGE gels and blotted onto PVDF membrane (Millipore). Blots were probed with primary anti-PKC antibodies (1 μg/ml) and HRP-conjugated anti-rabbit IgG or anti-sheep IgG secondary antibody (1:10,000). Immunoreactive bands were visualised by enhanced chemiluminescence (Amersham).

Autophosphorylation of PKC isoenzymes. Autophosphorylation of PKC isoenzymes was determined as a measure of activation, essentially as described previously (17). HL60 cells were incubated for 4h in medium containing 200 μCi/ml [^{32}P]orthophosphate prior to treatment with 50nM Bis A. Cells were then lysed and PKC immunoprecipitated with individual anti-PKC isoenzyme antibodies (The Binding Site). Immunoprecipitates were electrophoresed on 8% SDS-PAGE gels. Gels were dried, prior to autoradiography at −70°C using pre-flashed X-ray film (MAS Stirling, Scotland, UK).

RESULTS

HL60 Cell Growth and Differentiation

50 nM Bis A produced a rapid and sustained growth arrest of HL60 cells (Fig. 1A), with the majority of cells arrested in G2/M (Fig. 1B). The effect of Bis A on cell growth was dose-dependent. Bis A treatment increased adherence of HL60 cells to tissue culture flasks, from 3.4 ± 0.2% in control to 92.7 ± 5.2% and induced expression of the cell surface adhesion molecule CD11c ($\alpha_x\beta_2$ integrin) in the majority (73.5 ± 4.9%) of treated cells. The fraction of HL60 cells able to phagocytose complement coated yeasts was not significantly different in control (3.9 ± 0.5%) and Bis A-treated cultures (5.2 ± 0.8%). Bis A-treated HL60 cells did not stain positive for the monocyte enzyme ANAE (data not shown).

In Vitro PKC Activation by Bis A

PKC extracted from HL60 cells eluted as four main fractions from hap columns (Fig. 2A), using DAG as co-factor. With 50 nM Bis A replacing DAG, only one sharp peak of activity was detected using synthetic peptide MBP_{4-24} (Fig. 2B) or histone as substrate (data not shown). This region contained PKC-δ (Fig. 2C) with only minor co-elution with PKC-β isoenzymes.

PKC Isoenzymes Activation in Whole Cells

50 nM Bis A induced a rapid translocation of PKC-δ from the cytosol (c) to the membrane (m) fraction, with no effect on other PKC isoenzymes in HL60 cells (Fig. 3). Indirect immunostaining (Fig. 4) showed that PKC-δ was present in both the cytosol and nucleus of control cells (left panel), after a 10 min treatment with 50nM Bis A, PKC-δ immunofluorescence was patchy and localised to the nuclear and perinuclear regions (right panel). Bis A did not affect the distribution of other PKCs (data not shown). Treatment of cells for 10 min with 50nM Bis A increased the phosphorylation of PKC-δ, with no effect on the other PKC isoenzymes tested (Fig. 5A). Scanning densitometry confirmed that PKC-δ was the only isoenzyme with an increased phosphorylation status following Bis A treatment of HL60 cells (Fig. 5B). Antisera to PKC-ϵ and ζ did not precipitate a labelled 80kDa band.

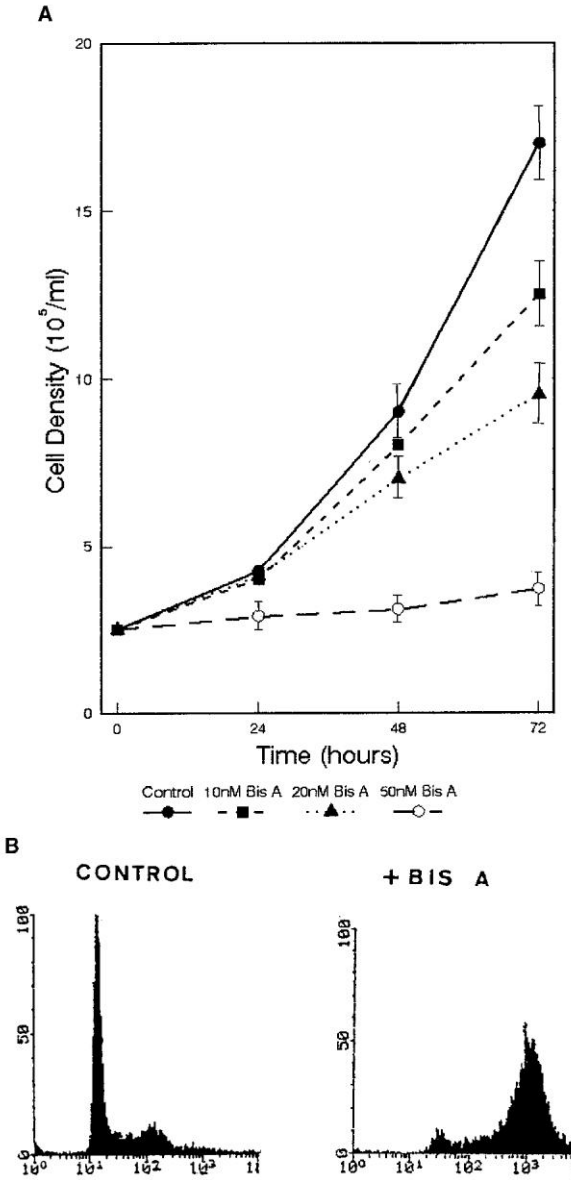

FIG. 1. Growth arrest induced by Bis A treatment of HL60 cells. (A) HL60 cells were incubated for up to 72h in medium alone or medium containing 10–50 nM Bis A. Cells were enumerated at the time intervals shown. Data are means ± s.e. of three experiments. (B) FACS analysis of control and 48h, 50 nM Bis A treated HL60 cells.

DISCUSSION

We have shown previously that Bis A could enhance the activation of a crude preparation of PKC, type 11 PKC (9), but only at micromolar concentrations. Here we show that Bis A activated only PKC-δ in HL60 cells, assessed in vitro and in whole cell assays. Furthermore, Bis A induced a predominant movement of PKC-δ towards the nuclear region. The activation of PKC-δ occurred within the concentration range able to induce growth arrest and adherence of HL60 cells to tissue culture plates.

How the activation of PKC-δ by Bis A could effect growth arrest in G2/M is not known. Bis A

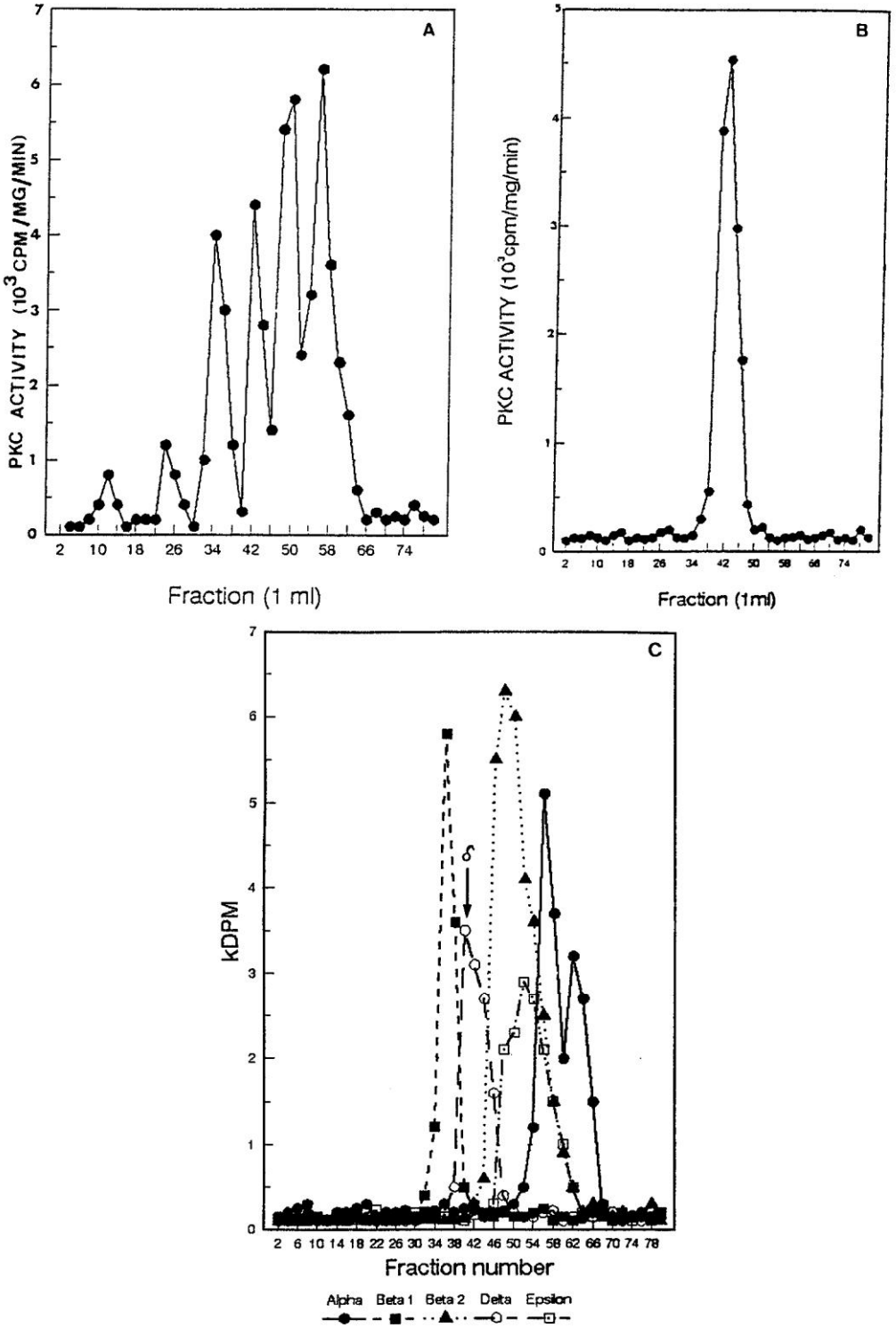

FIG. 2. Activation of purified PKC by Bis A. PKC was extracted from HL60 cells and alternate 1ml hap column fractions were assayed for PKC activity using either DAG (A) or 50 nM Bis A as co-factors (B), with histone-111s or a synthetic peptide MBP_{4-14}as substrates, respectively. (C) ^{3}H-labelled HL60 cells were extracted for PKC and hap fractions immunoprecipitated with anti-PKC isoenzyme antibodies, to reveal the elution position of individual PKC isoenzymes.

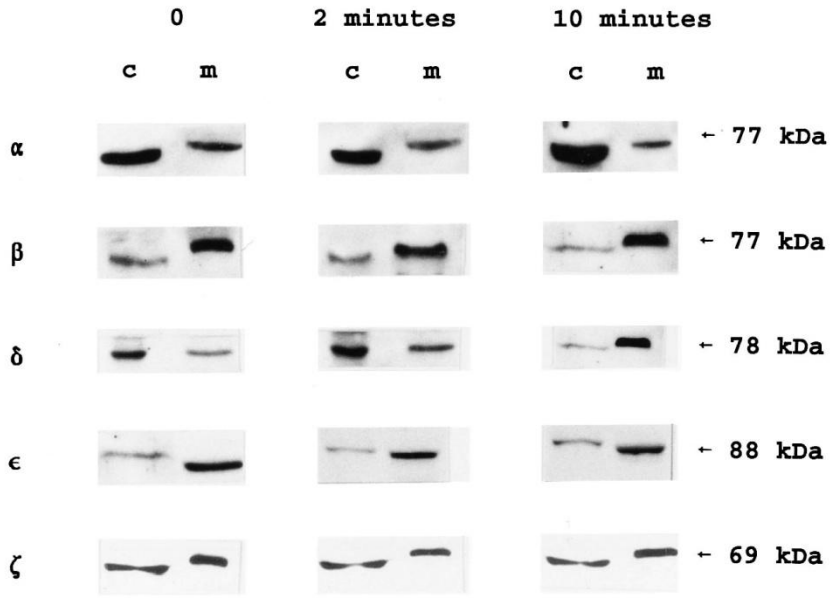

FIG. 3. PKC isoenzyme translocation induced by Bis A. HL60 cells were treated with 50 nM Bis A for 2 and 10 min and cytosol (c) and membrane (m) fractions screened for PKC isoenzyme content by Western blotting. The M_r of the precipitated bands, calculated from the position of reference standards, are shown.

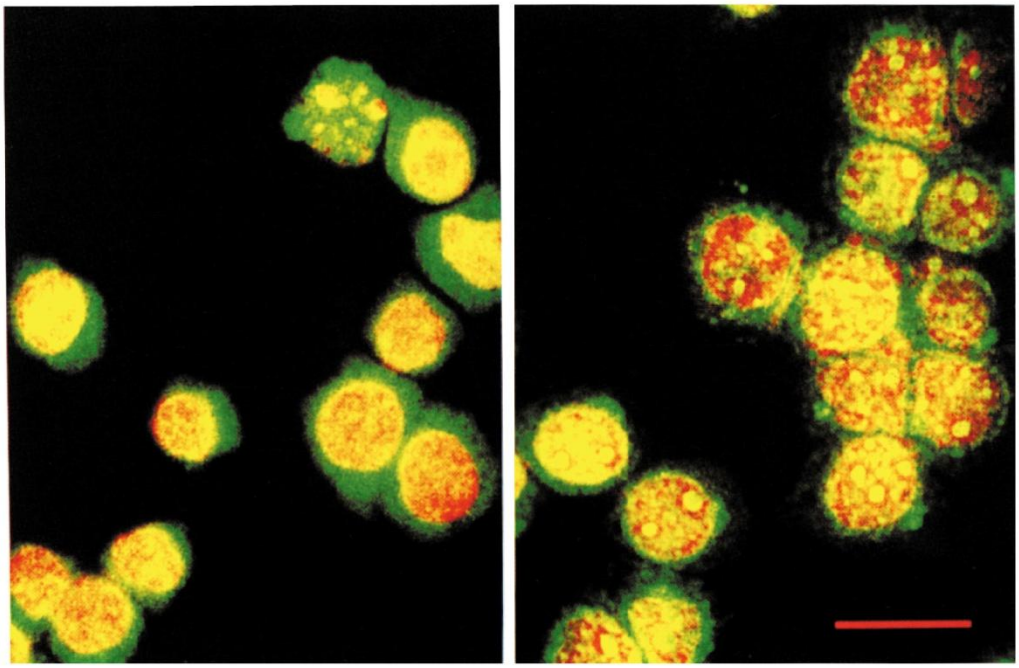

FIG. 4. Intracellular localisation of PKC-δ in Bis A treated HL60 cells. HL60 cells were incubated with medium alone (control) (left panel) or 50 nM Bis A (Bis A) (right panel) for 10 minutes prior to indirect immunostaining with anti-PKC-δ antibody. The secondary antibody was FITC-conjugated anti-sheep IgG and nuclei were counterstained with propidium iodide. Scale bar = 10 μm.

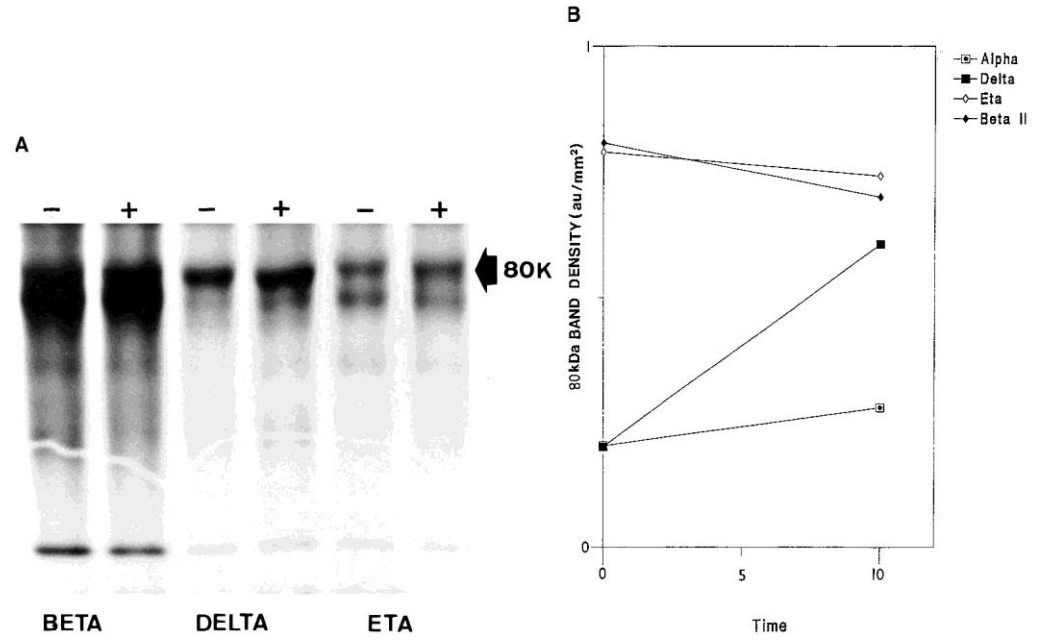

FIG. 5. Autophosphorylation of PKC isoenzymes following Bis A treatment of HL60 cells. HL60 cells were labelled with [^{32}P]orthophosphate prior to incubation with 50 nM Bis A for 10 minutes and immunoprecipitation with anti-PKC isoenzyme specific antibodies. Immunoprecipitates were separated on SDS-gels and ^{32}P-labelled PKC bands revealed using a phosphoimager (A). The mean density (au/mm^2) of PKC isoenzyme bands was determined in three separate experiments by scanning densitometry (B). Data are means ± s.d. Values significantly different from control ($P<0.05$), by Students t-test, are marked (*).

inhibits cytokinesis, but does not block DNA synthesis or nuclear division, leading to polyploidy and multinuclear cells (10). CHO cells overexpressing PKC-δ accumulate in G2/M in response to phorbol ester treatment (18), supporting a role for PKC-δ in normal cell cycle progression. Inhibition of cytokinesis may occur through phosphorylation of proteins involved in the regulation of cell cycle control or through modulation of the cytoskeleton. Whilst there is no evidence to suggest the phosphorylation of cdk's or cyclins by PKC in vivo, nuclear lamins have been shown to be substrates for PKC-β11 (19). The phosphorylation of nuclear lamins leads to their disassembly, which is required for the onset of mitosis. We have shown that PKC-δ associates with the intermediate filament (IF) protein vimentin, following activation by TPA (20). As nuclear lamins are also IF proteins, the translocation of PKC-δ to the nuclear and perinuclear region, following treatment with Bis A, may indicate association with nuclear lamins or other components of the cytoskeletal protein network.

With regard to adherence of Hl60 cells induced by Bis A, phosphorylation of cytoskeletal elements, including vimentin, by PKC-δ could mediate adherence tissue culture flasks. Activation of PKC-δ by Bis A also induced expression of the cell adhesion molecule CD11c or $\alpha_x\beta_2$ integrin, which would contribute to increased Hl60 cell adherence. Thus, Bis A appears to be a selective activator of PKC-δ and will be useful as a research tool in studies to clarify the specific involvement of PKC isoenzymes in cell function.

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Review

Tissue engineering and biotechnology in general thoracic surgery

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Summary

Public interest in the recent progress of tissue engineering, a special line of biotechnology, makes the current review on thoracic surgery highly relevant. In this article, techniques, materials and cellular processes are discussed alongside their potential applications in tissue repair. Different applications of tissue engineering in tracheo-bronchial replacement, lung tissue cultures and chest-wall reconstruction are also summarised in the article. Potential tissue engineering-based solutions for destructive, chronic lung-injury-related conditions and replacement of tubular structures in the central airways are also examined.

Keywords: Biotechnology; Tissue engineering; Lung surgery; Pulmonary regeneration; Trachea; Chest wall

1. Introduction

Tissue engineering is a branch of biotechnology and bedside medicine, a rapidly emerging chimera of two seemingly unrelated disciplines aiming to control pathologies through artificially facilitated tissue regenerative processes. At a first glance, this high-tech field has little to offer to surgery, an art where precise mechanical destruction simultaneously intends complete eradication of abnormal cell masses and reconstruction of derailed biological functions. In modern surgical practice, destruction and reconstruction are different sides of the same coin and they mutually depend on each other.

Replacing tissues or at least supporting certain biological functions has been the driving force behind the development of prostheses for lost extremities for a long time; however, mass production was only peaked during World War I. The case of Oscar Pistorius (the double-amputee Paralympic athlete) is a good example of the paradox that artificial limbs based on advanced biotechnology can be superior to the original ones, generating bizarre debates that overwrite sport rules. In addition to biomechanical targeted muscle prosthesis, iron lung and heart pumps are also examples of artificial organs developed by mechanically minded medics.

