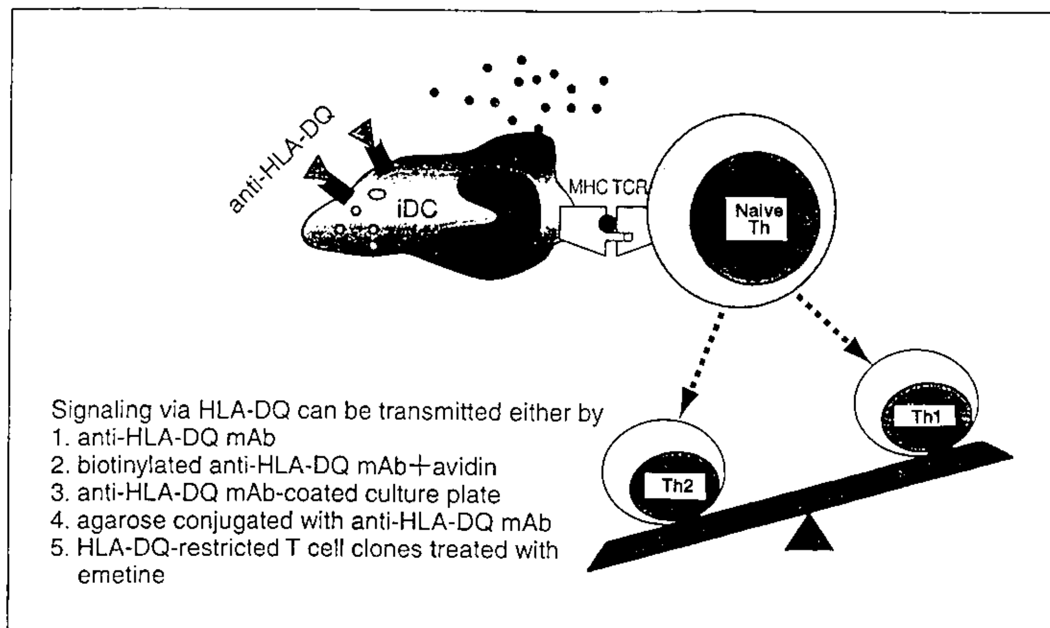

図5 HLA-DQを介した刺激はDC2を誘導する

を図4に示した．予想された通り，HLA-DQ分子を介した刺激はDC2を誘導することが明らかとなった(図5)．

活性化T細胞における MHC class II シグナルの効果

HLA classII分子は，マウス以外のげっ歯類やヒトの活性化T細胞上にも発現しているが，その機能の詳細については不明であった．しかし，T細胞に発現しているHLA-DR分子の架橋によって，PLC-γ1を含む種々の蛋白のチロシンリン酸化が誘導され，細胞質の遊離カルシウムイオン([Ca2⁺]i)の上昇が誘導されるという報告や，ヒト活性化T細胞上のHLA-DR分子を架橋すると，ZAP-70分子のチロシンリン酸化が誘導されるという報告からみても，T細胞上のclassII MHCを介したシグナルがT細胞自身になんらかの活性化シグナルを伝達していることは確実である．

T細胞はTCRとHLA-DR分子の両方を発現しているので，可溶性抗原ペプチドによって誘導されるアナジーの場合には，シグナルはTCRを介してだけでなく，classII HLA分子を介してもT細胞に伝えられる．CD28分子からの共刺激の欠損によって誘導されるアナジーにおいて，Rap1とCDKインヒビターp27Kip1の持続的な発現増加が関係しているという報告があるが，われわれが行った実験では，可溶性抗原ペプチドによって誘導される場合でも同様に，この現象が認められた[16]．しかし，この場合，CD28分子を介したシグナルを共存させてもアナジーを回避することはできない．そこで，活性化T細胞上のHLA-DR分子を抗体で架橋したところ，T細胞は一度増殖反応を起こした後アナジーに陥ることが明らかとなった[16]．この現象に伴って，やはりRap1とp27Kip1の発現が増強が観察された．以上より，たとえRap1やCDKインヒビターの挙動は一見同じでも，共刺激の欠損によって誘導されるアナジーとHLA-DR分子の架橋によって誘導されるアナジーとでは分子機構が異なると考えられる．DRの架橋によるアナジーの誘導は，可溶性抗原ペプチドによるヒトT細胞アナジーの誘導の際に貢献しているのかもしれない(図6)．

線維芽細胞における MHC class II シグナルの効果

線維芽細胞はIFN-γ存在下において，その細胞膜表面にclassII HLA分子を発現する．しかし，線維芽細胞が抗原提示細胞として機能するか否かについては議論の分かれるところであった．リンパ器官において，線維芽細胞がT細胞の増殖を誘導するとの報告もある[17]が，それには局所におけるIL-2などのサイトカイン，あるいはco-

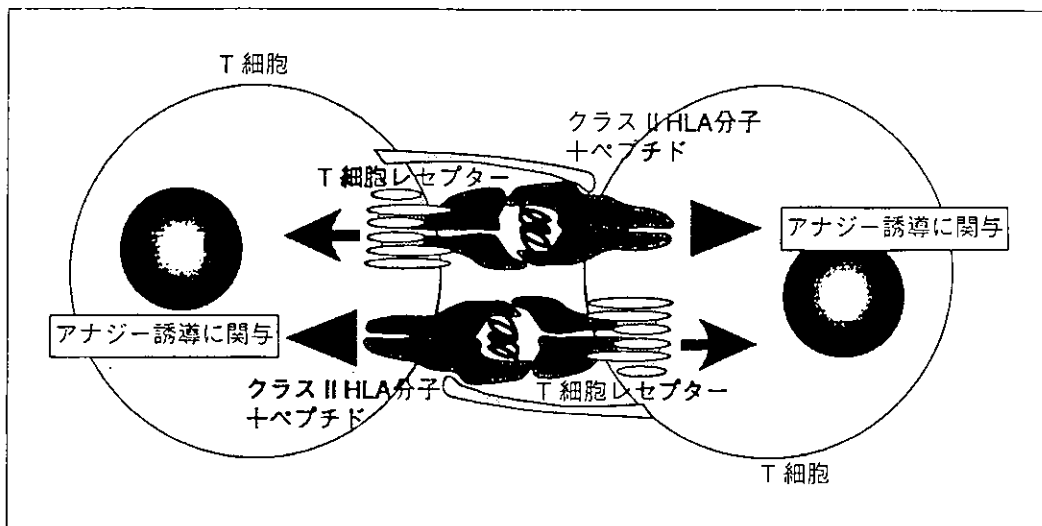

図6　可溶性抗原ペプチドによるヒトT細胞アナジーの誘導
T細胞を可溶性抗原ペプチドとともに培養すると，アナジーが誘導されることが知られている．可溶性ペプチドの添加により，T細胞上のclassⅡHLA分子により提示された抗原ペプチドをT細胞が認識すると，一度増殖した後にアナジーに陥る．　（文献[19]より引用）

stimulatory分子の発現量などさまざまな因子が関与すると考えられる．

抗原ペプチドを提示させた自己のclassⅡ分子を発現する歯根膜線維芽細胞(PDL)とその抗原ペプチドを特異的に認識するTh細胞クローンを共培養しても，それらTh細胞の増殖は誘導されない．

われわれは線維芽細胞上のclassⅡ分子を抗classⅡHLA抗体によって架橋することによって，線維芽細胞はRANTES, MCP-1やIL-8などのケモカインや，IL-6を産生することを明らかにした[18]．同様の現象はclassⅡ分子に抗原ペプチドを提示させた線維芽細胞をエメチン処理して*de novo*蛋白の合成を阻害したTh細胞クローンと共培養することによっても誘導される．この線維芽細胞のサイトカイン産生は，自己classⅡ分子拘束性のT細胞と共培養した時にのみ観察されることから，classⅡHLA分子－ペプチド－TCR複合体が形成された結果，細胞内にclassⅡ分子を介したシグナルが伝わることによって誘導されると考えられる．つまり，T細胞が増殖しないような特異的相互作用においても，線維芽細胞側にはサイトカインやケモカインの産生誘導が起こっているわけである(図7)．以上のことから，線維芽細胞上に発現するclassⅡHLA分子の生理的役割は，抗原提示分子というよりむしろ，サイトカイン産生を誘導するレセプター分子としての意味合いが強いといえよう．