During the Cold War, pressure was on to discover ways for haemopoetic cell replacement. Research in the era of nuclear war threat has led to the discovery of human stem cells (SCs) [1] that provide the basis for current tissue engineering developments. Tissue engineering as a widely accepted interdisciplinary field is <20 years old and is generally accepted since the ground-breaking publication of Langer and Vacanti in 1993 [2]. Combined principles of engineering and biosciences are applied in order to create biological substitutes to restore, maintain or improve tissue and organ function.

Although self-regenerative capabilities of various organs have been exploited in medicine for decades, medical sciences have been working hard to accelerate the search for novel ways to direct tissue regeneration. Progress is often slow, as regenerative potentials, structural and functional requirements vary from organ to organ and range from the highly regenerative liver to the ominously resistant central nervous system [3]. Clinical research in tissue engineering is steadily advancing towards applications in operating theatres. This can be exemplified where biotechnologically engineered heart valves and injection of myocardial SCs, following myocardial infarction [4,5], are ever more frequently used in cardiac therapy. In addition, bone-marrow or lymphatic SC replacement in haemopoietic malignancies [6] are relatively easily solved problems, as functions of these tissues are not dependent on mechanical structures. Meanwhile, reconstruction of structurally and functionally intact distal airways, which are capable of gas exchange, is not anywhere near the level of expertise required for therapeutical applications [7]. Nevertheless, solid or mesh chest-wall prosthesis and interposition of artificially made vascular grafts are relatively simple tasks that have been implemented and show continuous

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progress in clinical applications. Tubular structures, such as trachea and blood vessels, which have to fulfil both mechanical and biological functions, have recently reached clinical application stages [8].

Certainly, there is an increasing need for communication between thoracic surgery and the interdisciplinary science of biotechnology to meet demands in the field of pulmonary tissue replacement and/or directed regeneration. This article, therefore, aims to summarise the basic components and principles of tissue engineering around the needs of general thoracic surgery.

2. Glossary and definitions

Cells, scaffolds and signalling are the three main pillars which tissue engineering rests on. The successful process depends on the cell culturing, scaffolds that provide space and mechanical support for cellular growth and controlled direct and indirect cell-to-cell contacts in the form of signalling [9].

However, not all of these factors are always seemingly required to achieve complex tissue engineering tasks, as often *in vivo* tissue microenvironments provide one or more of the missing factors for tissue engineering to become successful.

For example, 'cell masses' without supporting mechanical structure are used to fulfil certain functions in the host body during SC implantation in bone-marrow treatments [6] or myocardial cell injection in post-infarct regeneration [4,5]. Spleen islet cell replantations, following splenectomy or SC therapy after cartilage injury, are additional eminent examples. In these treatments, the surrounding tissue microenvironment provides both the necessary mechanical support and the secreted signalling molecules that direct cellular growth and differentiation [9].

Scaffold-only treatment provides support for migrating autologuous cells that can grow on the artificial support and provide the necessary signals to one another. Mesh application in hernia repair and vascular grafts are clinically proven and properly established techniques. Stents and stentgrafts are in fact also scaffolds that maintain calibres in tracheal position and also in the biliodigestive tract, coronary artery or in the aortic position. As vascularisation of the implanted scaffold has eminent importance [10], vascular grafting or directed growth of endothelial-cell components into engineered tissues has a very high priority in biotechnology.

Cells, scaffolds and tissue functions are controlled by 'cell-to-cell signals' in a tissue engineering process in different ways and proportions [9]. Taking a wider approach, even cancer therapies interfering with inter- and intracellular signalling mechanisms can be interpreted as tissue engineering, as these treatments often result in the inhibition of vascularisation and induce cellular differentiation or apoptotic cell death rearranging existing tissue masses.

2.1. Cells

Tissue cultures are essential for engineering tissues [5,9]. The source of these tissue cultures can originate from non-differentiated (SCs), partly differentiated (tissue-specific progenitor cells) or tissue-specific differentiated cell types [9,11].

2.1.1. SCs and tissue-specific progenitor cells

The fertilised oocyte is regarded as totipotent as it is able to form an entire organism like a human being. Embryonic SCs (ESCs) are pluripotent as they are able to form all three germ layers. Multipotent SCs are able to develop mesenchymal, endodermal or ectodermal tissues. Oligopotent SCs can create two or more different cell lines.

Based on their origin, there are two basic types of SCs: ESCs and adult stem cells (ASCs). Plasticity, a very important feature of SC, refers to the ability that cells of one tissue are able to generate cell types of another tissue, even trespassing the borders of ectodermal–endodermal–mesenchymal cell lineages. Adult SCs are rare, undifferentiated cells within differentiated tissues located in the so-called 'SC niches', an anatomical location that is far from simple to find *in vivo* [5,7,11]. Recent studies have located residual pools of ASCs within the basal layer of the upper airways, within or near pulmonary neuroendocrine cell crests, at the bronchoalveolar junction as well as within the alveolar epithelial surface. In addition, it has been suggested that airway submucosal glands (SMGs) could serve as a protective niche for adult epithelial stem/progenitor cells of the proximal airways [12].

The two basic abilities, the capacity to self-copy as well as form into various other cell types of the body, make SCs primary players in tissue engineering [13]. Obviously, pulmonary SC types are key to rapid repair of the denuded alveolar surface after injury and therefore to survival. The term 'progenitor cells' although requires further consensus, it is broadly used to categorise the less immature, more lineage-committed postnatal cells [14].

2.1.2. SCs and tissue-specific progenitor cells in tissue engineering

Theoretically, ESCs are superior to ASCs and tissue-specific progenitor cells as ESC following directed steps of tissue-specific differentiation can generate any organ-specific tissue culture material. Unfortunately, while the usage of ESCs is often strictly regulated, ASCs and tissue-specific progenitor cell types are only available in limited numbers, making the growth of a full organ in *in vitro* conditions an almost impossible task.

The source of SCs in tissue engineering applications is generally the bone marrow, umbilical cord blood, circulating blood and foetal or adult tissues. Respiratory organs require at least two populations of epithelial stem/progenitor cells to give rise to the lung anlage, comprising the laryngo-tracheal complex versus the distal lung below the first bronchial bifurcation [15]. From a pulmonary point of view, resident SCs have been identified both in the proximal and in the distal airways, including the alveolar regions [12,14,16]. Lung parenchyma, like the brain and the heart, has a low cellular turnover and, consequently, a low regenerative potential. Not surprisingly, reconstruction and mastered regeneration of pulmonary tissue is an extremely complex and difficult task. To be able to achieve the hierarchical organisation of cells in an *in vitro* grown lung, a better

understanding of cellular and molecular interactions that regulate epithelial maintenance and repair is essential.

Recent attempts, using a directed SC differentiation approach, provide some hope for *in vitro* growth of pulmonary tissues. Using murine ESCs, it was possible to differentiate alveolar type II cells by provision of a serum-free medium supplemented with growth factors, designed for *in vitro* maintenance of mature alveolar epithelial cells (AECs; small airway growth medium (SAGM)) [17]. Samadikuchaksaraei et al. also provide evidence for *in vitro* differentiation of alveolar type II epithelial cells from human ESC [16,17], where the level of differentiation was supported both by cobblestone-like light microscopic morphology and by electron microscopic morphology, in addition to surfactant protein C expression, characteristic of type II epithelium. Although understanding ASC- and progenitor-cell-derived processes would particularly be important, currently embryonic lung progenitor cells are much better defined and characterised than their adult counterparts [18]. Nevertheless, cellular sources for lung tissue regeneration *in vivo* or lung tissue engineering *in vitro* include endogenous pulmonary epithelial SCs, extrapulmonary circulating SCs and ESCs. The role and application of each of the above cell types in the future of pulmonary tissue engineering is still uncertain [19] and problematic [20], with both identification and isolation of ASCs remaining as unsolved problems in pulmonary tissue engineering. In addition, although there are some existing *in vivo* labelling methods for selection, currently it would take a pair of healthy lungs to homogenise and harvest to obtain a sufficient number of ASCs to start cell culturing with an aim of building up a functioning engineered lung.

2.1.3. Tissue-specific differentiated cell types

Cellular differentiation is the process by which a less specialised cell becomes a more specialised cell type. Differentiation dramatically changes a cell's size, shape, membrane potential, metabolic activity and responsiveness to signals. These changes are largely due to the highly controlled modifications in gene expressions. Differentiation is a common process in adult tissues as well as during embryonic development. For example, depending on demand during normal cell turnover or tissue repair following injury, ASCs can divide and create one stem and one fully differentiated daughter cell, or two differentiated daughter cells.

2.1.4. Tissue-specific differentiated cell types in tissue engineering

As full organs and experimental tissue models need to be created on a regular basis according to therapeutical or experimental requirements, tissue-specific differentiated cells potentially represent a better source for primary cell material, as they are more freely available in large numbers. Differentiated cell types in traditional, two-dimensional (2D; i.e., Petri dish) culture conditions normally de-differentiate and can be forced to re-differentiate using the right additives, scaffolds or soluble factors. However, to create the optimal culture conditions for a directed re-differentiation process, intimate knowledge of extra- and intracellular signalling pathways as well as of the main features of endothelial–mesenchymal transition (EMT) is essential [21].

2.2. Scaffolds

As all organs are essentially 3D, no attempt at reconstruction of their biological function can ignore this requirement. However, most of our knowledge regarding extracellular interactions comes from studies using *in vitro*, 2D tissue cultures. This is understandable as experimentations, manipulations, imaging, etc., are easier in 2D culture systems. An added third dimension, however, is not only a simple spatial extension. While 3D culture is an absolute requirement for engineering a functional tissue, 3D cultures also create additional problems, including difficulties of gas and nutrient exchange in the third tissue dimension. To circumvent this problem, scaffolds [3,7,8,10,22–32] are purposefully engineered in a way to make tissue build-up and allow cellular interactions, nutrients and gas exchange to occur.

The type of the scaffold greatly varies, depending on the type of tissue we aim to create.

From a tissue engineer's point of view, the main groups of organs are the parenchyma (forming one's muscle and brain) or the tissue around a lumen (vessels, trachea and bowel) or the combination of the two (lung, heart and kidney). The size of a lumen is a crucial element, irrespective of function in subendothelial structures of the organ in question. Although scaffolds greatly vary, they share a number of basic physical properties that depend on the intended application. Mechanical support, for example, has priority in bone replacement, while in other tissues scaffolds provide only a temporary structure for cell populations to grow on until they reach and stabilise the third dimension. Such biodegradable scaffolds (polyglycolic or polylactic acids, or natural materials, including Gelfoam (porcine skin gelatin) sponges, collagen and Engelbert–Holms tumour basement membrane [7]) gradually degrade and the natural scaffold of extracellular matrix (ECM) takes over its function.

Despite the available scaffolds and the choice of cell types, creating a functional lung is still an unsolved problem. The human alveolar surface equals half of a volleyball field, that is compressed to the size of the thorax and capable of 250 ml min^{-1} oxygen and 265 ml min^{-1} carbon-dioxide transfers on an $\sim$2.8 m^3 surface area in adults. Such a large, easily compressible surface is difficult to find. So far, silicone or traditional degradable materials have been used to provide the skeleton surface for the blood–gas interface. Functional gas exchange, however, requires vascularisation of the complex lung parenchyma, which is currently still a challenge [10,33]. The directed growth of the vasculature requires endothelium, smooth muscle cells, secreted signalling molecules and ECM; therefore, creating the vasculature is just as difficult as growing the rest of the distal pulmonary tissue. Recent theories even look at the alveolar surface of the airways as a certain scaffold, mimicking vascular seeding on the basis of the natural process of haematogen metastasis or embedding of a Koch bacillus. Either way, as soon as vascular scaffolds are developed, a new generation of vascularised pulmonary tissue constructs can herald a new era in pulmonary tissue generation.

Meanwhile, tracheal scaffolds are made of either biodegradable polymers [23,24] or non-absorbable materials. De-cellularised grafts, made of the natural matrix of trachea or aorta, are also subjects of intense research [27–30]. Experimental studies of tracheal prosthesis have used pored, polyethylene terephthalate (Dacron) tubes [25] or titanium stents combined with either polyglactin, or a copolymer of lactide and caprolactone reinforced by polyglyceride fibres or a knitted mesh [26]. There are also successful applications of composite materials as possible scaffolds, such as polytetrafluoroethylene (PTFE). These hybrid scaffolds have been able to provide functional artificial tracheas in canine experiments with an overall survival >3 years [31,32].

2.3. Signalling

Both embryonic development and adult tissue homeostasis are controlled by intracellular signal transduction pathways that are activated or inhibited by cell-to-cell interactions and secreted factors. In the past, signal transduction was regarded as a linear process, but recent data from large-scale and high-throughput experiments revealed the existence of an extensive and complex inter- and intracellular signalling network [34]. In addition to direct cell-to-cell contacts, continuous reciprocal cellular interactions are maintained by secreted factors or ligands. Surprisingly, only a few ligand families are responsible for most cellular communications during tissue development and regeneration. These include bone morphogenic protein (BMP), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factor β (TGFβ), Hedgehog (Hh), Notch and the Wnt family of glyco-lipoproteins [21,34]. Fig. 1 summarises these pathways. Combinations of the above factors can activate downstream intracellular signalling pathways that control receptor and ligand expressions, leading to ultimate changes in cellular functions. These signalling networks are equally important during embryonic development, normal tissue regeneration processes and injury repair or wound healing following surgery [3,9,14].

Cellular interactions in the pulmonary system provide a good example for interactions amongst the main signalling pathways. Embryonic lung development, as well as epithelial injury repair, is tightly coordinated by a fine balance between stimulatory versus inhibitory genes that code proteins to co-regulate the function of SCs and adult progenitor cells in the lung. For example, *FGF* receptor tyrosine kinase signalling is essential for respiratory organogenesis and is negatively regulated by a family of inducible FGF pathway inhibitors [35]. In addition, FGF signalling is required for formation of new alveoli, protection of AECs from injury as well as for migration and proliferation of alveolar progenitor (stem) cells during lung repair.

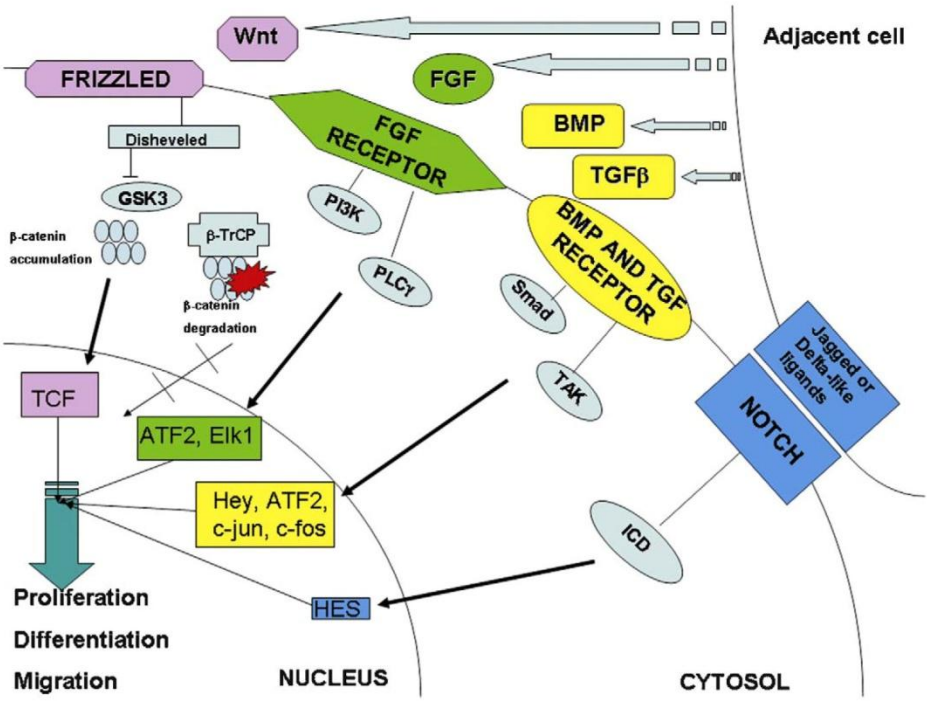

Fig. 1. Activation of the main signalling pathways regulating lung development and regeneration. Wnt, FGF, BMP, and TGF-β are secreted ligands with specific receptors and well characterised intracellular signalling pathways within the recipient cell. Activation of signalling pathways following ligand binding leads transcription factor activation that leads to specific gene transcription. For the Notch pathway activation, an adjacent cell expressing the ligands and another cell expressing the Notch receptor has to come into contact. Binding of ligand to receptor initiates a series of cleavage processes, leading to the liberation of the Notch intracellular domain (ICD). This Notch fragment migrates to the nucleus, and together with a transcriptional coactivator initiates transcription of the HES and HEY family of transcriptional repressors, which mediate Notch signalling.

Conversely, *TGFβ* receptor serine–threonine kinase signalling inhibits lung morphogenesis and can inhibit postnatal alveolar development, while excessive TGFβ signalling causes interstitial fibrosis. Similarly, while *Wnt-s* are essential in embryonic development and maintenance of the ASC pool in the pulmonary tissue, dysregulation of Wnt signalling cascades can result in cancer, fibrosis and persistent inflammation [21].

2.3.1. Branching and budding morphogenesis

The above-mentioned factors and their receptors are expressed during reciprocal molecular interactions between epithelium and mesenchyme [36]. FGFs, for example, bind to and activate four tyrosine kinase receptors (FGFRs) to regulate intracellular signalling pathways controlling cell proliferation, differentiation and migration. While one of the FGF receptors is expressed in epithelial tissues and is activated by mesenchymally expressed ligands, another receptor (FGFR2c) is expressed primarily in mesenchyme, and is activated by FGF ligands expressed in adjacent epithelia [37]. In the developing lung, FGF9 and FGF10 form an especially tight reciprocal pair of ligands that regulate branching and budding morphogenesis.

2.3.2. Vascularisation

Vascularisation during normal lung development is another critical step for successful survival at birth and in postnatal life. For normal gas exchange, the growth and maintenance of an intricate system of airways and vessels, including the thin yet vast blood–gas interface, are essential [38,39]. Observations on mechanisms that regulate development of the pulmonary circulation revealed that lung vasculature actively promotes normal alveolar growth during development and contributes to the maintenance of alveolar structures throughout postnatal life. Furthermore, increasing evidence suggests that the disruption of angiogenesis during lung development can impair alveolarisation [40,41,42].

To form blood vessels, the remarkably heterogenic endothelial cells of the lung require the presence of vascular endothelial growth factor (VEGF) and their specific receptors. As VEGF is a well-known target gene of the Wnt family [21] of secreted ligand proteins, it demonstrates how the complex network of inter- and intracellular signalling molecules is important in all aspects of pulmonary development and regeneration.

As a functional 3D lung model would require a continuous blood flow and the supplying network of vasculature, avascular seeding of cells on a scaffold is not a viable solution for an engineered lung despite working for many fields in tissue engineering [33].

2.4. Cells, scaffolds and signalling in the practice of tissue engineering

Implementation of the accumulated knowledge about the cell–scaffold–signalling network triad into clinical practice is possible through five potential ways. All potential utilisation can be categorised into one of three general strategies stated by Langer and Vacanti in 1993 [2], that is, *in vitro*, *in vivo* or hybrid as the combination of the previous two methods.

First, the artificially built scaffolds can be implanted into the body exposing it to autologous cells and the natural repair and regeneration processes of the host. Vascular prosthesis, hernia meshes, artificial pericardium, foams, films covering pleural dehiscences and chest-wall replacements fall into this category.

Second, where function dominates over mechanical structures, the original dysfunctional organ can be duplicated by implantation of the new structure into a different place of the body. (Extra transplant kidney and a 'piggyback' heart to support the function of the original organ are good examples.) In an ideal scenario, a transient function relief is achievable, resulting in regained function by the original organ. (This application is not unknown for liver transplants and during heart failure.) [43]

The third potential method is where the engineered organ is maintained operational *ex vivo*. Extracorporeal membrane oxygenation (ECMO) is a good example where a biologically built structure maintains function in a different body. This bypassing function is similar to what economics refers to as outsourcing. Bio-ECMO or engineered artificial kidney would make anti-thrombotic measures unnecessary and would be free of other damages caused by mechanical tools [44].