おわりに

本稿でご紹介した概念の追求は，HLAが多重遺伝子族として存在するのはなぜか，異なる遺伝子座の対立遺伝子間に密な連鎖不平衡が存在するのはなぜか，といった疑問に解決の糸口を与えるだけではなく，ペプチド収容溝の多型性のみに着目することで分子レベルで確認されてきた免疫応答遺伝子の概念を，根本から見直す必要性をも提示することになるかもしれない．さらには，特定のHLA対立遺伝子と免疫関連疾患の相関について細胞・分子レベルでより深い理解が進むと期待できる．関節リウマチはその代表であるが，特定のclassⅡHLA対立遺伝子との相関が明らかな他の自己免疫病(インスリン依存性糖尿病，多発性硬化症，原田病，高安病，原発性胆汁うっ滞性肝硬変，自己免疫性炎症性腸炎，自己免疫性甲状腺疾患，一部の糸球体腎炎，抗リン脂質抗体症候群など)においても，標的側の応答を理解するという意味において，魅力的な作業仮説を提供するであろう．また，CD40KOマウスにおいてもgerminal centerの形成やクラススイッチが完全には障害されない点，CD40(－)B細胞とT細胞の相互作用でも胸腺

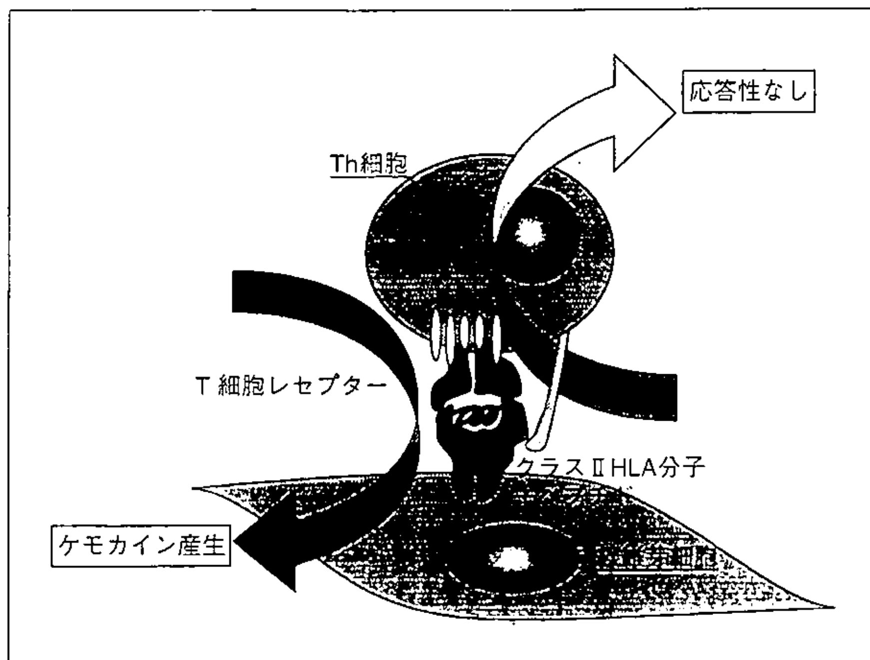

図7　線維芽細胞上に発現するclass II HLA分子の役割
ClassⅡHLA分子に抗原ペプチドを提示させた菌根膜線維芽細胞が，T細胞の増殖を誘導することはない．しかし，HLA-ペプチド-T細胞レセプター複合体の形成によって線維芽細胞側にシグナルが入り，線維芽細胞はケモカインをはじめとしたサイトカインを産生する．（文献[19]より引用）

依存性応答が誘導できる点などからも，MHCを介したシグナルの重要性は再考の余地が十分にあると考えられる．

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話題

Th2アジュバント*

松下　祥**

Key Words: adjuvant, Toll-like receptor, Th2 cells, phospholipids, glycolipids

はじめに

近年，Th2細胞の分化において，特定の性質をもった樹状細胞(dendritic cells; DC)が重要な役割を演じていることが明らかになった．さらに，アレルギー誘導物質には蛋白アレルゲン以外にも重要な活性物質を含んでいることがわかってきた．その多くは低分子物質であり，アジュバント様活性を有している．そのような物質と生体のinterfaceにあって重要な監視役を担っているのがDCである．つまり，DCは環境要因と免疫応答の接点になっている重要な細胞のひとつである，ということができる．この点において，DCはT細胞やB細胞とは大きく異なっているといえよう．アレルギー誘導物質に含まれるアジュバント様物質は従来のアレルゲンとしてではなく，多数のアレルゲンに対するIgE免疫応答の成立に抗原非特異的に関与する，と考えられる．この分野はアレルギー学とその応用の，新たなホットスポットになろうとしている．

Th1アジュバント

近年，プロフェッショナル抗原提示細胞としての樹状細胞(DC)に関する研究が急速に進展してきた．その中で得られた重要な情報の中に，DCに発現するToll-like receptor(TLR)がアジュバント受容体として機能しているという知見がある[1]．細菌などに特徴的な構造をもつ核酸[2)3)]，lipopolysaccharide[4]，さらには真菌由来のユニークな糖蛋白[5]などは，TLRの特定のアイソフォームと結合することにより，DCの形質を変化させ，Th1細胞を誘導しやすくする．このようなDCをDC1細胞と呼ぶ．誘導されたTh1細胞は炎症反応を媒介する重要な細胞である．このようにDC1細胞の誘導を介してTh1細胞を誘導する物質はTh1アジュバントと呼ばれている(図1)．

Th2アジュバント

同様な機序でDCの形質を変化させ(DC2細胞)，これを介してTh2細胞を誘導するような物質もしだいに知られるようになってきた．Whelanらは2000年に発表した論文の中で，初めてTh2アジュバントES-62を物質として記載した[6]．ES-62はフィラリアのnematode由来であり，phosphorylcholine含有糖蛋白である．骨髄由来の未熟DCにGM-CSFとES-62を加えて24h培養後，DO11.10-Tgマウスのnaive CD4 T cellsとOVAペプチドを加え3日間培養した．これをPMA+ionomycinで刺激し，上清のサイトカインを測定する系を用いて，ES-62のTh2アジュバント活性を証明した．この活性はCD80/86の発現バランスには依存していなかった．

Kleijらは2002年に発表した論文の中で，住血吸虫由来のphosphatidylserineがDCを直接刺激し，その結果T細胞応答がTh2寄りに傾くことを示した(図2)．合成されたphosphatidylserineや哺乳類由来のphosphatidylserineにはこのような活性がない．おそらく住血吸虫由来のphosphatidylserine

* Th2 adjuvant.

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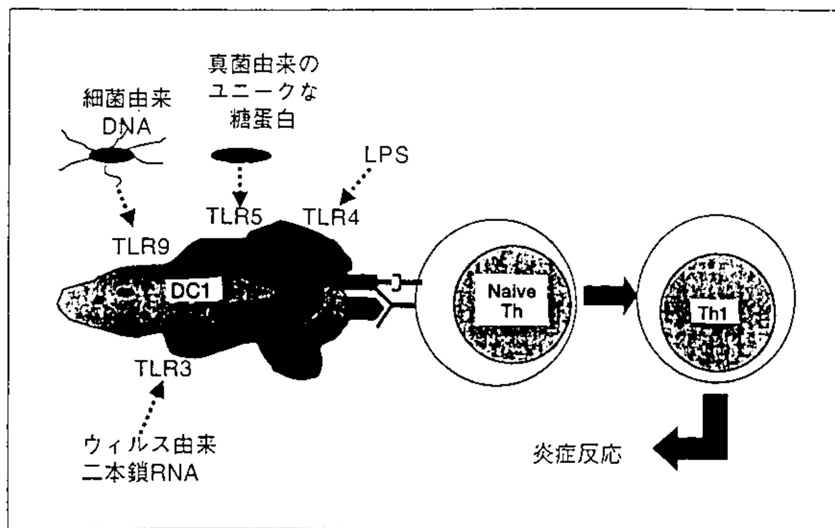

図1　Th1アジュバント
微生物由来のユニークな分子パターンをTLRを介して認識したDCは，ナイーブT細胞を活性化してTh1分化を促進するような性質に変化する．図中には示していないが，TLR3, TLR7, TLR8, TLR9はエンドソームに，ほかのTLRアイソフォームは形質膜上に発現している．

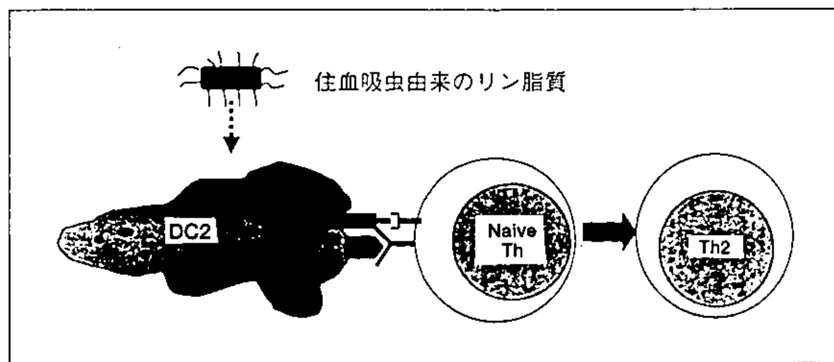

図2　Th2アジュバント
寄生虫由来のユニークなリン脂質を認識したDCは，ナイーブT細胞を活性化してTh2分化を促進するような性質に変化する．

がユニークな構造のアシル基をもっているからであると考えられる[7]．すなわち，寄生虫は抗原特異的な免疫応答を誘導する以前に，抗原提示細胞の応答を抗原非特異的に動かすことによって，Th2アジュバント活性を発揮できるがゆえに「アレルゲン」なのである．

住血吸虫は*de novo*の脂肪酸合成を行っていない．脂肪酸の供給はホストの側から受けており，寄生虫自らがもつ酵素によって，アシル基の伸長を行うことでユニークな構造の脂肪酸を作り出している．興味深いことに，アシル基が1本で不飽和のlysophosphatidylserine (lyso-PS) はIL-10産生性のregulatory T細胞(Tr)を誘導するようなDCを誘導する．この現象は抗TLR2抗体により部分的に阻止される．前述のようにアシル基が2本のphosphatidylserineはTh2を誘導するようなDCを誘導するが，これに関与するレセプターは不明である[8)9)]（図3）．Lysophosphatidic acid (Lyso-PA)やsphingosine-1-phosphate (S1P)も同じようにTh2を誘導するが，lysophosphatidylcholine (Lyso-PC)はTh1を誘導する．構造から考えると，head groupが重要なのであろう（図4）．

具体的に，DCが発現するどのような分子がT細胞分化を制御しているかについては，不明な点が多い．Monocyte chemoattractant protein-1 (MCP-1)はCCL2とも呼ばれており，DCからも産

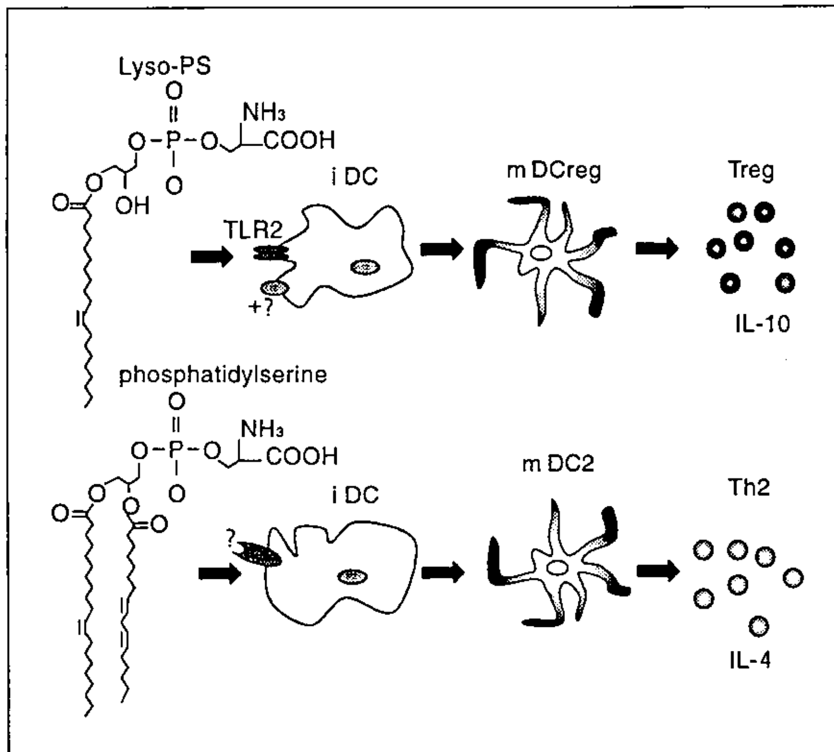

図3 Trを誘導するLyso-PSとTh2を誘導するphosphatidylserineの構造

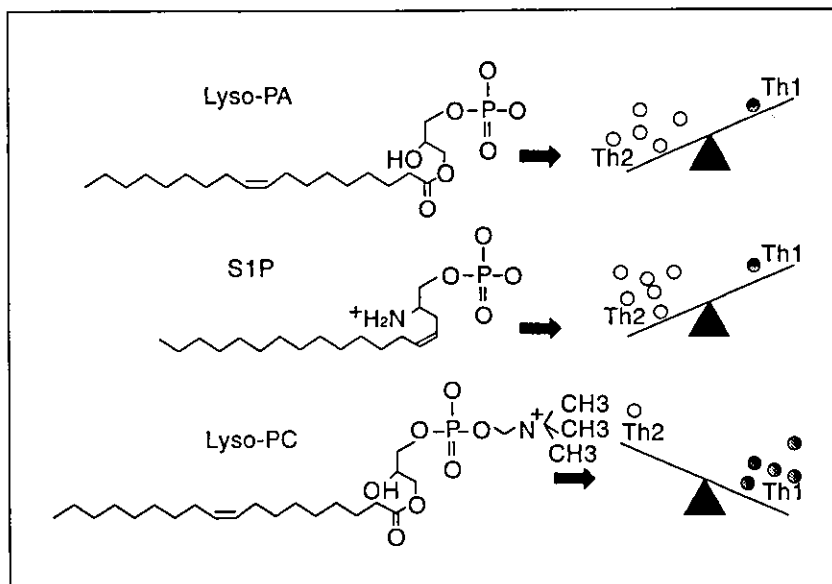

図4 Lyso-PA, S1P, Lyso-PCの構造比較

生される．Guらによれば，CCL2$^{-/-}$マウスではTNP-OVAを免疫してもTh2応答が激減し，IgG1応答も下がる．リーシュマニア感染に対しても抵抗性となる．リンパ球のtrafficingには異常がないため，CCL2はT細胞分化をTh2に傾ける方向に直接影響を与えていると思われる[10]．しかし，ヒトにおける活性は未知数である．Thymic stromal lymphopoietin（TSLP）受容体を発現する細胞種がヒトとマウスで大きく異なるという例もあるため，このような実験結果の解釈には注意を要する．

プロスタグランジンE2（PGE2）やプロスタグランジンD2は昔からTh2アジュバント様の活性を

有していることが知られていた．やはりDCに直接作用できるようである．Kalinskiらによると，TNFα＋PGE2でiDCを刺激すると，p40は産生されるがp70は産生されない．LPSやCD40L刺激ではp40もp70も産生されるが，これらの系をPGE2は抑制する．実はp40のmonomerやhomodimerはp70のantagonistであることが知られており，PGE2刺激ではこれらが産生されるためにTh2アジュバント活性を発揮できるようである．このような機序以外にも，PGE2は，Tに直接働いてIFNγを抑制する，IL-12Rの発現を抑制する，monocyte分化の初期にIL-10産生を誘導する，などの作用も有している[11]．興味深いことに花粉の表層に存在する極性脂質には，ヒトのロイコトリエンB4やPGE2と交差反応をする物質が存在し，受粉において重要な機能を担っているらしい[12]．

ほかに注目すべきTh2アジュバントとして，*C. albicans*の構成成分，ヒスタミン，G protein-R, PRR glycolipids, OX40L, Jagged 1(Notchリガンド)，Pam3Cys-Ser-Lys4(Pam3Cys)などもある．Pam3Cysはmycoplasma-associated lipopeptide 2の活性部分であるが，Erkの活性化とc-Fosの安定化を介してTh2アジュバント活性を発揮できる[13][14]．