The fourth option is when the tissue or the organ to be implemented can be fully built up *ex vivo* in a bioreactor [45] and, when it is functional, then implanted. This was how tissue-engineered skin and heart valves or the very first clinically applied tissue-engineered central airway was created and applied [8]. However, building a fully functional lung under laboratory conditions is not anywhere near realisation.

Finally, the fifth way is to generate the tissue within the host organism itself from the very beginning. SC therapy of lymphoproliferative diseases is an established method of challenging a permanent interaction between the graft and the host [46]. Theoretically, in case of more complex organs, an implanted micro-size tissue can be followed by a controlled growth to full size under the supervision of the surrounding tissue microenvironment and additionally administered growth and differentiation factors.

Whatever method is chosen, implantation is an extremely complex process as the host and the graft are in permanent and active interaction not without dangers, which requires continuous monitoring.

3. General thoracic surgical applications of tissue engineering

The present stage of leading-edge research in airway tissue engineering can be categorised according to anatomical locations: trachea [8,23–33,47,48], bronchial tree [49], lung tissue [7,11,12,14–17,50] and chest wall [22,51]. The construction of lung parenchyma is distinguished according to proximal or distal airways. From the point of view of a tissue engineer, the vascular bed of the lung parenchyma and its connective tissue network require the same approach, while the pleural surface and the external air barrier also constitute an issue impossible to neglect and

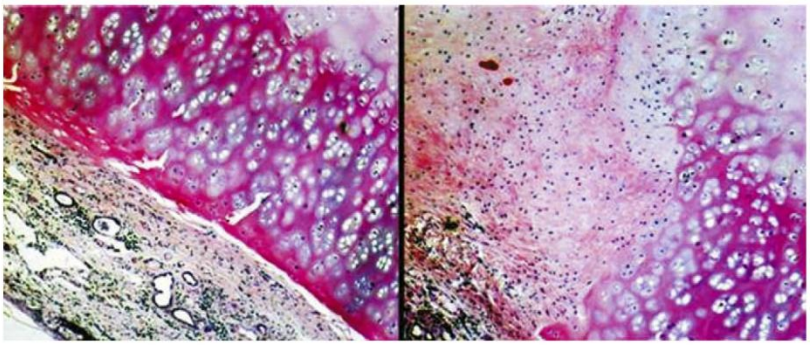

Fig. 2. Neochondrium, generated by chondrocytes around the polytetrafluoroethylene (PTFE) ringed vascular prosthesis matrix in neotrachea position in dog survived over 3 years (animal studies detailed in References [31,32]). Haematoxylin–eosin dying, amplification: 100×.

must be solved, if functional lung tissue is ever going to be engineered *in vitro*.

3.1. Trachea and central bronchial tree

The windpipe represents an old challenge to modern thoracic surgery, as the length of the trachea limits the resection and biology makes allo-replacement impossible [52]. As early as the 1950s, Belsey laid down the basics for proper trachea reconstruction [53]. Presently, we know that air tightness, lateral rigidity, vertical flexibility and anastomosis healing have priority over immediate build-up of mucosal integrity [32]. The importance of geometrical relation of the anastomosed edges was recently revealed, highlighting the combination of biological and purely mechanical factors in the success. So far, telescopic immersion of the neotrachea provided the best long-term results [31].

Two incidental and completely unexpected observations [27,31] of chondrocytes in and around the neotrachea (Fig. 2) can now be explained by the existence of SCs in the region [44]. Validation and potential exploitation of the surprising, 'out of nowhere' appearance of the 'magic neochondrocytes' is still awaiting further research. Research into reliable scaffold material that could solve the problem for creating a permanent hollow tube even after cellular colonisation must be given priority.

Replacement of the main bronchi is also a viable possibility, as it has been proved by numerous successful canine models [49,54]. In these experiments, the prosthesis frame – usually a polypropylene mesh tube – was reinforced by rings and collagen extracted from porcine skin conjugated to the frame. The inner surface was completely covered with ciliated columnar epithelium or nonciliated squamous epithelium in these experiments. However, the tendency of polypropylene for deformation is a limiting factor. Furthermore, there are potential adverse side effects, including infection, sputum retention, dehiscence and narrowing or occlusion due to inflammation or scar formation. Nevertheless, the very first replacement of a human bronchus has been performed and reported in 2008 [8].

In order to generate a tissue-engineered organ, a tracheal segment was retrieved from a human organ donor and the tissue was completely de-cellularised using detergents and enzymes to prevent subsequent immune rejection. Autologous epithelial cells and SC-derived chondrocytes were isolated from the recipient, expanded and characterised in the laboratory. The cells were seeded onto the donor matrix using a novel bioreactor system [48] designed to allow the co-culturing of different cell types, optimal nutrient transfer and 3D maturation. The graft was then used to replace the recipient's left main bronchus. The transplanted organ immediately provided the recipient with a fully functional airway, which was rapidly vascularised, had normal mechanical properties and was free from the risk of rejection [8].

3.2. Lung parenchyma

While the practical value of artificial lung tissue is unquestionable, the cellular source of an artificial pulmonary tissue remains an onward challenge [7]. Even if we simplify the cellular structure of the lung parenchyma, four basic cell types would be absolutely necessary to create a functional lung, which are discussed in the following.

'Fibroblasts' that are the most versatile of the connective tissue cell family. They participate in repair and regenerative processes in almost every human tissue and organ, including the lung. Their primary function is to secrete ECM proteins that provide a tissue scaffold for normal repair events, such as epithelial cell migration. They are also necessary to provide the essential signalling environment for the participating cellular elements.

'AECs' would also be necessary. Within the distal airways of the lung, the normal alveolar basement membrane is lined with AECs, which can be subdivided into type I and type II pneumocytes. Of the alveolar surface, 95% is covered with type I pneumocytes. These cells are metabolically active and harbour cell surface receptors for a variety of substances, including ECM proteins, growth factors and cytokines. The remaining 5% of alveolar lining cells consist of cuboidal epithelia (type II pneumocytes). Type II pneumocytes secrete surfactant (including surfactant protein C), facilitate trans-epithelial movement of water (via members of the aquaporin protein (AQP) family), function as antigen-presenting cells and represent a reservoir of cellular progenitors that regenerate the alveolar epithelium following lung injury [55].

'Smooth muscle cells' build an involuntary non-striated muscle tissue type. Apart from within large and small arteries and veins, the bladder, uterus, gastrointestinal tract, etc., these cells are found in the respiratory system. Smooth

muscle ensures mechanical support as well as movement during breathing.

'Endothelial cells' provide the thin lining of the interior surface of all blood vessels, including in the extensive vasculature of the airways. Recent studies suggest that endothelial cells are also critical components of the normal pulmonary microenvironment. Interestingly, microvascular endothelial-cell-mediated haematopoietic regeneration also occurs at the level of the haematopoietic SC renewal, suggesting a potential therapeutic role of microvascular endothelial cells not only in self-renewal and repair of adult haematopoietic SCs [56], but potentially also for the cuboidal epithelial cells of the lung.

Despite some remarkable advances, the phenotypic and functional characterisation of SCs and progenitor cells, and their derivatives, along with an understanding of molecular cues and pathways underlying differentiation into specific respiratory lineages is required and still unclear at large.

Nevertheless, the potential cellular sources for generation of lung tissue include (1) endogenous pulmonary SCs, (2) extrapulmonary circulating SCs and (3) SECs [57], or (4) leave the possibility for de-differentiation and re-differentiation of specific differentiated pulmonary tissues open as a potential option.

As de-differentiation and re-differentiation processes require intricate knowledge of signalling pathways and signalling interactions, further investigation using differentiated cell types is essential to understand how to maintain differentiation levels in primary human pulmonary tissues. Whichever way is followed, utilisation of ESCs or ASCs versus more differentiated cell types heralds two distinctive approaches.

4. Chest-wall structures and pleural surfaces

A field, which is ideal for scaffold-only techniques, saw a stealth development in the past 10 years [58,59]. Chest-wall and pericardium prosthesis, and application of biodegradable or nonabsorbable materials are *per se* tissue engineering issues. Surprisingly, further development of absorbable foams and bio-seal coverage of troublesome lung surfaces [60] are still unexplored areas in bioengineering research so far [61,62]. Currently, vascularisation of neo-structures is solved by microvascular techniques, while bony chest structure replacement requires a combined work with orthopaedics. Chest-wall defects – following tumour resection or due to extensive trauma – are additional fields of tissue engineering that require collaborative research work in several disciplines [63–65]. Artificial coverage or neogenesis of injured or destroyed pleural surfaces has an intense clinical priority but is neglected by basic research, highlighting the need for better communication between basic and clinical sciences.

5. Mediastinum

Based on their previous experience with tracheo-mediastinal fistula [66] Alvarez et al. are ready to initiate a phase I clinical trial on the use of autologous adipose-derived SCs grown *in vitro* for local treatment of tracheo-oesophageal fistulae.

6. Pulmonary models beyond surgery

Biotechnology in reconstructive lung surgery is still a test-tube-based laboratory issue and no tissue engineering laboratory has been able to put all the necessary cell types together to mimic a functional lung. Nevertheless, attempts are being made to generate artificial human pulmonary tissues and while these tissues are still incapable of fulfilling the function of a normal lung, they can provide useful models for lung development and pulmonary diseases. Due to the latest events of disastrous phase II clinical drug trials (e.g., TGN1412), further goals of tissue engineering include generation of human tissue models for safety and efficacy testing of pharmaceutical compounds. Results of these studies can aid directed modification of tissue regeneration and wound-healing processes, aiming at improved therapeutical potential for currently untreatable diseases.

One of these specific test models is the bronchial mucosa model that was created as a co-culture using fibroblast, epithelial and Tcells. This model proved to be a useful system to study the regulation and signalling mechanisms of inflammation in asthma [67].

Lung models also provide a useful test system for lung toxicology. Carbon-nanotube-based nanotechnology, for example, has emerged at the forefront of scientific research and technological development. Despite its widespread applications, no effect was tested on human tissues for a long time. A recent study estimated human exposure of single-walled carbon nanotubes using a tissue-engineered human lung model. In co-cultures of normal human bronchial epithelial cells and normal human fibroblasts, carbon-nanotube exposure has proved to be a health risk, increasing inflammatory and cell death markers within the respiratory system [68].

Understanding signalling interactions is also important and has therapeutical consequences in lung injury and repair. To decipher intercellular and consequent intracellular communication networks, specific differentiated pulmonary tissue types and/or SCs can be grown in 2D or 3D co-cultures. Although understanding signalling mechanisms does not immediately solve all the problems of pulmonary tissue engineering, in the long run it will help with development. An example of this is with tissue culture media with the right kind of supplements to aid 3D tissue growth [15].

There are also attempts to speed up artificial lung tissue development *in vivo*. The subcutaneous plug model belongs to this category. Mixed populations of isolated murine embryonic pulmonary cells containing epithelial, mesenchymal and endothelial cells were added to Matrigel and injected subcutaneously into the abdominal wall of adult mice. Vascularisation was enhanced by the administration of FGF2-saturated polyvinyl sponges. Exogenous FGF2 in combination with pulmonary cells resulted in enhanced capillary density and abundant interfacing between developing epithelial and vascular structures. According to expectations, distal pulmonary epithelial differentiation

(tested by prosurfactant protein C expression) was maintained *in vivo* [69].

7. Discussion

Various aspects of biotechnology are gaining entry into the medical disciplines, each at a different pace. This is particularly true for thoracic surgery, which heavily depends on sophisticated technology. While video-assisted thoracic surgery (VATS) and robotics are implemented to solve certain well-defined clinical problems, biotechnology is also expected to find its right niche in thoracic surgery. However, while engineered tissues had a relatively easy entry into vascular or cardiac surgery, the complex tissue environment of pulmonary surgery needs a more orchestrated approach to solve medical problems. Currently, the trachea and the main bronchus replacement are in promising, clinical phase trials [8]. Biotechnologically developed artificial oesophagus, another tubular, often neglected intrathoracic organ, is in wet-lab phase [70].

However, as neither VATS nor tissue engineering is a universal remedy for chest pathologies, continuous communication between surgeons and laboratory researchers is essential to reach optimal clinical applications of biotechnology in surgery. Certainly, it has to be clear for all involved that both practical and ethical issues need careful consideration if this novel and sophisticated technique will ever be successfully applied in medical practice. While medical problems are highlighted by clinicians, solutions have to be thought out together with research scientists. During this research process, the potential long-reaching negative effects have to be considered. For example, genetic manipulation that might aid vascularisation could trigger malignant transformation. The application of SCs in engineered tissues might also hide similar dangers, unless SC research can prevent undirected proliferation and differentiation. Potentially, one can also theorise that, malignancy in the long run might be the price to pay for immediate survival, as malignant outgrowth could often be controlled by cytostatic treatments.

Overall, without understanding signalling networks and code-transmission mechanisms in complex tissues, airway and lung parenchyma engineering, transplantation of the engineered tissues cannot be conducted safely, efficiently and successfully. To reach the necessary level of molecular understanding, however, vast resources have to be accumulated.

In order to optimise resources and knowledge flow, as well as to increase research efficacy, a forum was created under the aegis of the European Society of Thoracic Surgeons (ESTS) titled 'Use of homograft and regenerative medicine in thoracic surgery' in Barcelona, Spain on 28th, 2009 during the 6th International Meeting in General Thoracic Surgery. This congress provided many opportunities to present and discuss the state of the art in tissue engineering. As a result, am ESTS Working Group was created to include thoracic surgeons and basic scientists as well. This close collaboration is the way forward to develop tissue technology without evoking overenthusiasm and unrealistic expectations, which would cause serious damage to an otherwise promising field.

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Establishment and functioning of intrathymic microenvironments

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Summary: The thymus supports the production of self-tolerant T cells from immature precursors. Studying the mechanisms regulating the establishment and maintenance of stromal microenvironments within the thymus therefore is essential to our understanding of T-cell production and ultimately immune system functioning. Despite our ability to phenotypically define stromal cell compartments of the thymus, the mechanisms regulating their development and the ways by which they influence T-cell precursors are still unclear. Here, we review recent findings and highlight unresolved issues relating to the development and functioning of thymic stromal cells.

Thymus organogenesis

As T-cell precursor maturation is a noncell autonomous process, interactions with cells of the thymic microenvironment are required throughout T-cell development (1–3). Thymic stromal cells represent a heterogeneous mixture of cell types including cortical and medullary epithelium, fibroblasts, endothelial cells, dendritic cells (DCs), and macrophages (4, 5). In particular, as epithelial cells are known to play an essential role at multiple stages of thymocyte development (6–8), we focus our review where appropriate on the development and functioning of the thymic epithelium.

Origins of thymic epithelial cells

The thymus in mice initially develops from the third pharyngeal pouch, such that by embryonic day 10–11 (E10–11), the first signs of the budding and outgrowth of the epithelial rudiment are morphologically detectable (reviewed in 9). It is now well established that the forkhead transcription factor FoxN1 plays an essential role in thymic development (10–12). FoxN1 expression in the third pharyngeal region is first detected at low levels by E10.5 of gestation (13). By E11.5, FoxN1 is strongly expressed in the third pharyngeal pouch endoderm, a stage occurring after formation of the common primordium of the thymus and parathyroid (14)

but before these two structures become morphologically distinct. Induction of FoxN1 expression can be mediated by the soluble glycoprotein Wnt4 (13), which presumably acts by binding to Frizzled receptors on epithelial precursors. In the absence of FoxN1, the initial stages of thymus organogenesis occur normally, leading to the formation of epithelial anlagen (10). However, further thymic development does not occur, and the thymic epithelial rudiment remains uncolonized by lymphoid precursors (15), which persists in the adult as a cystic epithelial structure (16). The precise role played by FoxN1 in the development of thymic epithelium is not clear. Recently, Dooley *et al.* (17) showed that epithelial cells in the nude thymic rudiment phenotypically and genotypically resemble respiratory-type epithelial cells, raising the possibility that FoxN1 may play a role in regulating lineage choice in multipotent epithelial progenitors. Deletion of the N-terminal domain of FoxN1 to create a hypomorphic mouse strain resulted in thymic-specific defects, indicating a tissue-specific role for this domain in thymus development (18). Importantly, although some aspects of T-cell development occur, epithelial cells in the FoxN1 hypomorphic thymus are predominantly of a keratin (K) $5^{+}K8^{+}$ phenotype, which is suggestive of a blockade in epithelial cell differentiation at an immature progenitor stage (18). Although it is clear from these studies that FoxN1 plays an essential role in the formation of a functional thymic epithelial microenvironment, a key unresolved issue is whether FoxN1 is also required to maintain the functions of differentiated thymic epithelial cells. Indeed, it is not clear whether all epithelial cells in the adult thymus express FoxN1 or whether expression is restricted to particular epithelial subsets. Mouse strains carrying FoxN1 reporters and inducible gene knockouts will be required to address these questions.

The precise contribution that each of the individual germ layers makes to the developing thymus has been studied over a number of years. Although neural crest-derived mesenchymal cells of neuroectoderm origin are known to make an important contribution to the developing thymus, the origins of thymic epithelial cells are less certain. Two models of thymic epithelial cell development have been proposed, one in which all epithelial cells are endodermally derived, while another model argues in favor of a dual origin for the thymic epithelium, with contributions from both endoderm and ectoderm (reviewed in 19). While support for a physical contribution from ectoderm comes largely from descriptive histological studies (20, 21), there is now direct functional evidence in favor of a model of thymus development in which all thymic epithelial cells are endodermally derived. Thus, transplantation of pharyngeal endoderm under the kidney capsule of athymic nude mice was shown to develop into a functional thymus, while fluorescent labeling of pharyngeal ectoderm revealed no evidence in favor of an ectodermal contribution to the developing thymus (22). Such observations are in agreement with earlier studies using chick–quail chimeras demonstrating that cortical and medullary epithelial cells could be generated from isolated endoderm (23). Several important issues arise from these experiments. First, although these latter studies argue against a direct physical contribution from ectoderm during thymus development, pharyngeal endoderm and ectoderm are in physical contact by E10.5 (22), and so inductive interactions between these two cell types could still influence events early in the formation of the thymus anlagen. In addition, evidence in favor of a single origin for all thymic epithelial cell subsets has implications for the nature of epithelial cell precursors in the developing thymus.

The identification and isolation of epithelial cells in the thymus with progenitor activity is not only important in gaining a better understanding of mechanisms of thymus development but also is key to strategies aimed at enhancing thymus regeneration in the elderly or following chemotherapy and radiotherapy, where new T-cell production from transplanted bone marrow is critically dependent upon thymus function. Direct evidence for the existence of thymic epithelial precursors came from Rodewald *et al.* (24), who showed that distinct islands of medullary epithelial cells are derived from single precursors. A logical assumption regarding thymic epithelial cell progenitors is that they would make up a larger proportion of thymic epithelium in the early embryonic thymus when compared with the adult, where most of the epithelium is comprised of mature cortical and medullary subsets. Thus, the identification and use of reagents that identify dominant epithelial cell populations in the embryonic thymus and rare epithelial populations in the adult have been used as a strategy to study candidate epithelial progenitors. Mature thymic epithelial cells in the adult can be characterized by differential expression of keratins, such that medullary epithelial cells are predominantly $K5^{+}K8^{-}$ while cortical epithelial cells are largely $K5^{-}K8^{+}$. Interestingly, early in thymus development, epithelial cells are predominantly $K5^{+}K8^{+}$, raising the possibility that such 'double-positive' epithelial cells are a precursor on the single-positive cortical and medullary subsets (25–27). Moreover, $K5^{+}K8^{+}$ epithelial cells are rare in adult thymus, suggesting the possible persistence of a progenitor population. However, patterns of cytokeratin expression allow only phenotypic

characterization of epithelial subsets, and so reagents are required which recognize cell-surface antigens that are expressed in a developmentally regulated manner in order to purify and study the developmental potential of defined epithelial cells. The rat monoclonal antibodies Mts20 and Mts24 raised by Godfrey *et al.* (28) meet both of these requirements, and they have been used in studies aimed at identifying thymic epithelial progenitors. So far, experiments have shown that Mts24$^+$ epithelial cells from either E12 or E15 of gestation are able to generate a functional thymus upon transplantation into nude mice (25, 26). Of key importance is the observation that Mts24$^-$ stromal cells are unable to develop into a thymic structure, suggesting that progenitor activity is limited to the Mts24$^+$ compartment. Despite the evidence from studies on the embryonic thymus, there is no direct functional evidence for the existence of thymic epithelial progenitors in the adult thymus. The turnover and lifespan of epithelial cells in the thymus is not known, and it will be important to determine whether thymic epithelial microenvironments are maintained by continued progenitor maturation, or whether their persistence reflects the presence of long-lived differentiated cells. Further studies on the precursor–product relationships of thymic epithelial subsets and their persistence during ontogeny will continue to be important in understanding the mechanisms of thymus development. Moreover, the identification of new reagents and the establishment of clonal assays will aid in determining whether cortical and medullary epithelial cells share a common precursor or arise from distinct precursor pools.

Epithelial–mesenchymal interactions during thymus development

The development of several organs involves interactions between epithelial cells and mesenchymal cells (29). The thymus shares similarities to these other organs in that at around E10–12, the thymus anlage is an epithelial bud surrounded by mesenchymal cells of neural crest (NC) origin (30, 31). The presence of NC-derived mesenchymal cells has been shown to play an essential role in thymus development. In initial experiments in birds, ablation of the NC resulted in a failure of normal thymus organogenesis (32), while removal of thymic mesenchyme from mouse embryonic thymus prevented normal thymus development *in vitro* (31, 33). More recently, mutant mice have been used to study the importance of the NC in thymus organogenesis, with mice deficient in a variety of members of the Pax and Hox transcription factor families demonstrating abnormalities in thymus organogenesis (9). Of particular interest in this context is the impaired thymus development observed in Pax-3 (splotch) or Hoxa3 mutants, genes that are expressed in the NC mesenchyme. One possibility for the abnormal thymus development in Pax-3 mutants is that defective migration results in an absence of NC-derived cells in the developing thymus, although there is conflicting evidence on the migratory abilities of Pax-3 mutant NC cells (34). Interestingly, NC migration occurs normally in Hoxa3 mutants (35). While these findings indicate that Hoxa3 is not required for NC migration, whether Hoxa3 expression specifically in NC is important in thymus development is not clear, as Hoxa3 is expressed both in epithelial cells and mesenchymal cells in the thymus (36). Further studies using tissue-specific gene knockout or tissue recombination experiments are required for a better understanding of the role of Hoxa3 in thymus development.

Although the aforementioned studies demonstrate a role for NC mesenchyme during the initial stages of thymus organogenesis, it is unclear whether these cells or their descendents are involved in later stages of thymus development. Indeed, a recent study by Yamazaki *et al.* (37) using myelin protein 0-Cre reporter mice to trace NC-derived cells demonstrated their presence in the thymus at E11.5–16.5 but only rarely after this stage. Such findings are in agreement with another study using Wnt1-Cre reporter mice that demonstrated NC-derived cells in the early fetal thymus but not in the postnatal thymus (30). Moreover, Yamazaki *et al.* (37) showed that NC cells in the early fetal thymus represent multipotent progenitors, capable of giving rise to melanocytes, neurons, and/or glial cells. Thus, one possibility is that NC-derived mesenchyme represents a transient population of cells in the thymus that diminishes after early stages of thymus organogenesis. The precise origins of other mesenchymal cells that make up the septae, trabeculae, and capsule of the late-stage embryonic and postnatal thymus, and how they relate to the NC-derived mesenchyme are not known.

How NC-derived mesenchymal cells influence thymus development is poorly understood. However, from analysis of mice deficient in fibroblast growth factor-receptor 2iiib (FGF-R2iiib), it was noted that the thymus is present but remains small during development (38). Further analysis of these mice showed a thymus containing mature cortical and medullary epithelial cells capable of supporting a normal program of T-cell development (39), suggesting that FGF-R2iiib signaling may be required for growth but not development of thymic epithelial cells. We have shown that two of the ligands for FGF-R2iiib, FGF7 and FGF10, are expressed by mesenchymal cells but not epithelial cells in the E12 thymus and that immature epithelial progenitors express FGF-R2iiib

(40). Moreover, removal of the mesenchyme from E12 thymus lobes resulted in a loss of proliferation in immature thymic epithelial cells, which could be restored by the addition of either fetal mesenchyme or a combination of recombinant FGF7 and FGF10 (40). Thus, one role of NC-derived mesenchyme in early thymus development may be, via its production of FGF7 and FGF10, to induce proliferation of early thymic epithelial progenitors expressing FGF-R2iiib. FGFs have also been shown to directly influence mature thymic epithelium. Erickson *et al.* (41) showed that treatment of thymocyte-depleted fetal thymic lobes with FGF7 resulted in reduced expression of major histocompatibility complex (MHC) class II-invariant chain and cathepsin-L concomitant with an increase in the medullary epithelial compartment, while Rossi *et al.* (42) showed that FGF7 administration *in vivo* protected the thymic microenvironment and its ability to support thymocyte development during graft-versus-host disease. How FGFs regulate mature thymic epithelial cells and whether they are able to directly induce their proliferation, as is the case with embryonic thymic epithelium, is not clear. Proliferation of embryonic thymic epithelial cells has been shown to decrease during thymus development with few proliferating epithelial cells being detectable by E18 (43), a stage where a paucity of thymic NC-derived mesenchymal cells has been reported (37). Thus, the correlation between the presence of NC-mesenchyme in the thymus and proliferation of immature thymic epithelial cells supports the idea that NC-derived cells represent a transient thymic population required to expand epithelial cell numbers during early stages of thymus development.

As well as playing an important role in the expansion of embryonic thymic epithelial cells, mesenchymal cells have also been shown to play a direct role during T-cell precursor development. For example, $CD4^-CD8^-$ T-cell precursors require a dual combination of mature MHC class II^+ thymic epithelial cells and mesenchyme to reach the $CD4^+CD8^+$ stage, while the generation of $CD4^+$ and $CD8^+$ cells from $CD4^+CD8^+$ intermediates can occur in the presence of thymic epithelial cells alone (44). Thus, mesenchymal stromal cells provide essential signals during the early stages of T-cell development. In particular, transition from the $CD4^-CD8^-CD25^+CD44^+$ double-negative 2 (DN2) stage to the $CD4^-CD8^-CD25^+CD44^-$ DN3 stage maps to the transition from mesenchymal-dependent to -independent stages of T-cell precursor development (45). How mesenchymal stromal cells influence the development of DN2 thymocytes is not clear. However, pretreatment of mesenchyme with the enzyme hyaluronidase to disrupt the extracellular matrix (ECM) abrogated their ability to support T-cell development (45). Thus, production of ECM components by thymic mesenchyme may partly explain the importance of these cells in the thymus, with ECM enabling interactions with integrins expressed by T-cell precursors or by influencing T-cell development through the presentation of soluble growth factors such as IL-7 (46).

Lymphostromal interactions during thymus development

Once lymphoid precursors enter the thymus from the blood stream, they come into contact with thymic stromal cells that guide their maturation into functionally competent T cells. However, at stages in development when initial thymus colonization occurs, mature microenvironments typical of those within the postnatal thymus are yet to form (2, 25–27). So, just as T-cell precursors undergo a program of proliferation and differentiation, immature thymic epithelial cells undergo a developmental sequence resulting in the establishment of mature cortical and medullary epithelial cells, which are organized within a three-dimensional network. Thus, it appears that the fetal thymic microenvironment is capable of supporting T-cell development as it continues its own maturational program. Importantly, key mediators of T-cell development such as interleukin-7 (IL-7) (47) and Notch ligands (48) are expressed in the early thymic rudiment, which may partly explain the ability of immature thymic epithelial cells to support T-cell precursor development.

As mentioned earlier, ontogenetic analysis of epithelial cells during thymus development has established a model in which epithelial progenitors, characterized by dual expression of K5 and K8, proliferate and differentiate into mature cortical $K5^-K8^+$ and medullary $K5^+K8^-$ epithelial cells (25–27, 49). The signals responsible for triggering this differentiation are unknown. However, studies on the adult thymus of several mutant mouse strains have reported abnormalities in the organization of thymic epithelium, leading to the idea that reciprocal signaling between thymocytes and stromal cells not only results in T-cell production but also development and organization of thymic microenvironments (50, 51). For example, mice displaying a blockade in T-cell development resulting in the absence of T-cell receptor (TCR)-expressing cells results in defects in organization of the thymic medulla, with only scattered medullary epithelial cells being detected (52–54). Importantly, thymic medullary organization can be restored by the addition of mature T cells (53–55). In contrast, adult CD3εtg26 mice, in which T-cell development is blocked at the earliest $CD25^-CD44^+CD4^-CD8^-$ DN1 stage, show an absence of both cortical and medullary epithelial cells and an abundance of $K5^+K8^+$ cells (56). Taken together, these findings form the basis

of the thymic crosstalk model, in which signals from early $CD4^-CD8^-$ T-cell precursors are required for the formation of the thymic cortex, while signals regulating development of the medulla are derived from $CD4^+$ and $CD8^+$ thymocytes (57, 58).

While the above studies demonstrate that interplay between thymocytes and stromal cells can result in changes in the thymic microenvironment, they do not necessarily support a model in which the development of mature thymic epithelial cells from immature progenitors is dependent upon thymocyte-derived signals. Instead, analysis of the thymic microenvironment of adult mice with defective T-cell development may indicate that thymocyte-derived signals are required for the maintenance of thymic epithelial microenvironments. Accordingly, analysis of embryonic thymus development allows direct study of the mechanisms regulating the establishment of the thymic microenvironment and the maturation of immature epithelial progenitors. Indeed, analysis of embryonic recombination-activating gene/common γ chain ($RAG2/\gamma_c$)-deficient mice and Ikaros mutant mice, both with an absence of T-cell precursors during the fetal period, demonstrate three-dimensional organization of thymic epithelial cells together with the appearance of $K5^+K8^-$ epithelial cells, suggesting that initial development of thymic epithelial cells does not require thymocyte-derived signals (27). We have also shown that $K5^+K8^-$ and $K5^-K8^+$ epithelial cells are present in the embryonic CD3εtg26 thymus, despite T-cell development being blocked at the earliest stage (59). Moreover, proliferation of embryonic thymic epithelial cells still occurs in the absence of normal T-cell precursors (40), in agreement with the role of mesenchymal cells in regulating thymus growth (39, 40). Interestingly however, once $RAG2/\gamma_c$-deficient mice and CD3εtg26 mice reach adulthood, the thymus consists largely of disorganized $K5^+K8^+$ epithelial cells. The reasons for this difference in fetal and adult thymic epithelial cells are not clear, although it is possible that following normal thymic epithelial cell patterning in the embryo, the presence of thymocytes is required for the continued survival of $K5^+K8^-$ and $K5^-K8^+$ cells. As a consequence, $K5^+K8^+$ cells, which are normally present in the adult thymus as a small population (25), would preferentially accumulate (*Fig. 1*).

Alternatively, the prolonged absence of both thymocytes and the normal three-dimensional framework may result in changes in keratin gene expression, such that $K5^-K8^+$ and

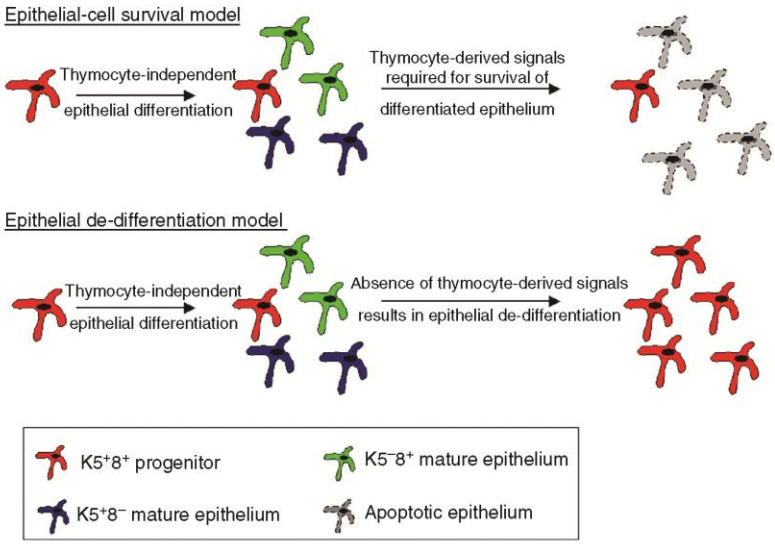

Fig. 1. Lymphostromal interactions in the maintenance of the thymic microenvironment. Current views on thymic epithelial cell development support a model whereby immature $K5^+K8^+$ progenitors are signaled to differentiate into mature $K5^-K8^+$ cortical and $K5^+K8^-$ medullary epithelial cells. It is now clear that this initial patterning of thymic epithelial cells can occur independently of signals from developing T-cell precursors. However, in the continued absence of a normal program of T-cell development, thymic epithelial phenotypes and normal thymic architecture become altered, often resulting in the predominance of $K5^+K8^+$ epithelial cells, indicating that thymocyte-derived signals are required to maintain the thymic epithelial microenvironment. In the 'Epithelial Survival' model shown here, it is suggested that thymocytes play a role in microenvironmental maintenance by the provision of survival signals to mature epithelial cells. In the absence of such signals, cortical and medullary epithelial cells are lost, resulting in a dominant $K5^+K8^+$ epithelial population. Alternatively, in an 'Epithelial De-Differentiation Model', the dominance of $K5^+K8^+$ epithelial cells that occurs in the continued absence of normal T-cell precursors may be a result of alterations in keratin gene expression in mature cortical $K5^-K8^+$ and medullary $K5^+K8^-$ epithelial cells, a process that could occur following loss of the normal three-dimensional thymic epithelial network.

$K5^+K8^-$ cells undergo a de-differentiation process and reacquire a $K5^+K8^+$ phenotype. An implication of the latter model is that the $K5^+K8^+$ epithelial cells in the adult thymus may not necessarily represent a population of epithelial progenitors. As mentioned earlier, further experiments are required to formally prove the existence of epithelial cells with progenitor activity in the adult thymus.

Thymus organization ensures an ordered program of T-cell development

Thymus colonization

Hemopoietic precursors are required to colonize the thymic microenvironment in order to efficiently generate T cells. Importantly, the site of production of T-cell precursors changes during ontogeny, such that the early fetal thymus is colonized by fetal liver-derived precursors, while the postnatal thymus is colonized by precursors from the bone marrow (2, 60). Several studies have now shown that fetal precursors are different from postnatal and adult precursors in a number of ways. T-cell production from fetal but not adult precursors can occur in the absence of IL-7 (61, 62). In addition, analysis of RAG-1 green fluorescence protein (GFP) knock-in mice has shown that fetal precursors have less myeloerythroid potential when compared with their adult counterparts (63). The reasons for these differences are not clear, and one possibility is that they reflect an intrinsic yet distinct developmental program of fetal and adult precursors. An alternative explanation is that fetal and adult precursors receive distinct prethymic maturational signals from the fetal liver and bone marrow, respectively, which may impact on their later developmental requirements and lineage potential. We have analyzed fetal liver and fetal thymus microenvironments for their ability to trigger Notch activation in T-cell precursors, a process that has been strongly implicated in T/B lineage choice. Our findings suggest that the fetal liver microenvironment contains discrete Notch ligand-bearing stromal cells that can activate Notch signaling in fetal precursors prior to thymus colonization (48). Although Notch activation is clearly important during the development of adult lymphoid precursors, the precise timing of Notch activation in relation to adult thymus colonization is not clear, and so it will be important to determine whether, as in the embryo, a prethymic Notch signal occurs in the adult that may influence the timing of T/B lineage choice.

As well as developmental heterogeneity in lymphoid precursors, the mechanisms of thymus colonization in the early fetal and late fetal/postnatal thymus are likely to differ. The lack of vascularization of the thymus at the initial stages of colonization means that at E12 of gestation, precursors must exit nearby blood vessels, travel through perithymic mesenchyme, and enter the thymus by migrating across an epithelial basement membrane (15, 64). In contrast, precursors in the adult enter the thymus directly by migrating from blood vessels at the corticomedullary junction (CMJ) (65, 66). Despite this difference, it is likely that in both fetal and adult life, the recruitment of precursors to the thymus involves a chemotactic mechanism. Indeed, thymic epithelial cells have been reported to express mRNA for several chemokines, including CCL21, CCL25 [thymus-expressed chemokine (TECK)], and CXCL12 [stromal cell-derived factor 1 (SDF-1)] (67, 68). Moreover, these chemokines are capable of inducing migration of fetal precursors using *in vitro* assays, which can also be blocked using pertussis toxin, which inhibits chemokine receptor signaling (67, 68). Despite data from *in vitro* systems indicating a possible role for CXCL12 in precursor recruitment to the thymus, mice deficient in either CXCL12 or its receptor CXCR4 show no impairment of precursor recruitment a the initial stages of thymus colonization (69). By contrast, CCR9 knockout mice show lower thymocyte numbers, which perhaps supports a role for TECK in thymus colonization (70), although effects on thymocyte viability cannot be ruled out. Moreover, *plt/plt* mice (deficient in CCL21) show diminished thymic precursor recruitment, which is reduced further by neutralizing anti-CCL25 antibodies (68). Overall, such studies suggest that recruitment of progenitors to the thymus is regulated by multiple chemokines including CCL21 and CCL25, which are produced by the thymic epithelium and act to establish a gradient along which cells migrate. Interestingly, mel-18, a member of the mammalian Polycomb group genes, has been reported to regulate expression of a number of chemokine receptors, including CCR9, with thymocytes from mel-18-deficient mice displaying reduced migration in response to CCL25 (71). Although it is clear that chemokines play a role in precursor migration, whether recruitment of precursors toward the thymus from the bone marrow/fetal liver is regulated by mechanisms distinct to those controlling entry to the thymic epithelial microenvironment is not clear. Itoi *et al.* (15) have shown that precursors are recruited to the fetal thymus of nude mice, but unlike wildtype mice, such precursors do not enter the thymic epithelial anlagen. Interestingly, the nude thymic anlagen has been shown to lack expression of CCL25 (72), raising the possibility that the initial recruitment of precursors from extrathymic sites to the surrounding perithymic mesenchyme occurs independently of TECK, which then plays a role in the migration of precursors into the thymic epithelium.

Although the precise mechanisms enabling precursors to exit blood vessels and gain entry to the thymus are unclear, several molecules have been implicated in this process. For example, thymic blood vessels have been shown to express a range of adhesion molecules including CD34, MECA79, vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and P-selectin (73). Moreover, mice deficient in the P-selectin glycoprotein ligand-1 (PSGL-1) show a reduction in early thymic resident progenitors (74), suggesting that thymic endothelial expression of P-selectin facilitates migration into the thymus. However, it is also important to note that recruitment of precursors to the thymus is a temporally regulated process, with periods where precursors can enter the thymus being separated by longer periods where the thymus is refractory to precursor recruitment (75, 76). Such a gated mechanism of precursor entry to the thymus may be regulated by changes in expression of adhesion molecules by the thymic vasculature, as has been shown for the endothelial marker MECA-79 (73) and also by intrathymic competition for stromal cell niches, which has also been shown to occur during the early $CD4^{-}CD8^{-}$ stages of thymocyte development (77).

Intrathymic migration

By the time of birth, the thymus is made up of an organized network of stromal elements. The positioning of developing T-cell precursors within the thymus reflects this microenvironmental organization, with immature $CD4^{-}CD8^{-}$ and $CD4^{+}CD8^{+}$ thymocytes dominating in the outer cortical regions and mature $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ cells residing within the inner thymic medulla. Such a defined positioning of thymocyte subsets within the thymus supports the notion that specific microenvironments are present within the thymus that provide the appropriate interactions and signals for particular stages of T-cell development. Evidence comes from studies analyzing the localization of T-cell precursors following their introduction into recipient mice. Thus, the thymic cortex has been subdivided into stratified layers on the basis of the distribution of the DN1–DN4 stages of thymocyte development, with $CD4^{-}CD8^{-}$ precursors undergoing maturation as they migrate outward from the CMJ, the point of entry to the postnatal and adult thymus, toward the capsule (66). Once $CD4^{+}CD8^{+}$ thymocytes are generated, they presumably then migrate back through the cortex, and following positive selection, their $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ descendents cross the CMJ to enter the medulla.