では，Th2アジュバントは二次リンパ器官に到達する可能性があるのだろうか？Cokerらは，アレルギー性鼻炎患者の粘膜を用いてnested RT-PCRを行い，VH領域のhypermuationにgenealogical treeを描くことができた．その中にはVDJがそっくりなIgEとIgAも含まれていた．傍証として，局所のactivation-induced cytidine deaminase (AID)の発現上昇もみられた．このような現象は，鼻炎のないアレルギー患者にはあてはまらなかった．つまり，二次リンパ節だけではなく末梢においても，somatic hypermutationとclass switchは起きているということになる[15]．もちろん，活性化されたDCはリンパ節に移動するわけではあるが，局所における相互作用でもアレルギー応答は成立するということを示している．

局所での反応に関連して，Soumelisらは興味深い知見を得ている[16]．TSLPはIL-7様サイトカインである．マウスTSLPはT/Bの初期分化に重要であるが，DCには影響を及ぼさない．しかしヒトではCD11cDCを活性化するにもかかわらず，T/B分化には影響を与えない．TSLPはIL-7Rα＋TSLPRのヘテロダイマーに受容されるが，ヒトではこれがT/BではなくDCに発現しているからである．TSLP, LPS, CD40Lなどで24h刺激したDCをヒトprimary T cell response(アロ認識反応)に加え，その6日後に洗浄後，CD3とCD28を介した刺激を入れた後に産生されるサイトカインを測定するという系を用いて実験した結果，TSLPで刺激されたDCはTARCやMDCを産生し，ヒトT cell分化をTh2寄り(ただしTNFαは作りIL-10は作らない)に片寄らせることが判明した．TSLPは骨髄系ではほぼmast cellsのみに発現している点も興味深い．そのほか，lung fibroblasts, bronchial smooth muscle cells, skin keratinocytes, などへの発現が重要である．実際，皮膚，とくにアトピー性皮膚炎患者の皮膚では，DC-LAMP陽性DC(活性化DC)とTSLP陽性keratinocytesが共存している．おそらく，抗原が侵入した皮膚細胞においてTSLPが産生されてDCを活性化し，このDCがTh2-attracting chemokinesを産生し，所属リンパ節に移動したDCはアレルゲン特異的T細胞の分化をTh2に向かわせ，このTh2はTARC/MDCのgradientに従って局所に戻ってアレルギー炎症を起こしているのでは，と考えることができる．このように環境要因とDCとの間にkeratinocytesが介入しているわけであり，このような中間的存在は肥満細胞，NKT細胞などを用いた系でも検討されるべきであろう．

Th2アジュバントのアッセイ法

われわれは，ほかのアレルギー誘導物質の中にも同様のTh2アジュバント活性を有するものがある，という作業仮説のもと，食品，環境ホルモン，ダニ抽出物，スギ花粉抽出物を用いて研究を進めてきた．その結果，複数の物質にDC2誘導活性があることが明らかとなった．具体的には，PBMCsより，抗ヒトCD14マイクロビーズを用いてCD14陽性細胞を分離し，IL-4, GM-CSFで6日間培養したものをモノサイト由来樹状細胞(Mo-DC)として用いた．この細胞を回収し，DCを成熟させる刺激としてTNFα，ならびに試験したいアジュバントを加えた．2日後に培養上清を回収した後，Mo-DCを洗浄した．この培養

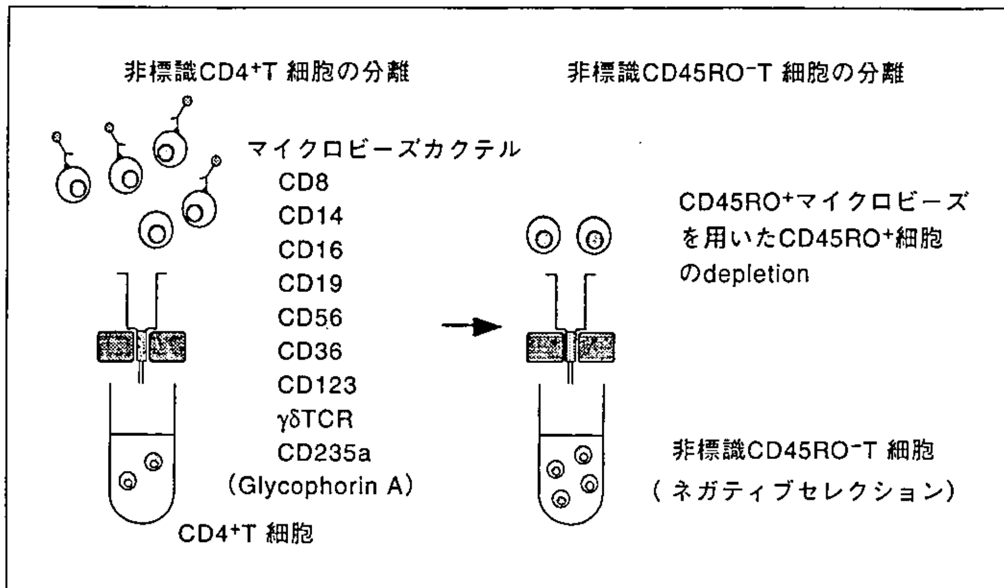

図5 Naive CD4 T細胞の調整

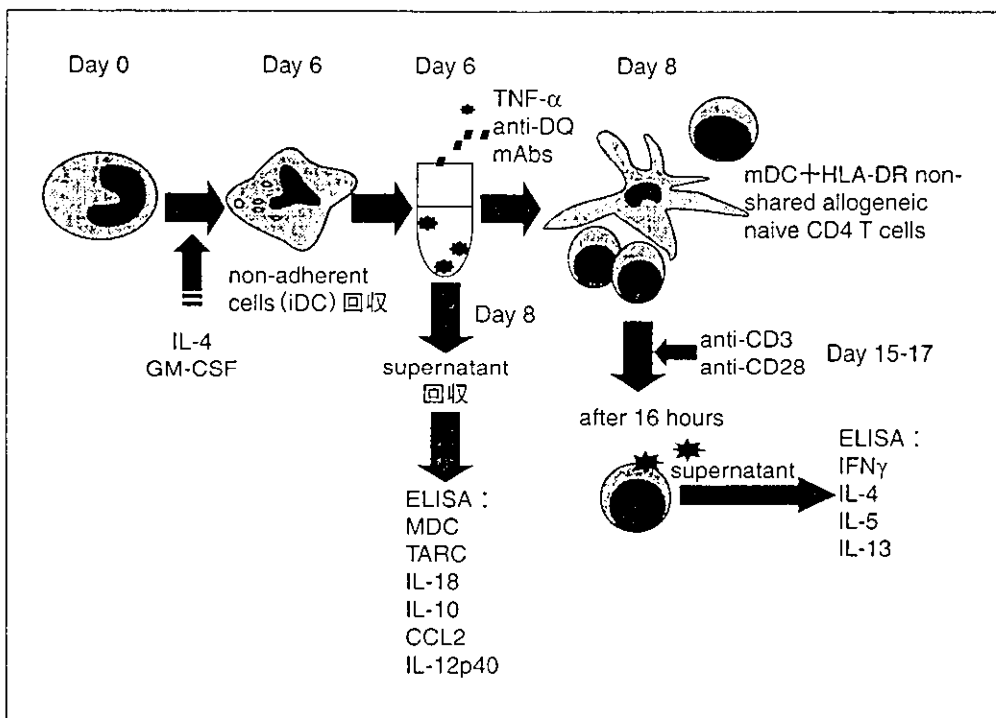

図6 アジュバント活性アッセイの流れ

上清については，IL-12p40, MDC, TARC, CCL-2, IL-10などを定量した．一方，HLA-DRタイピング済みのアロPBMCsから，negative selection法によりnaive CD4 T細胞を調整した(図5)．これを洗浄済みのMo-DCと共培養し，MLRを誘導した．これにより，naive CD4 T細胞にはTh1またはTh2への分化圧が加わることになる．7〜9日後，分化したT細胞にanti-CD3とanti-CD28で再刺激を加え，その16時間後に培養上清を回収した．培養上清中のIL-4, IL-5, IL-13, IFNγを測定し，Th1またはTh2への分化を判定した．方法の全体の流れ図を図6に示した．