Despite a clearer understanding of the positioning of defined thymocyte subsets in the thymus, very little is known about the stromal microenvironments that support transition between stages in T-cell development. Moreover, the molecular mediators regulating precursor movement between intrathymic microenvironments are not clear. However, evidence does exist that implicates a role for chemokines in the positioning of defined precursor populations in the thymus. Analysis of the development and thymic distribution of T-cell precursors deficient in CXCR4 has provided evidence that CXCR4 signaling via CXCL12 binding is important to insure that immature T-cell precursors reside within the thymic cortex. In the absence of CXCR4 expression, developmental arrest occurs at the DN1 stage, with precursors present at the CMJ but absent from the cortex (78). Thus, CXCL12 production by cortical thymic epithelial cells may play an important role in insuring that the earliest $CD4^{-}CD8^{-}$ precursors are able to gain access to microenvironments within the cortex that insure a normal program of T-cell development. In contrast, experiments analyzing T-cell precursors lacking CCR9 have shown that although the positioning of CCR9-deficient precursors in the thymus is abnormal with precursors notably absent from the subcapsular region, their development still follows an apparently normal program (79). In addition, the chemokine receptor CCR7 has been shown to play an important role in intrathymic migration of thymocytes at various stages of development. Thus, immature $CD25^{+}CD44^{+}$ precursors normally positioned within the thymic cortex accumulate at the CMJ in CCR7-deficient mice, suggesting that CCR7, like CXCR4, is important in cortical migration (80). At later stages of development, Ueno *et al.* (81) showed that CCR7 expression controls migration of thymocytes from the cortex to the medulla, with single positive thymocytes accumulating in the thymic cortex of CCR7-deficient mice. Despite this arrest in migration, CCR7-deficient thymocytes were shown to undergo a normal program of development including negative selection (81). Overall, these studies raise the possibility that while some stages of precursor maturation require entry to certain highly specialized thymic microenvironments, the ability to support other aspects of T-cell development may be a property shared by several thymic microenvironments.

In addition to chemokines, it is likely that cell–cell interactions between developing thymocytes and thymic stroma are important in the directed movements of precursors in the thymus. Indeed VCAM-1, which binds α4 integrin expressed by early thymocytes, has been shown to form part of a stromal matrix in the thymic cortex (82), while thymocytes devoid of the guanine nucleotide-binding protein Rho showed impaired binding to VCAM-1 (83). As Rho has been shown to play a

role in integrin-mediated cell adhesion, these findings suggest that regulation of intrathymic migration involves interplay between stroma-expressed cell surface adhesion molecules and thymocyte-expressed integrin receptors. In addition to cell–cell interactions, it is likely that thymocyte migration and development is influenced by interactions with ECM components such as fibronectin, laminin, and collagen, all of which are expressed within the thymic microenvironment (84). While ECM components may act as a facilitator of thymocyte migration by providing a noncellular scaffold enabling integrin-mediated thymocyte adhesion, ECM has also been shown to have other functions relevant to thymocyte development, including the binding of secreted proteins such as chemokines and cytokines, which may play a role in increasing local concentrations of key molecules such as IL-7 (46, 85) or in the establishment of chemokine gradients for directed migration (86).

Thymic stromal cells regulate key signaling pathways in thymocyte development

Regulation of Notch signaling by the thymic microenvironment

In recent years, it has become clear that signaling through the cell surface receptor Notch plays a key role during T-cell development in the thymus (87–89). Notch, a transmembrane receptor shown to be important in cell fate decisions in many tissues, interacts with members of the Jagged and Delta-like ligand families, which results in Notch cleavage and translocation of the intracellular domain to the nucleus where it acts as a transcriptional coactivator (87). Thus, cell–cell interactions regulate Notch activation. In the thymus, Notch has been shown to be expressed by developing T-cell precursors and has been implicated in various aspects of thymocyte maturation including commitment to the T-cell lineage (48, 90, 91), αβ/γδ T-cell commitment (92, 93), pre-TCR-mediated development (94, 95), positive (96, 97) and negative (98) selection of $CD4^+CD8^+$ thymocytes, and CD4/CD8 lineage choice (99). The importance of Notch signaling in T-cell development is covered in other articles in this issue. As Notch activation requires binding of Notch ligands that occur as a result of cell–cell interactions, understanding the cellular expression patterns and functions of individual Notch ligands is critical to our understanding of the regulation of Notch signaling during T-cell development. Importantly, members of both the Jagged and Delta Notch ligand families are expressed by thymic stromal cells (100, 101), including MHC class II^+ thymic epithelium (101), indicating that the thymic microenvironment regulates Notch signaling in developing T-cell precursors. Indeed, removal from the thymic microenvironment results in the downregulation of Notch target gene expression (101), indicating that Notch activation in T-cell precursors requires the presence of intrathymic Notch ligands.

A major factor limiting analysis of the patterns of Notch ligand expression in the thymus has been the lack of appropriate antibodies recognizing individual ligands, and so studies have been limited to mapping mRNA expression in isolated cells (100, 101). However, analysis of Notch ligand distribution in the context of an intact thymic microenvironment has recently been investigated. For example, analysis of β-galactosidase expression from a lacZ reporter cassette driven by the Delta-like 4 (Dll-4) promoter has demonstrated that while Dll-4 expression is readily detectable in embryonic thymic epithelial cells, Dll-4 expression in the adult thymus is weaker and appears present only in scattered cells (102). Immunohistochemical analysis has shown differential expression of Jagged-1 and Delta-like-1 (Dll-1) proteins in the thymus, with Dll-1 being expressed by most cytokeratin-positive cells in the thymic cortex (103), while Jagged-1 is only expressed by a cortical epithelial subset (104). Although it has been shown that Jagged-1 is expressed by almost all $ERTR5^+$ medullary epithelial cells (104), the expression pattern of Dll-1 in the thymic medulla is not clear. Thus, distinct intrathymic microenvironments may contain epithelial cells expressing distinct Notch ligands. However, whether compartmentalization of expression also occurs at a cellular level is not clear. For example, it is not known if Dll-1-expressing cortical epithelial cells also express Jagged-1. This expression may be of functional significance, as there is evidence suggesting that Jagged and Delta ligands play different roles during T-cell development. Thus, dual expression of Jagged/Delta ligands by individual stromal cells could allow these molecules to bind Notch either individually or in combination, which may result in the generation of distinct Notch signals to T-cell precursors. Indeed, while Jagged-1 or Dll-1 can both influence T/B lineage choice, Dll-1 but not Jagged-1 is able to promote T-cell development of $CD4^-CD8^-$ precursors, although Jagged ligands may influence αβ/γδ lineage choice (104, 105). That Dll-1 is an efficient regulator of early T-cell development is clear from many studies utilizing OP9 bone marrow stromal cells, which have been engineered to express this Notch ligand (106). Such cells are able to efficiently support the production of $CD4^+CD8^+$ thymocytes from a range of precursor populations, including hematopoietic progenitor stem cells (107), $CD34^+$ human cord blood cells (108), and embryonic stem cells (109). Despite emerging functional

differences between Delta and Jagged Notch ligands, functional redundancy also appears to exist between different Notch ligands, as indicated by the normal program of T-cell development seen in Dll-1-deficient mice (110), which is most likely due to overlapping functions with other Delta family members. Likewise, the finding that the later stages of T-cell development can occur normally in the absence of thymocyte Notch-1 expression (111) suggests that redundancy also occurs with Notch receptors. Although it is clear that Notch activation results in induction of target gene expression including *hes-1*, *pta*, and *deltex* (88, 89), with regard to thymocyte development, it is unclear whether interactions with Delta and Jagged ligands result in the activation of distinct downstream genes. A major goal in future research is to clarify the potential functional significance of distinct Notch ligands that may differentially regulate Notch signaling at various stages of thymocyte development.

Wnt–Frizzled interactions in thymocyte development

In addition to the cell–cell interactions between thymocytes and stromal cells that regulate T-cell migration and maturation, intrathymic microenvironments also provide signals in the form of secreted molecules. Although it is clear that these molecules can be in the form of well-characterized chemokines and cytokines, there is also increasing evidence to suggest that other families, such as the Wnt family of secreted glycoproteins, have roles in T-cell development in the thymus. Intrathymic signaling as a result of Wnt binding can initiate several distinct signaling cascades, including the canonical Wnt signaling pathway (112). This pathway results in the stabilization of β-catenin through inhibition of its phosphorylation, which would normally target β-catenin for degradation. Stabilized β-catenin can then translocate to the nucleus, where binding to members of the TCF/LEF families of transcription factors results in gene expression of Wnt target genes (113, 114).

Several studies have reported expression of Wnt genes in the thymus, supporting a role for Wnt signaling in T-cell development (115, 116). Evidence that Wnt signaling in T-cell precursors is regulated by the thymic microenvironment comes from studies analyzing expression of Wnts and their Frizzled receptors, in defined cellular compartments. We have shown that thymic epithelial cells but not thymocytes express several Wnt family members, including Wnt4, Wnt7a, and Wnt7b (116). Moreover, removal of T-cell precursors from the thymic microenvironment results in phosphorylation and degradation of β-catenin, providing further support that Wnt signaling in thymocytes is a consequence of interactions with thymic stromal cells (116). The precise roles that Wnts play in influencing the maturation of thymocytes are not clear. Despite a normal program of T-cell development, Wnt1/Wnt4 double-deficient mice show reduced overall thymocyte numbers, indicative of a role for Wnt signaling in the regulation of thymocyte survival and/or proliferation (117). In addition, treatment of fetal thymus organ cultures with soluble Frizzled receptors to block Wnt signaling was shown to perturb thymocyte development (115). However, while this finding is suggestive of a direct role for Wnt signaling in T-cell precursor development, it is also important to note that thymic stromal cells also express Frizzled receptors (116). Thus, the observed effects with soluble Frizzled receptors on T-cell development could be at least in part a consequence of altered thymic stromal cell function. Indeed, Wnts have been shown to influence epithelial cells in several tissues, and in the thymus, Wnts have been shown to regulate expression of FoxN1 (13), a key gene in thymic epithelial cell development.

As canonical Wnt signaling induces target gene expression as a result of TCF/LEF1 transcription factor activity, perhaps some of the strongest evidence supporting a role for the canonical Wnt signaling pathway in T-cell precursor maturation comes from studies of TCF/LEF1-deficient T-cell precursors. Maturation of T-cell precursors is blocked at the immature $CD4^{-}CD8^{+}$ stage in TCF/LEF1 double-deficient mice, suggesting that Wnt signaling may play a role in the survival and differentiation of immature thymocytes after the TCR β-selection checkpoint (118). Although several other reports have implicated β-catenin and canonical Wnt signaling at multiple stages of T-cell development including the generation and survival of $CD4^{+}CD8^{+}$ thymocytes, positive selection (119–122), other studies have shown that β-catenin is dispensable for a normal program of T-cell development (123). The reasons for this discrepancy are not clear, although one possibility is that functional redundancy occurs between β-catenin and its homolog γ-catenin during T-cell development. This possibility is supported by a recent report showing that T-cell development occurs normally in γ-catenin knockout mice (124). Further experimental approaches to simultaneously target both β-catenin and γ-catenin functioning during T-cell development will provide important information on the roles of these molecules and the importance of canonical Wnt signaling during T-cell precursor maturation.

Regulation of bone morphogenetic protein and Hedgehog signaling in the thymus

As is the case with Notch and Wnt signaling, in recent years, it has become clear that evolutionarily conserved signaling pathways that regulate a variety of developmental systems also play a

role during development in the thymus. For example, it is clear that bone morphogenetic protein (BMP) and Hedgehog (Hh) family members, as well as their receptors and components of their respective signaling pathways, are expressed intrathymically (125–127). Direct evidence that BMP signaling influences thymocyte development comes from studies demonstrating that BMPs act to inhibit the proliferation and differentiation of T-cell precursors (125, 128). Accordingly, inhibition of signaling by the BMP antagonists as Noggin and Chordin was associated with the maturation of $CD4^-CD8^-$ thymocytes to the $CD4^+CD8^+$ stage (125, 128). In these studies, expression of BMPs, Chordin, and Noggin was shown to be limited to thymic stromal cells, while BMP receptor expression was detectable in T-cell precursors (129), suggesting that the thymic microenvironment plays a key role in the regulating BMP-mediated events during T-cell development. Similarly, several studies have now shown that Hh family members are expressed intrathymically by thymic stromal cells (125), and that their receptors are expressed by developing T-cell precursors, which can act to influence T-cell development, including maturation to the $CD4^+CD8^+$ stage (130, 131).

Whether BMP and Hh families of molecules also play a role in the development and functions of thymic stromal cells is less clear. In support of this possibility, semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that thymic stromal cells, including purified thymic epithelial cells, express several receptors for BMP molecules, such as BMP-R1a, BMP-R1b, and BMP-R2 (Fig. 2), raising the possibility that thymic epithelial cells can respond to BMPs.

He *et al.* (132) have shown that overexpression of SMAD-7 results in blockade of BMP-mediated signaling (133) and causes defects in multiple epithelial tissues, including the thymus. In addition, BMPs have been shown to influence gene expression in thymic epithelial cells, including regulation of the transcription factor FoxN1 (134). Relevant to the role of Hh in the thymus, the Hh receptors Ptc1, Ptc2, and Smo are expressed by both T-cell precursors and thymic epithelial cells (130). Thus, several of the molecules outlined above, including BMPs, Hh, and Wnts, may have a dual role in the thymus, and as well as regulating the development of T-cell precursors, they may also act directly on thymic stromal cells to influence the development and functioning of thymic microenvironments.

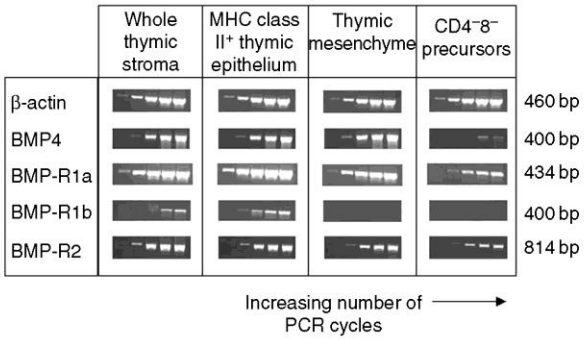

Fig. 2. Thymic epithelial cells express receptors for bone morphogenetic proteins. Reverse-transcription polymerase chain reaction was performed on cDNA samples prepared from either E14 $CD4^-CD8^-$ thymocytes, or various stromal cell preparations obtained from 2-deoxyguanosine-treated thymus lobes. Note that BMP4 expression is restricted to thymic stromal cells, including MHC class II^+ thymic epithelium. In contrast, analysis of expression of the BMP receptors BMP-R1a and BMP-R2 shows expression by both T-cell precursors and thymic epithelial cells, while expression of the BMP-R1b is limited to MHC class II^+ thymic epithelium. Such findings raise the possibility that BMP signaling in the thymus may play a role in the development of both T-cell precursors and thymic epithelial cells.

Thymic stromal cells and T-cell selection

A key stage during T-cell development in the thymus is when immature $CD4^+CD8^+$ thymocytes undergo positive and negative selection events based on the specificities of the αβTCR complexes they express. Thus, thymocytes which recognize self-peptide/MHC complexes on thymic stromal cells at a low avidity undergo a positive selection process, which results in rescue from cell death and the induction of a differentiation program leading to the generation of functionally competent MHC class I-restricted cytotoxic $CD8^+$ T cells and MHC class II-restricted helper $CD4^+$ T cells (135). In contrast, thymocytes bearing potentially autoreactive αβTCR specificities that recognize stromal cell-expressed peptide/MHC complexes at a high avidity undergo negative selection by the induction of apoptosis (136). Through these mechanisms, T-cell development in the thymus results in the generation of a self-tolerant T-cell pool capable of recognizing foreign antigens in the context of self-MHC molecules. A key aspect in understanding the mechanisms underlying T-cell selection lies in the identification of the cell types that are able to regulate T-cell selection. As recognition of self-peptide/MHC complexes is essential for both positive and negative selection, analysis of the distribution of MHC class I and class II expression in the thymus identifies candidate cell types for these processes. In the thymus, both epithelial cells and bone marrow-derived DCs coexpress MHC class I and class II molecules, and some models of TCR repertoire selection support the notion that thymic epithelial cells and DCs show some specialization in their abilities to support positive and negative selection, respectively (137–139). As discussed

below, whether this reflects a distinct nonoverlapping compartmentalization of thymic stromal cell function or whether there is promiscuity in the ability of stromal cells to mediate both types of intrathymic selection events is not fully understood.

Are thymic epithelial cells unique mediators of positive selection?

Initial experiments using MHC-mismatched bone marrow chimeric mice supported the idea that the generation of functionally competent T-cells in the thymus is mediated by nonbone marrow-derived, radioresistant thymic stromal cells (140, 141). Such conclusions are also supported by further experiments where restricting MHC expression in the thymus to thymic epithelial cells still allows positive selection to occur (142, 143). To analyze further the ability of thymic epithelial cells to mediate positive selection of thymocytes, we have adapted a cell conjugate assay in which freshly isolated thymic epithelial cells are allowed to interact with preselection $CD4^+CD8^+$ thymocytes. Cell–cell conjugate formation in this system is critically dependent upon TCR–MHC interactions, and interactions result in maturation to the $CD4^+$ and $CD8^+$ stages (144). Moreover, upon binding to thymic epithelium, we find that thymocytes undergo rapid changes in the distribution and polarization of several integral membrane proteins such as CD3, leukocyte function-associated antigen-1 (LFA-1), and CD45, at the point of contact with thymic epithelium. Such changes in cell surface molecule distribution are accompanied by a redistribution of key intracellular signaling molecules, such as linker for activation of T cells (LAT) and $p56^{lck}$ (144), and a polarization of the thymocyte actin cytoskeleton toward the thymic epithelial cell. Interestingly, thymic epithelial cells also undergo actin polarization following thymocyte contact, suggesting that contact induces changes in both sides of the cell–cell interaction. Thus, thymic epithelial cells are able to induce rapid changes in the cellular distribution of key cell-surface and signaling molecules in thymocytes, which accompany their maturation to the single positive stage.

While the above studies are supportive of a specialized role for thymic epithelium in positive selection, such findings do not necessarily show that the provision of the signals driving positive selection is a unique property of thymic epithelium. Whether there is an underlying requirement for thymic epithelial cells in positive selection has been addressed most recently using an embryo fusion approach to generate tetra-parental chimeric mice in which expression of MHC molecules of particular haplotypes can be confined to either thymic epithelial cells or nonthymic epithelial cells (145). Following viral infection, $CD8^+$ T-cell responses were found to occur at similar levels, regardless of whether the appropriate MHC class I molecules were expressed by thymic epithelial cells or nonthymic epithelial cells. Such findings are in agreement with several other studies, which have shown that when MHC expression is limited to nonthymic epithelial cells, including bone marrow-derived cells and mesenchymal fibroblasts, positive selection can still occur (146, 147). Importantly however, as these experiments were performed *in vivo* in the presence of host thymic epithelium, these findings could be the result of a three-cell system in which TCR ligands can be provided by any cell type, while the accessory interactions required to promote positive selection are still provided by thymic epithelial cells. Moreover, measuring the relative efficiency of positive selection in these systems, particularly following viral challenge which would operate via a postselection mechanism and result in the expansion of mature T cells irrespective of their initial frequency, means it is difficult to directly measure the efficiencies of different cell types to support positive selection *in vivo*. In contrast, *in vitro* experiments performed in the absence of any thymic epithelial cell support have shown that there is an essential requirement for thymic epithelial cells to provide the signals required to induce differentiation of $CD4^+CD8^+$ thymocytes during the positive selection process (148). Following such observations, it is proposed that thymic epithelial cells are specialized for their ability to support efficient positive selection because of their expression pattern of accessory molecules. This specialization may be in the form of cell type-specific cell-surface receptors that are uniquely expressed by thymic epithelial cells or may be in the form of a unique combination of cell-surface molecules that are individually expressed on other tissues. Clarification of the specialization of thymic epithelial cells for positive selection requires further studies to identify the cell-surface molecules they express and the subsequent identification of the accessory interactions they provide that are necessary to promote maturation of $CD4^+CD8^+$ thymocytes.