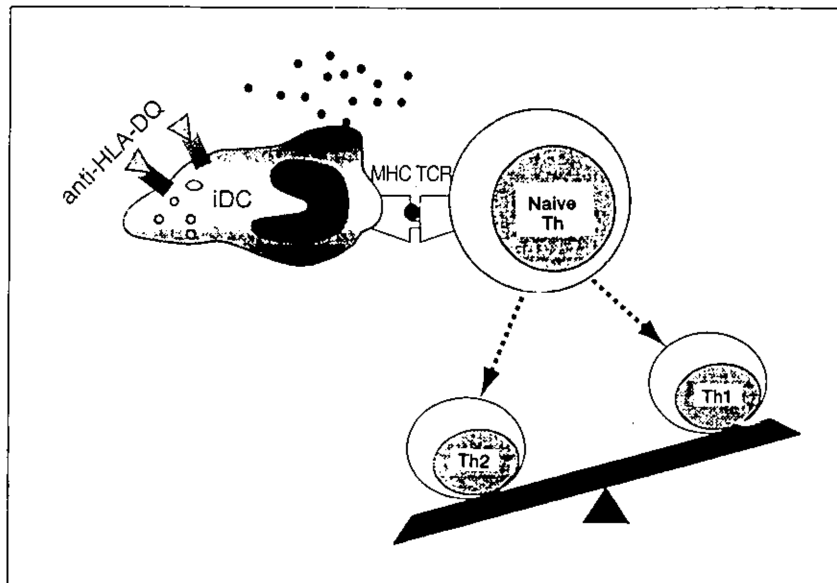

図7　HLA-DQを介した刺激はDC2を誘導する
HLA-DQを介したシグナルは以下の方法により細胞内に伝達される．①抗HLA-DQ抗体．②ビオチン化抗HLA-DQ抗体＋アビジン．③抗HLA-DQ抗体でコートした培養プレート．④抗HLA-DQ抗体アガロース．⑤エメチン処理したHLA-DQ拘束性T細胞．

抗原提示分子とアジュバント活性

われわれは以前の研究から，通常の蛋白抗原を認識するT細胞クローンにおいてもその拘束分子(抗原提示分子)によって性質が異なっており，この性質の違いは，MHC-peptide-TCR複合体が形成された際にMHCを介して抗原提示細胞内に伝えられるシグナルの差に由来することを示していた[17]．そこで，抗HLA-DQ抗体を用いて抗原提示細胞に刺激を入れ，そのT細胞分化誘導能を観察した．予想された通り，HLA-DQ分子を介した刺激はDC2を誘導することが明らかとなった[18](図7)．

そこで，実際にPBMCから得られたshort-term T cell lineの拘束分子によってTh1/Th2シフトがみられるかを観察した．図8に示すようにHLA-DR拘束性クローンに比べると，HLA-DQおよびHLA-DP拘束性のT細胞クローンは，明らかにTh2にシフトしたパターンを示すことが明らかとなった．この一連の知見は，われわれが17年前に発表した，免疫抑制遺伝子を細胞レベルで説明するものである[19]．

環境ホルモンとアレルギー[20]

環境ホルモンの毒性が，単なる「メス化」を誘導するだけのものではないことも近年明らかになってきた．以前から，少量のエストロゲンはTh1を活性化させ，多量のエストロゲンはTh1を抑制すること，またプロゲステロンはTh2を活性化させ，男性ホルモンTh1とTh2をともに抑制することが知られていた．フタル酸エステルでリンパ球を刺激するとIL-10の上昇がみられる．エストロゲン受容体アンタゴニスト(ICI182,780)は無効なので，エストロゲン受容体を介さずに作用しているのかもしれない．Jurkat細胞では，刺激後瞬時に細胞内カルシウムイオン濃度が上昇し，蛋白リン酸化が起こる．また，IL-13の上昇はビスフェノールではみられるが，ノニルフェノールではみられない．MonocytesからiDCを誘導する過程にノニルフェノールを共培養すると，CD86の発現増強が起きる．トリブチル錫(TBT)，はB細胞に作用してIL-12のp40とp35の発現低下を誘導し，IL-10発現を上昇させる．T細胞のアポトーシス感受性は，Treg>Th1>Th2の順であるが，TBTはapoptosisを下げ，proliferationを上げることでTh2シフトに関与しているらしい[20]．

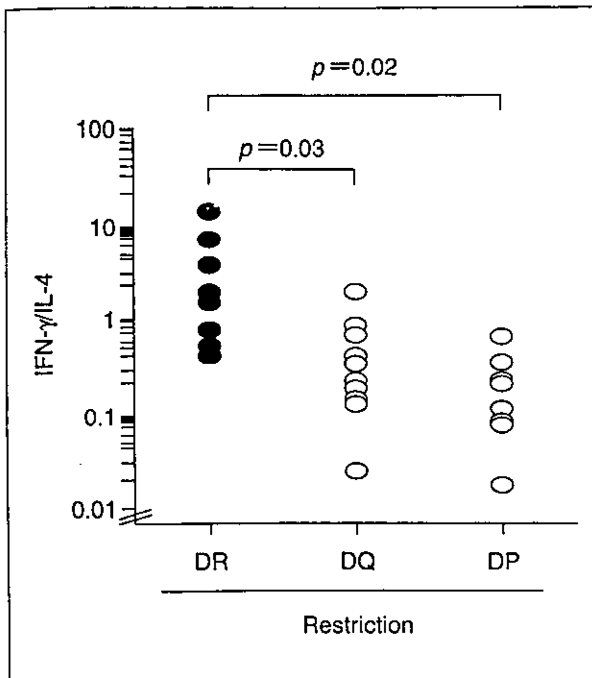

図8　T細胞の拘束分子とTh1/Th2分化
ランダムな19-mer(X_{19})に反応し，DR, DQ, DPに拘束されたT細胞をAPCとともにX_{19}(500μM)で刺激した．48時間後，培養上清を採取し，IFNγとIL-4の濃度をELISAで測定した．1つのスポットは1つのT細胞クローンを示している．

おわりに

著者が20年前に取り組んだ博士号のテーマは「スギ花粉症の免疫遺伝学的解析」であった．当時も今も同じであるが，花粉抗原の抽出の第一ステップはエーテルによる脂質の除去である．そうしなければ可溶性のアレルゲン蛋白を得ることができないし，そもそも当時，水に溶けない物質を*in vitro*の系にもち込むことは至難の技であった．当時の私は，エーテルに溶け出した黄色の脂質溶液を「これ，本当に捨てていいのだろうか？」と思いつつ，自分で採集した大量の花粉と格闘していたが，20年経ったいま，当時の「ゴミ」で研究を展開することになってしまった．

また，アレルギー誘導物質の研究の流れを振り返ると，糖脂質に対するグループ1CD1分子拘束性のヒトT細胞応答が完全に見落とされている可能性もある．応用面では，免疫治療学，環境医学，予防医学など，幅広い可能性を有しており，今後の展開が楽しみな分野である．

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Research article

A serine/threonine kinase, Cot/Tpl2, modulates bacterial DNA–induced IL-12 production and Th cell differentiation

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A serine/threonine protein kinase, Cot/Tpl2, is indispensable for extracellular signal–regulated kinase (ERK) activation and production of TNF-α and PGE_2 in LPS-stimulated macrophages. We show here that Cot/Tpl2 is also activated by other Toll-like receptor (TLR) ligands. Bacterial DNA rich in the dinucleotide CG (CpG-DNA), unlike LPS or synthetic lipopeptide, activated ERK in a Cot/Tpl2–independent manner. Peritoneal macrophages and bone marrow–derived DCs from $Cot/Tpl2^{-/-}$ mice produced significantly more IL-12 in response to CpG-DNA than those from WT mice. Enhanced IL-12 production in $Cot/Tpl2^{-/-}$ macrophages is, at least partly, regulated at the transcriptional level, and the elevated IL-12 mRNA level in $Cot/Tpl2^{-/-}$ macrophages is accompanied by decreased amounts of IL-12 repressors, such as c-musculoaponeurotic fibrosarcoma (c-Maf) and GATA sequence in the IL-12 promoter–binding protein (GA-12–binding protein; GAP-12) in the nucleus. Consistently, $Cot/Tpl2^{-/-}$ mice showed Th1-skewed antigen-specific immune responses upon OVA immunization and *Leishmania major* infection in vivo. These results indicate that Cot/Tpl2 is an important negative regulator of Th1-type adaptive immunity, that it achieves this regulation by inhibiting IL-12 production from accessory cells, and that it might be a potential target molecule in CpG-DNA–guided vaccination.