Thymic epithelial cells support the development of multiple lineages from $CD4^+CD8^+$ precursors

The precursor–product relationship between immature $CD4^+CD8^+$ thymocytes and conventional $\alpha\beta$TCR expressing $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells is well established (149). In addition to these populations, it is now clear that $CD4^+CD8^+$ intermediates can also generate other distinct T-cell lineages. While invariant Vα14i natural killer T (iNKT) cells are generated from $CD4^+CD8^+$ thymocytes as a result of interactions with CD1d molecules expressed by thymocytes themselves

(150), other studies have shown that thymic epithelial cells induce positive selection of several lineages from $CD4^+CD8^+$ intermediates. With regard to $CD8^+$ T cells, expression of CD8α and CD8β chains distinguishes heterogeneity within this population, such that the $\alpha\beta TCR^+CD8^+$ T-cell compartment contains cells expressing CD8 either as an αβ heterodimer or a αα homodimer. These latter $CD8\alpha\alpha^+$ T cells have distinct properties from the conventional CD8αβ expressing T cells, including the ability to recognize the nonclassical MHC molecule TL (151). Using reaggregate thymus organ cultures, Yamagata *et al.* (151) showed that interactions with thymic epithelial cells expressing an MHC class I-restricted agonist peptide resulted in the positive selection of $CD4^-CD8^+$ cells expressing a CD8αα homodimer as well as FcεRγ and Id2, which are features typical of NK cells (151). Thus, thymic epithelial cell expression of a peptide/MHC ligand that, when expressed by bone marrow-derived cells promotes negative selection, results in the generation of a distinct $CD8^+$ T-cell lineage (151, 152).

A parallel to the above studies on $CD8^+$ T-cell-positive selection can also be drawn from the $CD4^+$ T-cell lineage, where analysis of expression of CD25 and the transcription factor FoxP3 allows the separation of $CD4^+$ T cells into conventional $CD4^+CD25^-FoxP3^-$ and regulatory $CD4^+CD25^+FoxP3^+$ (Treg) subsets (153). Cortical epithelial cells have been shown to be important for the positive selection of both these $CD4^+$ T-cell lineages (154), with interactions with agonist peptide ligands expressed by thymic epithelial cells being implicated in Treg development (155, 156). How interactions between $CD4^+CD8^+$ thymocytes and thymic epithelial cells can result in such distinct developmental outcomes is not clear. In addition to the importance of the nature of the TCR ligand, thymic epithelial cells may express particular cell-surface molecules, which bind to receptors on $CD4^+CD8^+$ thymocytes and directly induce a program of gene expression, which plays a role in lineage choice. With regard to $CD25^+$ Tregs, FoxP3 has been shown to play an important role in Treg development (157, 158), with recent evidence suggesting that CD28 signaling can induce FoxP3 gene expression (159). Interestingly however, thymic epithelial cells have been shown to be heterogeneous in their expression of the CD28 counter-receptors CD80 and CD86, with expression detectable in medullary epithelium but not cortical epithelium (160, 161). How such findings relate to observations suggesting that cortical epithelial cells support Treg-positive selection is not clear. One possibility is that while cortical epithelium provides agonist peptide/MHC ligands resulting in the initiation of Treg development, additional interactions with medullary epithelial cells expressing CD80/CD86 are required for the induction of FoxP3 expression and completion of Treg selection. Alternatively, induction of FoxP3 expression could also occur as a result of signaling through thymocyte-expressed receptors other than CD28, with cortical epithelial cells expressing the appropriate ligands. Indeed, in support of the latter, FoxP3 mRNA is detectable, albeit at lower levels, in $CD4^+CD8^-$ thymocytes in CD28-deficient mice (our unpublished observations). Again, a clearer understanding of the costimulatory and accessory molecules expressed by subsets of thymic epithelial cells will aid analysis of the specialization of these cells for the positive selection of various T-cell lineages.

Thymic stromal cells and negative selection

Thymic DCs

As well as mediating the positive selection and differentiation of $CD4^+CD8^+$ thymocytes, the thymus also ensures tolerance to self-antigens by supporting the removal of potentially autoreactive TCR specificities. It is now clear that tolerance to self-antigens in the thymus is achieved by several different mechanisms, including $CD25^+$ Treg generation and clonal deletion by apoptosis. While thymic epithelial cells are implicated in Treg development (155, 156), bone marrow-derived DCs have been shown to be potent mediators of thymocyte negative selection. The generation of mice expressing MHC molecules only on nonhemopoietic cells has shown that absence of negative selection by bone marrow-derived cells results in the generation of autoreactive T cells, presumably as a consequence of unopposed positive selection (142, 143). Addition of DCs to thymic epithelial cell reaggregate cultures has also been shown to dramatically reduce the overall efficiency of positive selection (138, 162), indicating that considerable overlap occurs between positive and negative selection events in the thymus.

Dendritic cell expressing MHC class I and class II molecules are located predominantly at the CMJ in the thymus (137), where they are thought to screen developing $CD4^+CD8^+$ thymocytes for their TCR specificities as they undergo positive selection and migration from the cortex to the medulla. While this finding suggests that the positioning of DCs in the thymus may be important for their ability to mediate efficient negative selection, it is important to note that negative selection can also occur when corticomedullary migration of thymocytes is abnormal (81). Interestingly, several studies have shown that the recruitment of new T-cell precursors to the thymus is accompanied by the migration of a cohort of DCs

(66, 75), suggesting that a new influx of DCs to the thymus occurs to ensure efficient negative selection during each wave of T-cell precursor development. The mechanisms of DC recruitment to the thymus are not clear. However, mice deficient in the NF-κB family member Rel-b lack normal thymic DC subsets, which has been shown to be a direct consequence of an absence of an organized thymic medulla (163), suggesting that medullary epithelial cells may play a role in the recruitment and positioning of DCs in the thymus.

Medullary thymic epithelial cells

T cells produced in the thymus are largely tolerant to the wide range of self-antigens expressed throughout the body, including peripherally expressed tissue-specific proteins. Until recently, whether tolerance to peripheral tissues is achieved during T-cell development in thymus has been poorly understood. The finding that the thymus supports development of $CD4^+CD25^+$ FoxP3-expressing T cells with the ability to regulate autoreactive T cells provides strong evidence that events in the thymus play a key role in preventing unwanted immune responses to peripheral tissues (153–156). In addition, it is now clear that a wide range of peripheral antigens, which are typical of many tissues such as the central nervous system, salivary gland, and pancreas, are expressed in the thymus (164, 165). These findings indicate that developing thymocytes can encounter tissue-specific antigens (TSAs) in the thymus, providing evidence that tolerance to peripheral tissues involves intrathymic mechanisms.

Many studies have now extended these initial observations and have shown that TSAs are expressed by thymic epithelial cells, including those in the cortex and medulla (166). Moreover, intrathymic expression of such antigens has directly been shown to induce T-cell tolerance (167), highlighting the functional significance of intrathymic TSA expression. Importantly, expression of the transcription factor Aire (autoimmune regulator) has now been shown to play a major role in the intrathymic expression of some but not all TSAs (168). Thus, Aire is expressed by medullary but not cortical epithelial cells in the thymus, with lower levels of Aire are also detectable in thymic DCs (169, 170). Aire-deficient mice have been shown to have reduced intrathymic expression of several TSAs, including salivary protein 1, casein α, and preproinsulin-2, and this alteration in intrathymic gene expression is associated with the onset of multiorgan autoimmune disease, a phenotype resembling the human condition autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), where expression of a defective form of AIRE occurs (171).

The mechanisms that regulate Aire expression in medullary epithelial cells therefore play a key role in ensuring that self-tolerant T cells are produced in the thymus. Recent evidence has suggested that lymphostromal interactions between thymocytes and stromal cells may play a role in this process. In particular, signaling through the lymphotoxin β receptor (LTβR) on medullary epithelial cells as a result of interactions with LT-expressing thymocytes has been shown to influence Aire expression in the thymus (172, 173). As LTβR signaling induces NF-κB activation, such findings correlate well with the demonstration of thymic stromal cell-dependent self- tolerance defects in mice deficient in TNF receptor-associated factor 6 (TRAF-6) (174), and NF-κB-inducing kinase (NIK) (175). While interactions between medullary epithelial cells and thymocytes may be important regulators in the maintenance of Aire expression in the adult thymus, we have shown that Aire is expressed in the thymus prior to thymus colonization (40) and that it continues to be expressed in epithelial cells from the thymus of CD3εtg26 mice, which is devoid of normal T-cell precursors (our unpublished observations). Thus, as stated earlier, the mechanisms that maintain patterns of gene expression in differentiated epithelial cells in the adult thymus may be distinct from those that mediate induction of gene expression during thymus development.

Concluding remarks

The generation of a functionally competent pool of $CD4^+$ and $CD8^+$ T cells is essential for the normal immune responses. The production of T cells in the thymus is known to involve a multistep developmental program that is becoming increasingly well defined. It is clear that many of the signals that are required for particular stages of intrathymic development are provided by thymic stromal cells in the form of cell surface molecules, secreted proteins and ECM components. While some of the mechanisms of thymic stromal cell functioning are beginning to emerge, we still have a poor understanding of the developmental mechanisms that result in epithelial cell heterogeneity in the thymus as well as the precise roles played by discrete stromal compartments during T-cell development. Gaining a clearer understanding of these issues is of increasing practical importance, such as the decline in thymus output with aging, and may aid in the treatment of conditions where the slow recovery of T-cell function following ablative therapy and bone marrow transplantation both reflect compromised function of the thymic microenvironment.

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Review

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Wnt signalling in lung development and diseases

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Abstract

There are several signalling pathways involved in lung organogenesis including Notch, TGFβ /BMP, Sonic hedgehog (Shh), FGF, EGF, and Wnt. Despite the widely acknowledged significance of Wnt signalling in embryonic lung development, the role of different Wnt pathways in lung pathologies has been slow to emerge.

In this review, we will present a synopsis of current Wnt research with particular attention paid to the role of Wnt signals in lung development and in pulmonary diseases.

Overview of Wnt signalling

The Wnt family of 19 secreted glycoproteins control a variety of developmental processes including cell fate specification, proliferation, polarity and migration. Consequently, mis-regulation of Wnt signalling during embryonic development cause developmental defects, while defective Wnt signalling in adult tissue results in the development of various diseases [1]. As Wnt-s have a diverse role in regulating cell functions, Wnt signalling is predictably complex. Wnt family members bind to cell surface receptors called Frizzleds (Fz) and trigger intracellular signalling cascades. The 10 Fz proteins are members of the seven-loop transmembrane receptor family, and are encoded by 9 genes. The assembly of an active receptor complex also requires the presence of the co-receptor low density lipoprotein related protein (LRP) 5/6.

There are at least three signalling pathways involved in the signal transduction process: the canonical or β-catenin dependent, and two non-canonical: the polar cell polarity (PCP) or c-Jun N-terminal kinase (JNK)/ activating protein (AP) 1 dependent and the Ca2+ or protein kinase C (PKC)/Calmodulin kinase (CaMK) II/ nuclear factor of activated T cells (NFAT) dependent signalling pathways. Wnt signalling is modulated by numerous regulatory molecules (for a review see [1,2]) and by frequent interactions amongst the pathways themselves [3]. Wnt molecules have been grouped as canonical (Wnt1, Wnt3, Wnt3a, Wnt7a, Wnt7b, Wnt8) and non-canonical pathway activators (Wnt5a, Wnt4, Wnt11) [4]. The ability of the two groups to trigger canonical or non-canonical signalling cascades, however, is not absolute. Promiscuity of Wnt-s and their receptors are a feature of this developmentally and pathologically important glycoprotein family making studies of Wnt signalling difficult.

Canonical Wnt-pathway

The canonical or β-catenin/Tcf dependent Wnt pathway was discovered first, studied most and as a result reviewed frequently [5,6]. Briefly, in the absence of Wnt signalling, glycogen synthase kinase (GSK-3) is active and phosphorylates β-catenin in the scaffolding protein complex of adenomatous polyposis coli (APC) and axin [7,8]. The phosporylated β-catenin is targeted for ubiquitination and 26S proteasome-mediated degradation, thereby decreasing the cytosolic level of β-catenin [9,10] (Figure

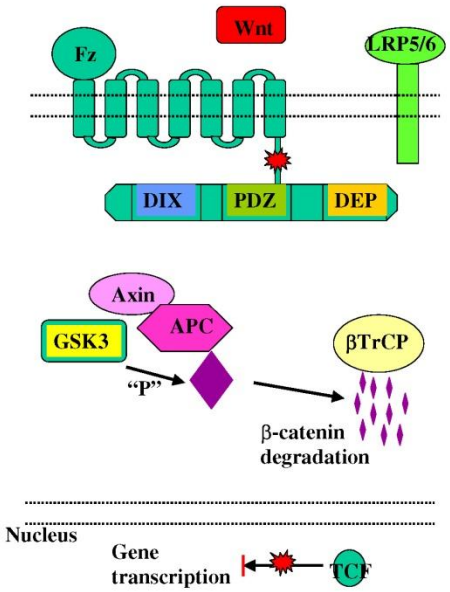

Figure 1
Inhibition of canonical Wnt signalling pathway in the absence of Wnt signals

1). A Wnt-Fz-LRP6 complex is formed in the presence of Wnt-s that leads to the phosphorylation of three domains of Dishevelled (Dvl), which is a family of cytosolic signal transducer molecules [11]. Activation of Dvl ultimately leads to phosphorylation and consequently inhibition of GSK-3. This process is summarised in Figure 2. Inhibition of GSK3 results in stabilisation and consequently cytosolic accumulation of β-catenin (Figure 2). The accumulated β-catenin translocates to the nucleus, where it forms an active transcription complex with members of the T Cell Factor (LEF1, TCF1, TCF3, TCF4) transcription factor family [12,13] and transcription initiator p300 [14]. Successful assembly of the transcription complex leads to target gene activation. Target genes of the canonical β-catenin pathway include matrix metalloproteinases (MMP2, MMP3, MMP7, and MMP9) [15], cyclin D1 [16,17], Cox-2 [18], c-myc [19], c-jun [20], Fra-1 [20], VEGFR [21], etc. (For a recent update see Nusse's Wnt website: http://www.stanford.edu/~rnusse/wntwindow.html).

Non-canonical Wnt-pathways

The non-canonical Wnt pathways, the JNK/AP1 dependent, PCP and the PKC/CAMKII/NFAT dependent Ca2+ pathway (just like the canonical Wnt pathway) become activated following Wnt-Fz receptor binding [22,23]. The non-canonical pathways differ from the β-catenin pathway in their dependency on the type of G-proteins [24] they require for activation. Further downstream, Dvl is critical for signal transduction in both [25] but in contrast to canonical Wnt signalling, phosphorylation of all three domains of Dvl, is not a requirement [26]. Although the Dvl family has long been accepted as cytosol based signal transducers for the three Wnt-pathways, recent studies have revealed the ability of Dvl to translocate into the nucleus where it regulates intranuclear stability of β-catenin [27,28]. How this new function of Dvl fits into the more traditional role of the molecule awaits further investigation.

Nevertheless, downstream of the cytosolic Dvl, the two non-canonical Wnt pathways can activate different signalling cascades and trigger the transcription of different gene-sets, although cross-pathway activation, signal integration, and consequently gene expression modification via complex formation between NFAT and AP1 [29] can also occur. The noncanonical pathways are summarised in figure 3 and 4.

Ca2+ pathway

Following Dvl activation, the Ca-dependent Wnt signalling pathway activates several downstream targets including protein kinase C (PKC), Ca-Calmodulin kinase II (CaMKII), and the Ca sensitive phosphatase, calcineurin [30] before the activation of NFAT [31] occurs. NFAT is a family of transcription factors that regulate activation-induced transcription of many immunologically important genes including interleukin(IL)-2, IL-4, IFN-γ, and TNF-α [32]. Whether the genes outlined above are directly regulated by Ca2+ dependent Wnt signals has yet to be clarified. A prominent member of the non-canonical Wnt pathway activators, Wnt 5a, has recently been connected to pro-inflammatory cytokine (IL6, IL8, IL15) production [33] implicating PKC and NFkB in the process [34], although the role for both PKC and NFkB requires further conformation.

JNK/AP1 dependent PCP pathway

In the PCP pathway, activation of Dvl leads to JNK, and in turn to AP1 activation [35]. AP1 is not a single protein, but a complex of smaller proteins, which can form homo- and heterodimers. The main components of AP1 are cJun, JunB, JunD, cFos, FosB, Fra1, Fra2, ATF2, and CREB. The composition of the AP1 complex is a decisive factor in the selection of genes targeted for activation. Therefore regulation of the individual AP1 components is just as impor-

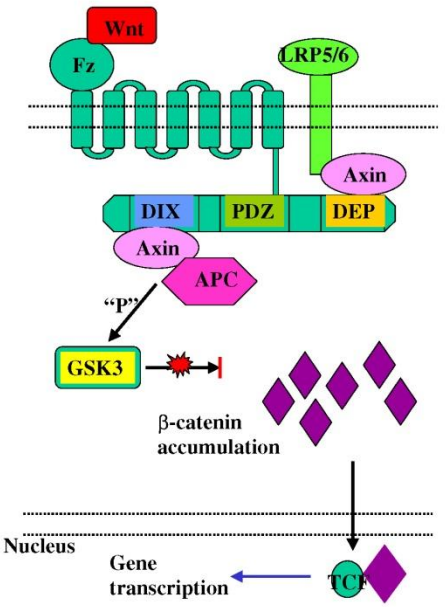

Figure 2
Activation of canonical Wnt signalling pathway in the presence of Wnt signals.

tant as the activation or inhibition of upstream members of the pathway. cJun and Fra1, two prominent members of the AP1 complex, have been identified as target genes of the canonical Wnt signalling pathway [20], indicating yet another potential for cross-regulation between the canonical and the non-canonical Wnt pathways.

Several genes including cyclin D1 [36], MMP-3 [37], Bim [38], GMCSF [39], which are also described as Wnt target genes, are activated by AP1. Although identification of Wnt-signal dependent AP1 target genes are awaiting further investigation, recent studies have implicated both cyclin D1 and MMP-3 as direct targets of JNK-dependent Wnt signalling [40]. Intriguingly, activation of cyclin D1 gene transcription is triggered by a cFos and cJun heterodimer of the AP1 complex [41], in which cJun is a canonical β-catenin pathway target gene. It certainly raises the possibility, that regulation of cyclin D1 expression by the PCP pathway is also influenced indirectly through canonical Wnt signalling.

Regulation of Wnt signalling

The highly complex Wnt signalling pathways are central to the regulation of a wide range of cell functions and therefore tightly controlled. An armada of secreted extracellular (DKK-s [42], sFRP-s [43,44], WIF [45], Cer [46]) and intracellular, both cytosolic (ICAT [47-49], Nkd [50]) and nuclear (Sox17 [51]), signal modulators make Wnt signalling difficult to decipher. Further to individual inhibitors, there is also cross-talk amongst different Wnt signaling pathways. The non-canonical pathways, for example, can also act as regulators of canonical Wnt signalling, often by influencing the phosphorylation and therefore activation state of GSK (one of the main enzymes of the canonical Wnt pathway) [52,53].

Furthermore, inhibitory Fz pathways have also been described. Fz1 [54,55] inhibits Wnt signal transduction via a G-protein dependent manner. The other inhibitory Fz, Fz6, [56], inhibits Wnt dependent gene transcription by activating a Ca dependent signalling cascade involving TAK1 and Nemo-Like Kinase (NLK) [57,58], and ends with the phosphorylation of TCF family members. The resulting structural changes in TCF-s inhibit β-catenin TCF binding and consequently activation of gene transcription [57] (Figure 5).

Wnt signalling in the developing lung

Modulation of Wnt expression in embryonic and adult mouse lung suggests that Wnt pathways are important for cell fate decisions and differentiation of lung cell types. The involvement of canonical Wnt signalling in lung development has been proven by several ways. A TCF promoter-LacZ based reporter system has shown, that canonical Wnt signalling is active throughout lung development in mouse embryos [59]. β-catenin, a central molecule of canonical Wnt signalling, has been shown to localize in the cytoplasm, and often also the nucleus of the undifferentiated primordial epithelium (PE), differentiating alveolar epithelium (AE), and adjacent mesenchyme [60]. Using a conditional knockout system for β-catenin in mice has also revealed that β-catenin dependent signalling is central to the formation of the peripheral airways of the lungs, responsible for conducting gas exchange, but is dispensable for the formation of the proximal airways [61]. Constitutive activation of the canonical Wnt pathway using a β-catenin-Lef1 fusion protein, produced a similar effect [59]. Although proximal airways developed, the lung was reduced in size and lacked alveoli [59].