Introduction

Macrophages play a central role in the innate immune response to a wide range of bacterial components such as LPS, lipoprotein, peptidoglycan, flagellin, and bacterial DNA rich in the dinucleotide CG (CpG-DNA). On activation by these components, macrophages produce cytokines, such as TNF-α, IL-12, and IL-10, and secondary mediators, such as PGs and NO (1, 2). DCs also sense the types of pathogens and deliver critical information to antigen-reactive T cells. Upon activation by pathogens, DCs express high levels of MHC class II and costimulator molecules, such as CD80 and CD86, on their cell surface and become a major source of IL-12, which greatly potentiates IFN-γ-producing Th1-type T cell differentiation (3, 4). Thus, the maturation and cytokine production profiles of DCs are critical for the linkage between innate and adaptive immunity.

Understanding how bacterial components activate macrophages and DCs was greatly expedited by the identification of the Toll-like receptors (TLRs). The phenotypes of animals with functionally defective TLRs (both naturally occurring and artificially manipulated) suggest that each TLR is essential for the recognition of specific pathogen-associated molecular patterns (PAMPs) (5). Although TLRs share some downstream signaling molecules, various reports have also suggested difference among the biological responses induced by TLRs (5).

A serine/threonine protein kinase, Cot, also known as Tpl2, was originally identified as an oncogene by the SHOK cell transfection assay (6, 7) and also identified as a target for proviral integration in MoMuLV-induced rat T cell lymphomas and MMTV-induced mammary carcinomas (8, 9). Cot/Tpl2 belongs to the mitogen-activated protein kinase kinase kinase family and can activate both the extracellular signal-regulated kinase (ERK) and the JNK signaling pathways (10, 11). Cot/Tpl2 has also been implicated in NF-κB activation through IκB kinase complex or degradation of the inhibitory p105 protein (12–14), which has recently been reported to stabilize and inhibit Cot/Tpl2 (15, 16). Recently, Tsichlis and colleagues reported that the Cot/Tpl2 kinase plays an important role in LPS signaling (17, 18). According to their reports, $Cot/Tpl2^{-/-}$ macrophages are defective in ERK activation induced by LPS, while the activation of other MAPKs remains intact. The lack of ERK activation in $Cot/Tpl2^{-/-}$ macrophages results in impaired secretion of TNF-α and PGE_2 — key regulators of both inflammation and immunity — in response to LPS (17, 18). A quite recent paper also indicated an essential role of Cot/Tpl2 in the

Nonstandard abbreviations used: c-Maf, c-musculoaponeurotic fibrosarcoma; CpG-DNA, bacterial DNA rich in the dinucleotide CG; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; GA-12, GATA sequence in the IL-12 promoter; GAP-12, GA-12-binding protein; HEK, human embryonic kidney; IFA, incomplete Freund's adjuvant; MD2, myeloid differentiation-2; MEK1, MAPK/ERK kinase-1; PAMP, pathogen-associated molecular pattern; RPA, RNA protection assay; TLR, Toll-like receptor.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 114:857–866 (2004). doi:10.1172/JCI200420014.

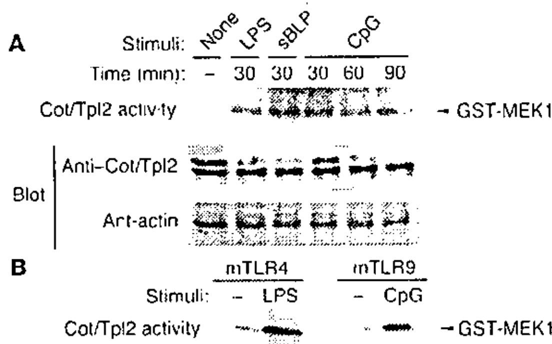

Figure 1
Cot/Tpl2 activation by TLR ligands. **(A)** RAW 264.7 cells were stimulated with 1 μg/ml LPS, 1 μg/ml synthetic lipopeptide, or 1 μM CpG-DNA for the indicated periods. In vitro kinase assays were performed with an Ab against Cot/Tpl2 and GST-MEK1 as the substrate. As a control, Western blot analyses were performed with Ab's against Cot/Tpl2 and β-actin. sBLP, synthetic bacterial lipopeptide. **(B)** HEK 293 cells were transiently transfected with Flag-tagged Cot/Tpl2 in combination with either mouse TLR4 plus CD14 plus MD2 (mTLR4) or mouse TLR9 (mTLR9). Transfectants were stimulated for 30 minutes with 1 μg/ml LPS or 1 μM CpG-DNA, respectively. In vitro kinase assay was performed with anti-Flag Ab on GST-MEK1 as the substrate.

downstream signals of CD40 and TNF-α receptor in B cells (19). However, it is largely unknown whether Cot/Tpl2 is also involved in macrophage responses to bacterial components other than LPS or in signal transduction of other types of immune cells.

In this study, using a separate line of Cot/Tpl2$^{-/-}$ mice, we demonstrate that Cot/Tpl2$^{-/-}$ macrophages undergo normal ERK activation and produce significantly more IL-12 in response to CpG-DNA than their normal counterparts. Similarly, Cot/Tpl2$^{-/-}$ DCs produced higher levels of IL-12 in response to LPS and CpG-DNA than WT DCs. Consistently, after OVA immunization and *Leishmania major* infection in vivo, Cot/Tpl2$^{-/-}$ mice showed antigen-specific immune responses polarized toward Th1 type.

Results

LPS, synthetic lipopeptide, and CpG-DNA induce Cot/Tpl2 activation in macrophages. To address the involvement of Cot/Tpl2 in various TLR signals, we measured the Cot/Tpl2 kinase activity using GST-MAPK/ERK kinase-1 (GST-MEK1) as the substrate in RAW 264.7 cells stimulated with LPS, synthetic lipopeptide, or CpG-DNA. All 3 TLR ligands rapidly activated Cot/Tpl2 (Figure 1A). To rule out the possible contamination of LPS in the CpG-DNA reagent we used, human embryonic kidney (HEK) 293 cells transiently transfected either with mouse TLR9 or with mouse TLR4 combined with myeloid differentiation-2 (MD2) and CD14 were analyzed. The transfectants were solely responsive to CpG-DNA or LPS, respectively, as determined by NF-κB activation assays (data not shown). When the expression plasmid for Flag-tagged Cot/Tpl2 was cotransfected in this cell system, in vitro kinase assays with Flag immunoprecipitates revealed that CpG-DNA induces Cot/Tpl2 activation in HEK 293 cells transfected with mouse TLR9 but not in those transfected with mouse TLR4 plus MD2 and CD14. Thus CpG-DNA, but not the contaminating LPS, is capable of Cot/Tpl2 activation through mouse TLR9 (Figure 1B).

Generation of Cot/Tpl2$^{-/-}$ mice. To address the physiological role of Cot/Tpl2 in TLR signaling, mice deficient for Cot/Tpl2 were generated by replacement of the 3' half of coding exon 2 and the 5' half of exon 3, encoding the ATP-binding domain (residues 123–224 of the total 467 amino acids of Cot/Tpl2 protein), with a neo-resistance gene cassette (Figure 2A). Mice heterozygous and homozygous for Cot/Tpl2 deletion were assessed by Southern blot analysis for a correct targeting event (Figure 2B). After heterozygous mating, Cot/Tpl2$^{-/-}$ mice were born at the expected mendelian frequency, with life expectancy no different from that of WT littermates. RT-PCR (Figure 2C) and Western blot analysis (Figure 2D) confirmed that Cot/Tpl2 mRNA and protein were absent in the generated Cot/Tpl2$^{-/-}$ mice.