Recent studies have related particular Wnt production to specific lung cell types. Wnt2 [62] for example has been mapped predominantly to the mesenchyme, Wnt11 to both epithelium and mesenchyme [63], while Wnt7b was exclusively expressed in the lung epithelium [64]. Additional studies have revealed that Wnt7b promoter activity

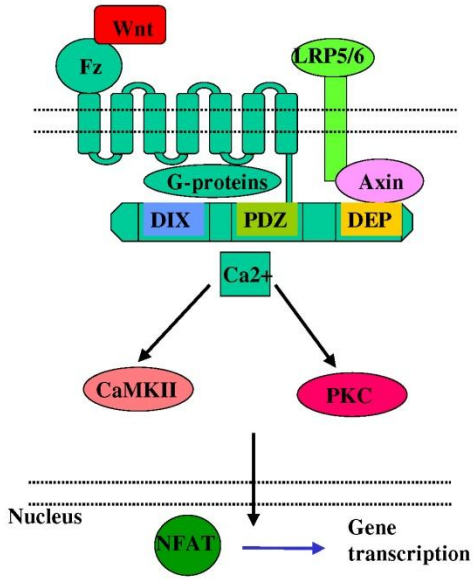

Figure 3
Activation of non-canonical Wnt signalling.

is regulated by a homeodomain transcription factor, TTF1, which is essential to the differentiation of lung epithelium, being especially important for the highly specialised Type II alveolar epithelial cells [65]. Since the TTF1 null mice have a lethal lung phenotype with increased epithelial and mesenchymal proliferation, which at the neonatal stage contains abundant mesenchyme and no functional alveoli [65], it is likely that the lack of functional alveoli is a result of dysregulated Wnt7b signalling [64].

Apart from β-catenin and Wnt-s, mRNA of Fz-1, -2 and -7 and several intracellular signalling molecules including Tcf-1, -3, -4, Lef1, and secreted Fz related proteins (sFrp-1, -2 and -4) have been found to be expressed in the developing lung [60] in specific, spatio-temporal patterns [60]. Wnt signalling has also been reported to be important in the regulation of spatial and distal branching of the lung [61].

While the importance of canonical Wnt signalling in lung development is well established, the role of non-canonical Wnt signalling is less clear. Wnt5a knock-out studies have shown, however, that non-canonical Wnt signalling is also important. In Wnt5a-/- animals the lung is morphologically smaller than in the wild type [66] and has thickened mesenchyme. Furthermore, alveolar development is delayed, although not prevented [66]. Lungs of Wnt5a knock-out animals also have increased expression of FGF10 and Shh [66,67] suggesting that the morphological changes might be related to dysregulation of other signalling pathways modulated by Wnt signalling (see below for further details).

Wnt-s in adult lung

Primary lung tissue and cell lines, derived from adult lung tissue, express a wide range of Wnt-s including Wnt-3, -4, -5a, -7a, -7b, -10b, and -11 [68], as well as Fz-3, -6 and -7 [68], Dvl [69], and Dkk [70]. Since, generally, Wnt signalling retains cells in a low differentiation state, the role of Wnt signalling in adult tissue may not be immediately clear. If we assume that the maintenance of adult organs is stem cell dependent and that stem cells rely on β-catenin and Tcf/Lef signalling to be maintained in the required low differentiation level, the role of Wnt signals in adult tissue becomes understandable. Stem cell niches in proximal and distal airways exist [71,72], similarly to intestine, hair follicle and dermis, and would need Wnt signalling to be able to fulfill their role in maintenance of adult lung structure.

Wnt in lung carcinoma

While lung cancer is one of the leading causes of cancer deaths worldwide [73,74] data regarding the role of Wnt pathways in human lung cancer is still limited. The most studied pathway mutations in cancer are the inherited and sporadic mutations in the tumour suppressor adenomatous polyposis coli (APC) and β-catenin. Since APC is part of the degradation scaffold for β-catenin, mutations of APC can result in reduced degradation and increased nuclear accumulation of β-catenin leading to activation of target genes such as oncogenes cyclin D1 and c-myc [75]. Degradation resistant β-catenin has similar effect on target gene activation [59]. Although increased levels of β-catenin have been reported in different types of lung cancers [76,77], mutations of APC [78] and β-catenin [79,80] are rare in lung cancers. However, proof of dysregulation of specific Wnt molecules leading to oncogenic signalling has emerged. While frequent loss of Wnt7a mRNA was demonstrated in some studies in lung cancer cell lines and primary tumours [81], elevated levels of Wnt1 [82] and Wnt2 [83] have been reported in non small cell lung cancer. Decreased levels of Wnt7a indicates that Wnt7a may function as a tumour suppressor in lung cancer. In support this concept, non-small-cell lung cancer cells transformed with Wnt7a showed inhibition of anchorage independent growth [68]. Although member of the canonical group, Wnt7a inhibits proliferation and induces differentiation via the JNK/AP1 dependent PCP signalling pathway [68]. The role of non-canonical Wnt signalling in the development of lung cancer remains con-

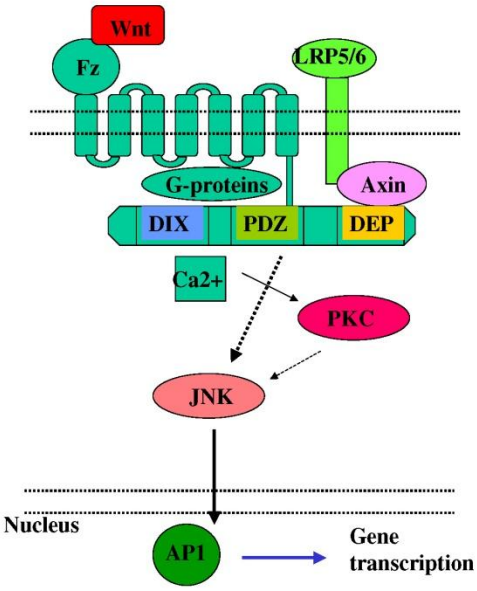

Figure 4
Activation of non-canonical Wnt signalling.

troversial despite recent findings. Although the non-canonical pathway activator Wnt5a is an important regulator of lung development, and generally is an inhibitor of canonical Wnt signalling, elevated levels of Wnt5a in lung metastases of human sarcoma [84] has been reported and thus questions the role of non-canonical Wnt signalling as a general inhibitor of lung cancer. In metastatic stage of any tumours including human lung carcinomas, epithelial-mesenchymal transformation (EMT) is typical [85] and generally linked to increased β-catenin dependent signalling [86]. As β-catenin mutations in lung cancers are relatively rare [79,80,87], another possible mechanism might be at place which regulates EMT and consequently tumour metastasis in the lung. Certainly, non-canonical Wnt5a the very molecule which has recently been reported to regulate fibroblast growth factor (FGF) 10 and sonic hedgehog (Shh) expression [67] has been found elevated in lung metastases [84]. Both FGF-s and the hedgehog family are well-known modulators of epithelial-mesenchymal interactions [88] and epithelial-mesenchymal transformations (EMT) [89-91]. Dysregulation of FGF and Shh signalling certainly raises the possibility that Wnt5a and perhaps non-canonical Wnt signalling in general, is indirect regulator of lung tumour metastasis.

Lung developmental studies have also provided support for the involvement of canonical Wnt signalling in lung cancer. Constitutive activation of the canonical pathway in the developing lung resulted in a non-differentiated lung phenotype resembling cancer [59]. Target genes of the canonical and PCP Wnt pathways include matrix metalloproteinases, which are essential for tissue remodelling and are elevated in invasive cancer [92,93], thus providing additional evidence for the involvement of Wnt signalling in lung cancer.

Overexpression of Dvl, a positive regulator of Wnt signalling pathways has been reported in 75% of non-small-cell-lung-cancer samples compared with autologous matched normal tissue [94]. Downregulation of Wnt pathway antagonists like Dkk3 [70], WIF [95,96] and sFRP [97] have also been reported in various types of lung cancers providing further evidence of the role of this complex pathway.

Wnt in lung inflammation

To date there is no direct evidence for the involvement of Wnt signalling in inflammation of the central airways. However, based on the general features of inflammatory diseases and evidence for Wnt regulated signalling in inflammation in the joint [34], we have addressed the potential involvement of Wnt signalling in inflammatory diseases of the lung.

Increased levels of pro-inflammatory and inflammatory cytokines such as IL1, IL6, IL8, and IL15, monocyte chemotactic protein-1 (MCP-1), TNFα and intercellular adhesion molecule-1 (ICAM-1) are general features of inflammation. The elevated expression of ICAM in the epithelium is important in leukocyte recruitment, adhesion and retention [98], while IL8 secreted by the bronchial epithelium [99], is thought to be central to the attraction of neutrophils. Neutrophils together with macrophages contribute to the pathogenesis of inflammatory tissue injury by reactive oxygen metabolites and proteinase release. Increased levels of tissue matrix metalloproteinases (MMP-s) are a feature of inflammatory conditions and may contribute to the overall evolution of the inflammation-induced tissue destruction. Several pulmonary cells including resident alveolar macrophages, neutrophils, parenchymal cells (including interstitial fibroblasts), type II epithelial cells and vascular endothelial cells are capable of elaborating MMPs [100], and numerous MMP-s, including MMP3 and MMP9, have been considered to have important pro-inflammatory roles in acute lung inflammation [101]. Activation of MMP gene transcription has been attributed to both pro-inflammatory cytokines [102,103] and canonical Wnt signalling [15], but it is still not clear whether they act in competition or in close connection to regulate the transcription of MMP

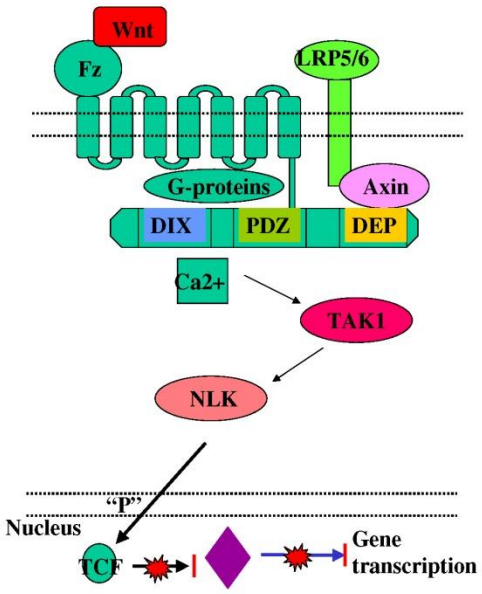

Figure 5
Inhibition of Wnt signalling by a Fz-dependent pathway.

genes. Certainly, the canonical pathway activator Wnt-1 has been linked to stimulation of pro-MMP3 transcription [104], which is implicated in lung inflammation [105]. Understanding of signalling pathway interaction is thus of importance in the study of pathogenic processes and hence disease modulation.

Studies of rheumatoid arthritis have accumulated evidence that Wnt5a-Fz5 mediated signalling can contribute significantly to the production of pro-inflammatory cytokines (IL6, IL8, IL15) [33] and that overexpression of Wnt5a leads to increased pro-inflammatory cytokine levels. Furthermore, dominant negative and antisense Wnt5a and anti-Fz-5 antibody block Wnt5-Fz5 signalling leading to decreased cytokine production [33].

Additionally, the inflammatory cytokine inducing Wnt5a has also been implicated in the down-regulation of Shh levels in the lung [67]. Elevated Shh signalling is well established in the regulation of inflammatory and fibrotic processes of the gut and lung [91]. This suggests a role for Wnt5a but further investigation would be necessary to clarify this in the central airways- in pulmonary inflammation.

Wnt in lung fibrosis

Lung diseases resulting in tissue damage activate a defence mechanism to repair the lesions. Tissue damage can result from several acute and chronic stimuli including inflammation caused by infections, autoimmune reactions (asthma, allergic alveolitis), and drugs and toxins (bleomycin, asbestos) or mechanical injury (surgery, and irradiation). Any tissue repair involves coordinated cellular infiltration together with extracellular matrix deposition and where appropriate, re-epitheliasation. In the first regenerative step, injured cells are replaced by cells of the same type, then normal parenchyma is replaced by connective tissue leading to fibrosis. Usually both steps are required for healing, however, when the fibrotic step becomes uncontrolled and pathogenic, the process can lead to organ failure and death. The interstitial lung disease (ILD) includes a wide range of disorders in which pulmonary inflammation and fibrosis are the final common pathway.

Generally, any activated state of tissue repair requires the stimulation of signalling pathways involved in proliferation, cell migration and differentiation. It is therefore understandable that the fibrotic process is influenced by a combination of growth factors (such as TGFβ, FGF), and cell adhesion molecules (such as integrins). Modulation of growth factor expression, loss of E-cadherin and activation of β-catenin dependent gene transcription leads to epithelial-mesenchymal transition (EMT) which is also an important feature of the fibrotic process. Direct involvement of canonical Wnt signalling in EMT has been confirmed in studies using Wnt1 and Lef-1 overexpression [106]. Furthermore, during cellular migration, which is an important factor in tissue repair, proteolytic degradation of the extracellular matrix is necessary to enable fibroblasts to migrate through the extracellular matrix to the site of the lesion. Proteolytic degradation of the extracellular matrix requires plasminogen and matrix metalloproteinases [107,108]. Gene transcription of MMP-s is regulated by Wnt signalling of both canonical and non-canonical pathways. Metalloproteinase matrilysin (MMP7), a target gene of the canonical Wnt signalling pathway [109], has recently been identified as a key regulator of pulmonary fibrosis [110,111]. In many cases of idiopathic pulmonary fibrosis, the levels of nuclear β-catenin are elevated [112], as are the levels of β-catenin target genes, cyclin D1 and MMP-s [112].

As Wnt-s have also been implicated in the modulation of proliferation and differentiation of many lung cells [59,60,66], the role of Wnt signalling in regulating cell proliferation and differentiation during idiopathic pulmonary fibrosis, is likely to be central rather than a consequence of the disease.

In summary, Wnt signalling may also be central to all causes of pulmonary fibrosis and requires further evaluation.

Interaction of Wnt pathways with FGF, TGFβ / BMP/Smad pathways

Although detailed discussion of interactions of Wnt with other signalling pathways is not the aim of the present review, it is still important to highlight some regulatory interactions, which might also play a role in development and control of pulmonary diseases. Certainly, the non-canonical pathway activator Wnt5a has been implicated in the regulation of several signalling pathways. In Wn5a-/- knockout animals there is increased FGF10 and BMP4 expression [66] suggesting a key role of Wnt5a in the regulation of both factors. Since FGF10 stimulates proliferation and branching in the developing lung and also induces delayed distal epithelial BMP4 expression, which eventually inhibits lung bud outgrowth [113], Wnt5a appears to be a key regulator of cellular proliferation in the lung.

The effect of Wnt-s as signal modulators of other signalling pathways has also been demonstrated. For example, the canonical Wnt pathway inhibitor, ICAT [47], regulates the expression of the BMP pathway inhibitor, BAMBI (BMP and activin membrane-bound inhibitor) [114]. Since ICAT functions by blocking binding sites of TCF-s and p300 on the armadillo domains of β-catenin [47] and therefore inhibiting β-catenin dependent gene transcription, this suggests that BAMBI is not only directly controlled by BMP4 [115] but also by canonical Wnt signalling.

Moreover, both the TGFβ and BMP pathways require Smad-s (reviewed in [116]) for signal transduction but Smad-dependent gene transcription can also be modulated by β-catenin [117,118], binding to Smad-nuclear complexes. A role for the Smad-system activator TGFβ 1 in pulmonary fibrogenesis has recently been confirmed [119]. It was shown that TGFβ 1 has a direct role in regulating EMT by promoting alveolar epithelial cell transition to form mesenchymal cells with a myofibroblast-like phenotype. As both TGFβ and β-catenin signalling induces EMT, a Wnt/TGF signal interaction became evident once again emphasising the need for further studies to define details of signal transduction and pathway coordination to fully understand the underlying processes of EMT.

Since FGF, Shh, TGFβ, and BMP signalling pathways are all important in tissue repair, fibrosis and cancer invasion, it appears, that Wnt signalling can modulate disease progression both directly and indirectly by activating gene transcription and modulating and cross-regulating signalling pathways.

Summary

The involvement of Wnt signalling in lung development, maintenance, cancer, and repair (including idiopathic pulmonary fibrosis) is supported by evidence, while based on indirect evidence a role for Wnt signalling in inflammatory lung diseases can also be postulated. Certainly, better understanding of Wnt signalling in the lung is likely to be important and provide information central to new treatment approaches for a wide variety of lung diseases.

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Regulation of neutrophil apoptosis: A role for protein kinase C and phosphatidylinositol-3-kinase

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Neutrophils play a central role in host defense and are recruited in vast numbers to sites of infection where they phagocytose and kill invading bacterial pathogens. Neutrophils have a short half-life that is extended at the inflamed site by pro-inflammatory cytokines and contact with bacterial cell walls. Normal resolution of inflammation involves the removal of neutrophils and other inflammatory cells by the induction of apoptosis. Spontaneous neutrophil apoptosis does not require Fas ligation, but is mediated by caspases 3, 8 and possibly caspase 9 and also involves activation of protein kinase C-δ. With chronic inflammatory disease, neutrophil apoptosis is delayed by pro-inflammatory cytokines, leading to persistence of neutrophils at the inflamed site and non-specific tissue damage. Here we discuss the evidence for inhibition of neutrophil apoptosis via signaling though PI-3-kinase and downstream pathways, including PDK-1 and PKB. Therapeutic strategies to resolve chronic inflammation could therefore usefully target neutrophil apoptosis and the PI-3-kinase or PKC-δ signaling pathways.

Keywords: apoptosis; inflammation; neutrophil; PI-3-kinase; PKC; T-cell.

Introduction

Neutrophils are short-lived, polymorphonuclear leukocytes that play a major role in the early stages of the inflammatory response to infection, phagocytosing and killing extracellular microbial pathogens. Human neutrophils are produced in the order of $1–2 \times 10^{11}$ cells per day and in the absence of infection they will survive in the circulation for only 24–36 hours before undergoing apoptosis.[1] During infection neutrophils leave the blood under the influence of chemotactic factors that include microbial products (fMLP), complement components (C5a) and chemokines (IL-8). They migrate into tissues and upon reaching the site of infection, begin to phagocytose and kill ingested pathogens, by a variety of mechanisms including release of lytic enzymes and generation of reactive oxygen and nitrogen species. The ingestion of microbes also induces the neutrophil to release pro-inflammatory cytokines that will attract additional inflammatory cells. After killing ingested microbes, neutrophils die by apoptosis and are themselves phagocytosed by macrophages, preventing loss of neutrophil contents and consequent tissue damage.[2] Once the pathogen has been eliminated the inflammatory response must be resolved by the elimination of residual inflammatory cells, including neutrophils, through the induction of apoptosis.[3] The correct regulation of the apoptotic programme is vital to ensure the maintenance of neutrophil numbers in the circulation, the efficient removal of invading pathogens and the rapid resolution of the inflammatory response.

Disregulation of apoptosis may lead to the persistence of immune cells at inflammatory sites and the development of chronic inflammatory disease.[4,5] Perturbation of neutrophil apoptosis has been proposed to contribute significantly to tissue damage associated with inflammatory diseases such as acute respiratory distress syndrome[5] and rheumatoid arthritis.[6] Novel anti-inflammatory therapies based on the restoration of neutrophil apoptosis have considerable promise, but to identify realistic targets it is important firstly to understand the precise pathways that regulate apoptosis in neutrophils, then identify survival factors for neutrophils at inflammatory sites and determine their mode of action. In this review we discuss the role of protein kinase C (PKC) and phosphoinositide-3-kinase (PI3K), signaling enzymes known to be major regulators of cell survival and apoptosis[7,8] in the control of spontaneous neutrophil apoptosis and their potential role in the inhibition of this process by pro-inflammatory cytokines.

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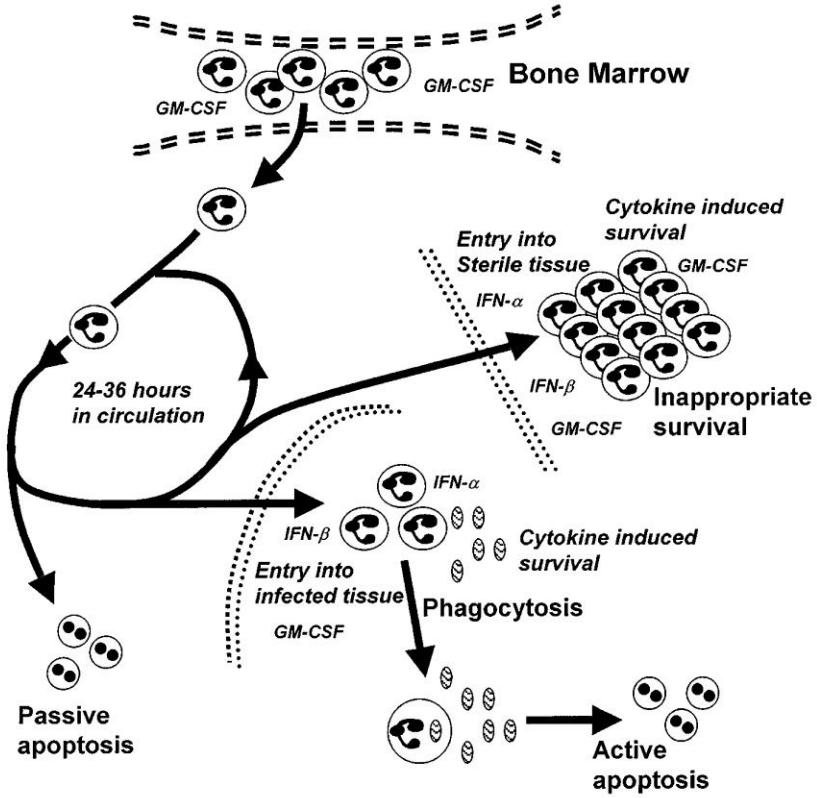
Figure 1. Neutrophils die by cytokine-deprivation induced apoptosis. In the bone marrow neutrophils receive survival signals from cytokines such as GM-CSF. Absence of these cytokines in the circulation limits the life-span of the neutrophil. Recruitment to sites of infection provides survival signals from pro-inflammatory cytokines and apoptosis is induced by neutrophil activation. Recruitment of neutrophils to sterile inflamed sites delays apoptosis inappropriately leading to neutrophil accumulation and tissue damage.



Neutrophil apoptosis

Neutrophils released from the cytokine rich environment of the bone marrow will circulate in the blood for approximately 1–2 days before dying by apoptosis. However, if neutrophils are recruited to a site of infection their life-span is extended by the actions of pro-inflammatory cytokines[5] and death is then induced as a consequence of phagocytosis of microbes and activation of microbicidal mechanisms.[9,10] If neutrophils are recruited to a sterile, chronically inflamed site apoptosis is also delayed by inflammatory cytokines.[4] Apoptosis can not be induced by microbes in this situation and neutrophils accumulate inappropriately leading to tissue damage.[6] We propose that as a normal homeostatic mechanism, effete neutrophils in the circulation die as a result of passive, cytokine deprivation-induced apoptosis rather than by an active induction pathway. Therefore cytokines produced at sites of inflammation can delay neutrophil apoptosis, which is beneficial for the elimination of pathogens, but will promote persistence of inflammation and tissue damage in the absence of infectious agents (Figure 1).

Induction of the apoptotic programme in effete neutrophils in the circulation and during the resolution of inflammation has been proposed to involve signalling through Fas/CD95/Apo-1,[11] reduced expression of anti-apoptotic members of the Bcl-2 family of proteins[12–14] and activation of pro-apoptotic members of the protein kinase C (PKC) isoenzyme family.[15,16] While neutrophils express both Fas and Fas ligand,[11] recent studies employing antagonistic anti-Fas antibodies have shown that signalling through Fas is not required for spontaneous neutrophil apoptosis.[17] Also neutrophils from Fas (*lpr*) or Fas ligand (*gld*) deficient, mice show a normal rate of spontaneous apoptosis.[18] Whether ligation of Fas operates during the resolution of acute inflammation, when a high concentration of neutrophils at the inflammatory site could promote neutrophil fratricide, remains to be established. In addition, the involvement of other death-inducing receptors, such as TNFRII or TRAIL

cannot be ruled out at this stage. However, neutrophil apoptosis is difficult to induce actively *in vitro* through either Fas[5,17] or TNF-α receptors,[5] arguing against a major role for active promotion of neutrophil apoptosis via membrane located death receptors.

In contrast, the data suggesting loss of anti-apoptotic proteins as a primary mediator of spontaneous neutrophil apoptosis is substantial. Firstly, promyeloid cell lines such as HL60 express several anti-apoptotic members of the Bcl-2 family, including Bcl-2 itself. The level of these proteins declines as they differentiate towards the granulocyte lineage.[19] Secondly, a majority of authors have reported that freshly isolated human neutrophils do not express the anti-apoptotic proteins Bcl-2 or Bcl-x_L,[12–14] though they do have detectable levels of the cytosolic Bcl-2 homologues Mcl-1[14] and A-1[20] and the pro-apoptotic proteins Bak[21] and Bax.[14,22] While levels of Bax remained constant as neutrophils were aged in culture and entered apoptosis, Mcl-1 expression declined concomitant with the increasing level of apoptosis.[14] In addition, neutrophils from A-$1^{-/-}$ mice have an accelerated rate of spontaneous apoptosis.[23] Thus the selective loss of anti-apoptotic proteins expressed in neutrophils, in the presence of a high level of pro-apoptotic Bax and Bak proteins, is likely to be a key factor in the promotion of neutrophil apoptosis. Furthermore, loss of Mcl-1 can be maintained by pro-inflammatory cytokines such as GM-CSF,[14] supporting our contention that cytokine deprivation is the primary cause of spontaneous neutrophil apoptosis.

The lack of expression of mitochondrial proteins involved in the inhibition of apoptosis, such as Bcl-2, is intriguing and may reflect a lack of involvement of mitochondria in spontaneous neutrophil apoptosis, bearing in mind their relatively low numbers in neutrophils and their proposed vestigial function in these cells.[24] Alternatively, as a key role of Bcl-2 is to maintain mitochondrial membrane integrity, loss of Bcl-2 may represent a key event allowing mitochondrial permeability transition.[25] Loss of mitochondrial membrane potential ($\Delta\psi$m) occurs as an early event in apoptosis and can be induced downstream of death receptor ligation and caspase 8 activation[26] or as a result of increased intracellular calcium, ceramide or reactive oxygen species.[27] Release of cytochrome *c* resulting from the decrease in $\Delta\psi$m leads to activation of caspase 9 and caspase 3. Caspase 3 can also be activated directly by caspase 8 independent of mitochondrial events,[26,28] though the current literature does not support this mechanism in neutrophils. Neutrophils express only a limited number of the 13 known caspases: caspases 1, 3, 8 and 9.[29,30] Caspase 10 may also be present.[30] Caspase 3 activation has been reported during spontaneous[15,16] and UV irradiation-induced[31] neutrophil apoptosis and inhibition of either caspase 3 or caspase 8 significantly delays spontaneous neutrophil apoptosis.[15,16] There are also preliminary reports showing loss of mitochondrial membrane integrity[32] and activation of caspase 9[29] during spontaneous neutrophil apoptosis. Taken together these data suggest that the activation of caspase 3 seen during spontaneous neutrophil apoptosis occurs via the mitochondria route. However, the initial triggers for both activation of caspase 8 and loss of $\Delta\psi$m remain to be identified, but do not appear to involve Fas ligation.

Protein kinase C and neutrophil apoptosis

Protein kinase C (PKC) is a lipid activated serine/threonine kinase and consists of a catalytic domain and a hydrophobic regulatory domain separated by a protease sensitive hinge region, V3.[33] PKC comprises a multigene family of 11 isoenzymes that are regulated independently and have been sub-divided into three classes according to their requirements for co-factors: the classical PKCs (α, β_I, β_{II}, and γ); the novel PKCs (δ, ε, η, θ and μ); and the atypical PKCs (ζ and ι/λ). Several lines of evidence suggest that PKC isoenzymes are differentially involved in the regulation of apoptosis and the advent of isoenzyme specific inhibitors now means that PKC is a realistic target for the therapeutic modulation of apoptosis.

PKC-α and the atypical PKCs appear to be predominantly anti-apoptotic, whereas novel PKC-δ and PKC-θ are pro-apoptotic.[7,34–36] Thus PKC-α is overexpressed in a variety of tumours[7,37] and its down-regulation or removal by anti-sense RNA,[37,38] both lead to increased apoptosis and tumour regression. Substrates for PKC-α include Bcl-2, with phosphorylation increasing the anti-apoptotic actions of this molecule.[39] In addition, PKC-α is inactivated by ceramide during stress-induced apoptosis[36] and atypical PKC-ζ is cleaved and inactivated by caspase 3 during apoptosis in HeLa cells.[40] In contrast, novel PKC-δ has been implicated in the promotion of apoptosis, initially by the work of Emoto *et al.*[41] who showed that PKC-δ was cleaved by caspase 3 in the V3 hinge region. In the case of PKC-δ, caspase 3 cleavage was not inhibitory as it resulted in the release of the active catalytic 40 kDa kinase domain fragment. Moreover, transfection of HeLa and NIH 3T3 cells with the caspase generated PKC-δ fragment was sufficient to induce an apoptotic morphology.[42] PKC-θ, which has a very high homology to PKC-δ, is also cleaved and activated by caspase 3 during apoptosis.[43] Thus altered expression or changes to the activation status of specific PKC isoenzymes could play a role in neutrophil apoptosis.

Neutrophils express a range of PKC isoenzymes, including pro- and anti-apoptotic members, namely PKCs-α, -β_I, -β_{II}, -δ and -ζ.[15,16,44–46] The classical PKCs are known to be involved in neutrophil activation during the immune response[47] and it is now clear that distinct PKC isoenzymes are involved in the regulation of neutrophil

apoptosis. Analysis of the activation status of PKC isoenzymes in healthy and apoptotic neutrophils revealed that association of PKC-β and δ with the cell membrane, an indicator of PKC activation, was increased in apoptotic cells.[15] In addition the caspase 3-generated catalytic fragment of PKC-δ was also detected in apoptotic neutrophils.[15,16] However, inhibition of PKC-β, using either Go6976[16] or LY379196,[15] did not reduce the rate of spontaneous neutrophil apoptosis,[15] suggesting that this isoenzyme is not directly involved in neutrophil apoptosis. However, neutrophil apoptosis was inhibited by the PKC-δ inhibitor Rottlerin.[15] Furthermore, in a cell free system comprised of healthy nuclei and apoptotic cytosol, nuclear DNA fragmentation was reduced when PKC-δ was depleted from the cytosol using antibody, whereas removal of the other PKC isoenzymes expressed in neutrophils had no effect.[15] Therefore activation of PKC-δ, rather than reduced activity of anti-apoptotic PKC-α, is involved in the promotion of spontaneous neutrophil apoptosis.

Exactly how activation of PKC-δ contributes to apoptosis in human neutrophils is not known. In proliferating cells PKC-δ has been shown to translocate to the nucleus[48–50] and mitochondria[51] during apoptosis. Whilst the significance of mitochondrial translocation is not known, nuclear targets for PKC-δ include proteins involved in DNA replication and repair, DNA-PK,[49] and maintenance of nuclear structure, lamin B.[50] Phosphorylation of lamin B by PKC-δ is required for disassembly of the nuclear lamina during apoptosis.[50] In the non-proliferating neutrophil, PKC-δ also localises to the nucleus during apoptosis[15] and as DNA replication and repair are not operational, it is possible that nuclear PKC-δ primarily regulates the disassembly of the nuclear lamina.

Neutrophils in the circulation are therefore committed to death by apoptosis, achieved by activation of caspases, including caspase 3, caspase 8 and possibly caspase 9 and activation of pro-apoptotic PKC-δ. Although neutrophils are committed to apoptosis, their death can be delayed at sites of inflammation by external factors including pro-inflammatory cytokines,[52] bacterial membrane components such as lipopolysaccharide,[52] and pro-granulocyte differentiation factors such as GM-CSF.[52,53] These mechanisms are important during acute inflammation responses when extension of neutrophil lifespan will contribute to accumulation of neutrophils at sites of infection and benefit microbicidal efficiency. However, inappropriate retention or recruitment of neutrophils to sterile sites (Figure 1) can lead to chronic inflammatory disease.[5,6] Knowledge of the signalling pathways employed by cytokines to delay neutrophil apoptosis will identify novel therapeutic targets for inflammatory diseases such as rheumatoid arthritis.

Inhibition of neutrophil apoptosis

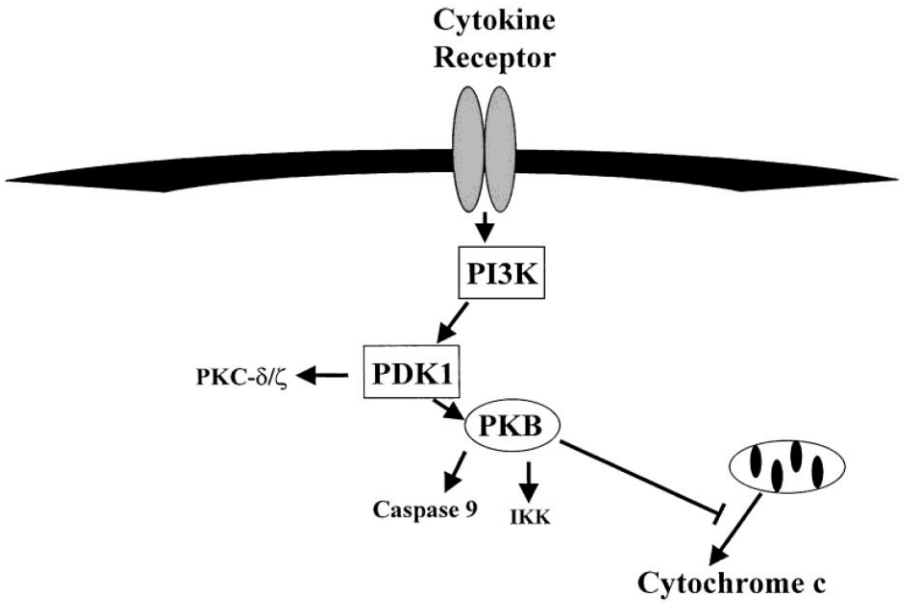
As stated above, several pro-inflammatory cytokines are able to delay neutrophil apoptosis.[52–55] In the normal immune response to infection many of these agents function to prime the neutrophil. Neutrophils in the circulation are relatively unresponsive to bacterial compounds such as fMLP and require prior contact with priming agents to induce optimal activation. Priming also influences neutrophil survival, which can be beneficial during infection, but can have pathological consequences if the primed state is inappropriate.[5] Neutrophils then persist inappropriately at inflamed sites and induce tissue damage.

Priming agents include the chemokine IL-8[56] that binds to G-protein linked receptors, as well as cytokines whose receptors signal through tyrosine kinase activation, e.g. GM-CSF[57] and G-CSF.[52] Neutrophil apoptosis can also be delayed by pro-inflammatory cytokines that are not involved in neutrophil priming. Our own studies have shown that Type-1 interferon, which delays T cell apoptosis in the synovial fluid of patients with rheumatoid arthritis,[58] also delays neutrophil apoptosis (Wang and Scheel-Toellner, unpublished observations). As neutrophils comprise a significant proportion of the cellular infiltrate in the rheumatoid synovium, Type-1 interferon may mediate their increased survival and promote chronic inflammation and neutrophil mediated joint damage. Whether there is a common link between the downstream signaling pathways activated by these agents that delay neutrophil apoptosis remains to be established, though we propose here that one possibility is the activation of the phosphatidylinositol-3-kinase (PI3K)–3-phosphoinositide dependent kinase-1 (PDK-1)–Protein kinase B (PKB) pathway.

PI3K is activated by a variety of growth and survival factor receptors[8,59] and phosphorylates inositol lipids at the 3′-OH position to generate the second messengers phosphatidylinositol 3,4-bisphosphate (PI3,4P_2) and phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5P_3). PI3,4,5P_3 binds to PKB inducing its translocation to the cell membrane and also activates PDK-1, which then phosphorylates and activates PKB (Figure 2). PDK-1 has a variety of downstream targets in addition to PKB, including PKC, cyclic AMP dependent protein kinase (PKA) and p70 S6 kinase.[60] PKB has been identified as playing a key role in transduction of survival signals by cytokine and growth factor receptors in many cell types[8,59] and may therefore also be involved in the delay of neutrophil apoptosis by pro-inflammatory cytokines.

Protein kinase B (Akt), is a 57 kDa serine/threonine kinase with a high degree of homology to PKA and PKC, that was first identified as the cellular homologue of the oncogene, *v-akt*, transduced by the retrovirus AKT8.[61] PKB comprises three isoforms in mammals, namely

Figure 2. Signaling pathways potentially involved in the prevention of neutrophil apoptosis by pro-inflammatory cytokines. Ligation of cytokine receptors recruits PI3K to the receptor, via G proteins or receptor associated tyrosine kinases. PI3K generates PI3,4,5P_3 which leads to the activation of PDK-1 and PKB. PKB targets include anti-apoptotic proteins caspase 9 and IκB kinase (IKK) and mitochondrial proteins. Downstream targets of PDK-1 include PKC and PKA.



PKBα/Akt1, PKBβ/Akt2, PKBγ/Akt3 (reviewed in [60]). Any differential functions of the three PKB isoforms have not yet been identified. Recent studies have identified several potential anti-apoptotic targets of PKB, including: the pro-apoptotic Bcl-2 family protein BAD, whose activity is reduced by phosphorylation at Ser^{112} or Ser^{136};[62] human caspase-9, which has reduced ability to cleave and activate caspase-3 in a phosphorylated state;[63] and IκB kinase, which is activated upon association with PKB,[64,65] leading to activation of NF-κB and transcription of anti-apoptotic genes.[66] More recently, PKB has been shown to inhibit upstream events in the apoptotic programme, most notably the loss of mitochondrial membrane potential.[67] Several of these survival mechanisms are potentially relevant to neutrophil apoptosis, which may involve loss of mitochondrial membrane integrity,[32] activation of caspase 9 [29] and loss of NF-κB activity.[68]

Despite the current interest in PKB-mediated cell survival, the majority of studies in neutrophils have concerned the role of PKB in neutrophil activation rather than apoptosis. Activation of neutrophils by fMLP and ligation of Fcγ-receptors results in PKB activation in a PI3K-dependent manner, indicating a role for PKB in respiratory burst and phagocytosis.[69] However, GM-CSF, which delays neutrophil apoptosis, has been shown to activate PI3K via recruitment and activation of the tyrosine kinase Jak 2.[70] Our own studies have shown that the inhibition of neutrophil apoptosis by Type-1 interferon is also PI3K-dependent (Wang and Scheel-Toellner, unpublished observations) and it is likely that PI3K is activated via association of STAT3 with the interferon receptor as reported by others.[71] However, the activation of PKB downstream of PI3K by either of these survival promoting cytokines has not yet been demonstrated and it is possible that PKB is involved primarily in neutrophil activation. If this is true then survival may be mediated directly by the lipid products of PI3K, independent of PKB. For example, PDK-1 is activated by PI3K-generated lipids and has substrates in addition to PKB that are also implicated in the prevention of apoptosis, for example PKC-ζ.[72] Also, in our studies in T cells, Type-1 interferon mediated inhibition of apoptosis was associated with a rapid reversal of the nuclear translocation of PKC-δ that occurred early in the apoptotic process.[48] As PKC-δ can also be phosphorylated by PDK-1,[72] it is possible that movement away from the nucleus is mediated by this kinase. Whether Type-1 interferon prevents nuclear PKC-δ translocation in neutrophils is currently under investigation.

Conclusions

Effete neutrophils die by apoptosis and are removed from the circulation by macrophages. Their spontaneous death does not require signaling through Fas, but does involve activation of caspase 8, caspase 3, PKC-δ and possibly

caspase 9. The initial stimulus for the constitutive apoptotic programme in neutrophils remains to be established. However, as neutrophil apoptosis can be delayed by factors such as GM-CSF, which are present at high levels in the bone marrow, we speculate that release of neutrophils from the marrow essentially removes survival factor signals. Spontaneous neutrophil apoptosis thus results from a constitutive growth/survival factor deprivation. Furthermore, the lack of expression of anti-apoptotic proteins such as Bcl-2, ensures that the cell dies rapidly unless death is delayed at sites of inflammation by pro-inflammatory cytokines.

Neutrophils are present in high numbers at inflammatory foci in chronic inflammatory diseases such as rheumatoid arthritis (RA) and their presence at these sites has been linked to tissue damage.[73] These inflammatory sites also contain high levels of pro-inflammatory cytokines able to delay the apoptosis of leucocytes,[6,58] including neutrophils,[6,52] by signaling through the PI3K pathway. The continued presence of pro-inflammatory cytokines at inflammatory foci in diseases such as RA ensures that inflammatory cell apoptosis is delayed and hypercellularity persists. Future therapeutic approaches to the treatment of chronic inflammatory disease may thus be usefully targeted at pathways regulating neutrophil apoptosis, such as PI3K and PKC-δ.

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