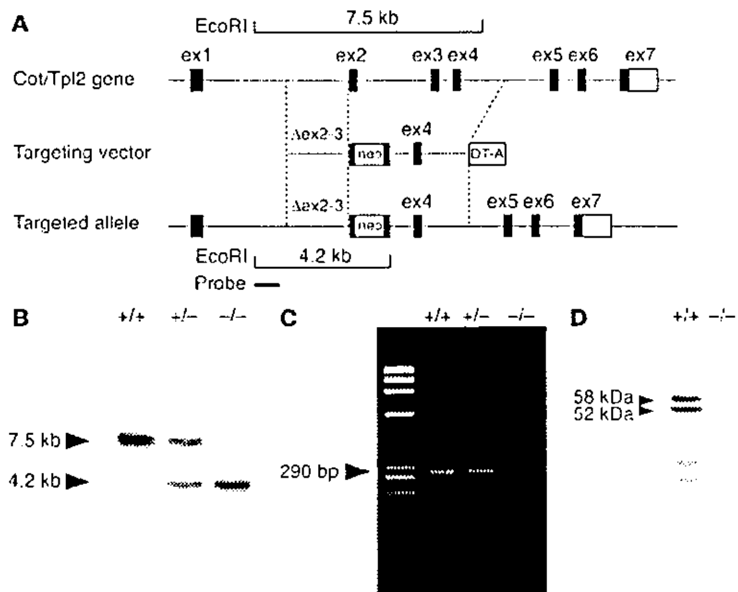

Figure 2
Targeted disruption of the Cot/Tpl2 gene. **(A)** The schematic structure of the mouse Cot/Tpl2 gene with exon 1 (ex1) to exon 7 is shown at the top. A targeting vector was designed to remove a part of exons 2 and 3, encoding the ATP-binding domain of Cot/Tpl2. Homologous recombination was verified using informative restriction fragments and diagnostic probes as indicated. **(B)** Southern hybridization using EcoRI-digested DNA extracted from mouse tails identified the expected genotypes (7.5-kb WT and 4.2-kb mutant fragments). **(C)** RT-PCR analysis by Cot/Tpl2–specific primers in the targeted region (290 bp) identified the expected genotypes. **(D)** Western blot analysis of proteins extracted from the heart using anti–Cot/Tpl2 Ab for the nontargeted region identified each genotype (the 58-kDa and 52-kDa forms of the Cot/Tpl2 protein).

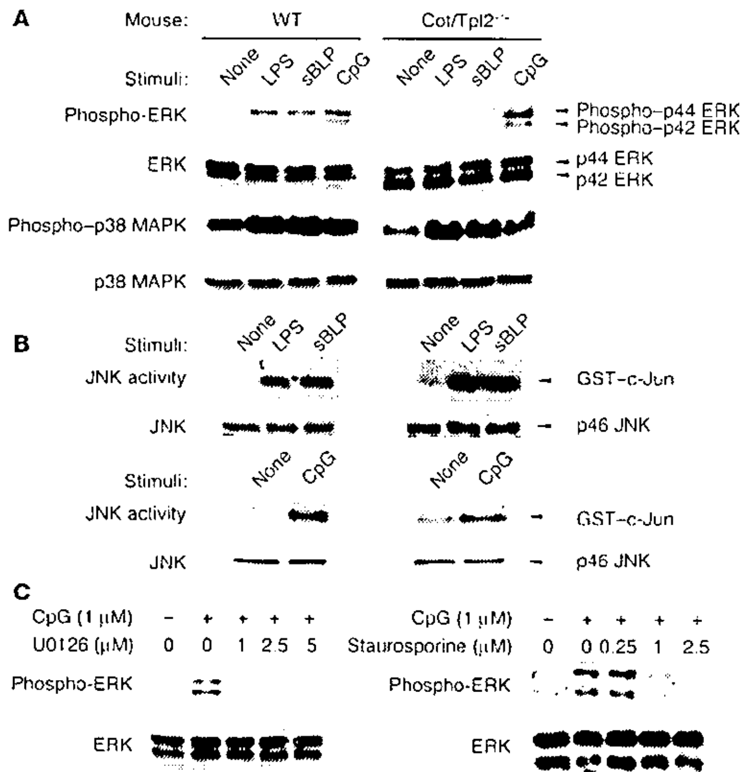

Figure 3
Cot/Tpl2 is not required for CpG-DNA–induced ERK phosphorylation. **(A)** WT and Cot/Tpl2$^{-/-}$ macrophages were unstimulated or stimulated with 1 μg/ml LPS, 1 μg/ml synthetic lipopeptide, or 1 μM CpG-DNA for 30 minutes. Western blot analyses were performed with Ab's against phospho–ERK1/2, ERK1/2, phospho–p38 MAPK, or p38 MAPK. **(B)** In vitro kinase assay of anti-JNK1 immunoprecipitates was performed on GST–c-Jun as the substrate. As a control, Western blot analysis was performed with anti-JNK1 Ab. **(C)** Cot/Tpl2$^{-/-}$ macrophages were pretreated with U0126 or staurosporine for 30 minutes, and then left unstimulated or stimulated with 1 μM CpG-DNA. Western blot analyses were performed with the Ab against phospho–ERK1/2 or ERK1/2.

CpG-DNA, but not LPS or synthetic lipopeptide, induces ERK activation in Cot/Tpl2$^{-/-}$ macrophages. Using peritoneal macrophages from the Cot/Tpl2$^{-/-}$ mice, we examined the phosphorylation of ERK1/2 before and after stimulation with synthetic lipopeptide, CpG-DNA, or LPS using a mAb specific to the phosphorylated forms of ERKs. As previously reported (17, 18), Cot/Tpl2$^{-/-}$ macrophages showed defective ERK phosphorylation in response to LPS (Figure 3A). They were also defective in ERK phosphorylation in response to synthetic lipopeptide. In contrast, Cot/Tpl2$^{-/-}$ macrophages showed normal ERK phosphorylation in response to CpG-DNA. JNK1, JNK2, and p38 MAPK were normally activated in response to all 3 TLR ligands (Figure 3, A and B).

As ERK appeared to be activated by CpG-DNA independently of Cot/Tpl2, we sought to analyze the molecular mechanisms of CpG-DNA-induced ERK activation in Cot/Tpl2$^{-/-}$ macrophages. We first tested the effects of a specific MEK1/2 inhibitor, U0126. ERK phosphorylation induced by CpG-DNA in Cot/Tpl2$^{-/-}$ macrophages was sensitive to pretreatment by U0126 (Figure 3C), which indicates that this signaling is MEK1/2-dependent. We obtained similar results from PD98059, another MEK1/2-specific inhibitor (data not shown). To explore the upstream activation mechanisms of the MEK/ERK pathway, we next utilized a PKC inhibitor, staurosporine. Pretreatment with staurosporine abrogated the ERK phosphorylation induced by CpG-DNA in Cot/Tpl2$^{-/-}$ macrophages, indicating that a protein kinase(s) sensitive to staurosporine, but not Cot/Tpl2, is critically involved in the MEK/ERK activation by CpG-DNA (Figure 3C).

Cytokine secretion profile of Cot/Tpl2$^{-/-}$ macrophages in response to LPS and CpG-DNA. To compare the regulatory roles of Cot/Tpl2 in cytokine production induced by LPS and CpG-DNA, both WT and Cot/Tpl2$^{-/-}$ macrophages were stimulated with LPS and CpG-DNA for 24 hours. As previously reported (17), the Cot/Tpl2$^{-/-}$ macrophages showed impaired TNF-α production compared with the WT macrophages in response to LPS. Although CpG-DNA induced a relatively small amount of TNF-α production, the induced TNF-α level was slightly lower in Cot/Tpl2$^{-/-}$ macrophages. In contrast, the CpG-DNA-treated Cot/Tpl2$^{-/-}$ macrophages showed significantly increased IL-12 production compared with the WT counterparts. Additionally, IL-10 production induced by LPS or CpG-DNA was enhanced in the Cot/Tpl2$^{-/-}$ macrophages (Figure 4A). To analyze the role of ERK activation in CpG-DNA-induced IL-12 production from macrophages, we pretreated the cells with U0126. At 1 μM of U0126, which was sufficient to inhibit ERK activation (Figure 3C), IL-12 production from both the WT and the Cot/Tpl2$^{-/-}$ macrophages was only moderately decreased (Figure 4B). Thus, ERK activation does not appear to be essential for CpG-DNA–induced IL-12 production in the presence or absence of Cot/Tpl2.

Increased IL-12 mRNA in Cot/Tpl2$^{-/-}$ macrophages stimulated with LPS and CpG-DNA. It has been reported that TNF-α production by macrophages in response to LPS stimulation is regulated at both transcriptional and posttranscriptional levels, whereas IL-12 production is regulated mainly at the transcriptional level (20). To assess the levels of TNF-α and IL-12 mRNA in response to LPS or CpG-DNA, peritoneal macrophages were treated with LPS or CpG-DNA, and total RNA was extracted and subjected to Northern blot analysis or RNA protection assay (RPA). Surprisingly, unlike in a previous report (17), LPS-induced TNF-α mRNA levels were consistently lower in the Cot/Tpl2$^{-/-}$ macrophages than in the WT macrophages (Figure 4C). CpG-DNA-induced TNF-α mRNA levels in the Cot/Tpl2$^{-/-}$ macrophages were similar to those in the WT macrophages. Thus, the reduction of TNF-α secretion induced by LPS in the Cot/Tpl2$^{-/-}$ macrophages seems at least partly regulated at the transcriptional level. In contrast, IL-12 p40 and p35 mRNA levels were significantly higher in the Cot/Tpl2$^{-/-}$ macrophages than in the WT macrophages in response to both LPS and CpG-DNA (Figure 4C).

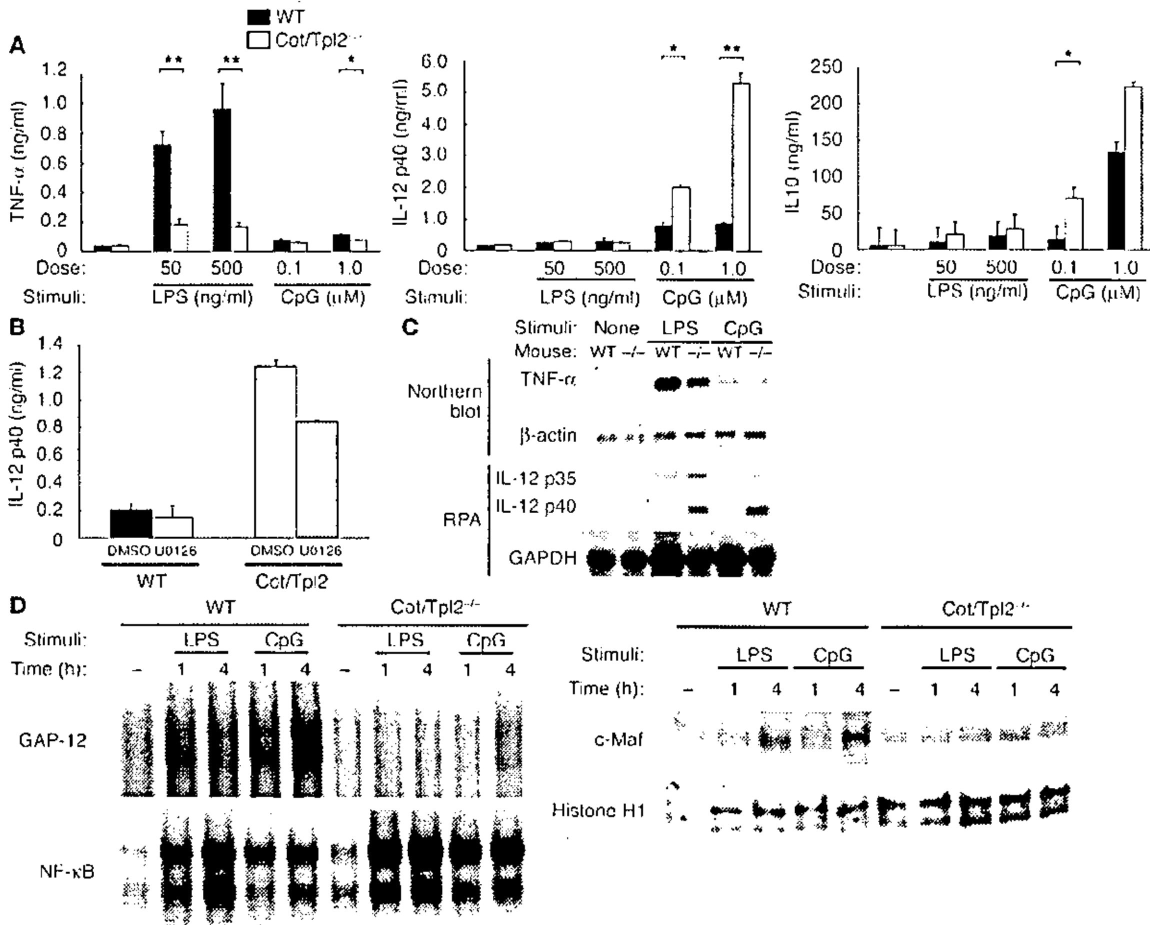

Figure 4
CpG-DNA–treated Cot/Tpl2$^{-/-}$ macrophages showed significantly increased IL-12 production. (**A**) WT (black bars) and Cot/Tpl2$^{-/-}$ (white bars) macrophages were stimulated with LPS or CpG-DNA for 24 hours. Levels of TNF-α, IL-12 p40, and IL-10 in culture supernatants were measured by ELISA. *$P < 0.05$ compared with WT. **$P < 0.01$ compared with WT. (**B**) WT and Cot/Tpl2$^{-/-}$ macrophages were pretreated with 1 μM U0126 and stimulated with 1 μM CpG-DNA for 8 hours. The level of IL-12 p40 in culture supernatants was measured by ELISA. Data are expressed as mean ± SD and are representative of 3 independent experiments. (**C**) WT and Cot/Tpl2$^{-/-}$ macrophages were treated for 2 hours with 1 μg/ml LPS or for 4 hours with 1 μM CpG-DNA. Total RNA extracted from peritoneal macrophages was subjected to Northern blot analysis and RNA protection assay (RPA). (**D**) WT and Cot/Tpl2$^{-/-}$ macrophages were unstimulated or stimulated with 1 μg/ml LPS or 1 μM CpG-DNA for the indicated times. Nuclear extracts were subjected to EMSA to evaluate DNA-binding activities of NF-κB or GAP-12. The same nuclear extracts were subjected to Western blot analyses with Ab's against c-Maf and histone H1.

Changes of nuclear transcription factors in Cot/Tpl2$^{-/-}$ macrophages. Transcription of IL-12 p40 is regulated by the binding of positive transcription factors, such as NF-κB, C/EBPβ, and PU.1, to the 5′ transcriptional regulatory region (21). Recently, 2 repressors have been reported for the IL-12 p40 gene expression, namely c-musculoaponeurotic fibrosarcoma (c-Maf) and GATA sequence in the IL-12 promoter-binding protein (GA-12-binding protein; GAP-12) (21, 22). We thus performed electrophoretic mobility shift assay (EMSA) to analyze DNA-binding activities of the 5′ promoter of the IL-12 p40 gene in Cot/Tpl2$^{-/-}$ macrophages. Stimulation of the Cot/Tpl2$^{-/-}$ macrophages with LPS or CpG-DNA induced DNA binding of NF-κB at a similar level to that in the WT macrophages (Figure 4D). We next performed EMSA using the GA-12 site of the mouse IL-12 p40 gene, which is the binding site for the putative negative transcriptional regulator GAP-12 (21). In the WT macrophages, stimulation with either LPS or CpG-DNA induced protein binding to the GA-12 probe, whereas the protein-DNA complex was scarcely increased in the Cot/Tpl2$^{-/-}$ macrophages (Figure 4D). We then examined the nuclear content of c-Maf protein and found that c-Maf protein was increased after LPS and CpG-DNA treatment in the nuclei of WT macrophages. This c-Maf induction in the nucleus was severely impaired in the Cot/Tpl2$^{-/-}$ macrophages (Figure 4D). We also found that c-Maf mRNA levels after LPS or CpG-DNA stimulation were lower in the Cot/Tpl2$^{-/-}$ macrophages (data not shown), which indicates that c-Maf expression was inhibited at the transcriptional level in the absence of Cot/Tpl2.

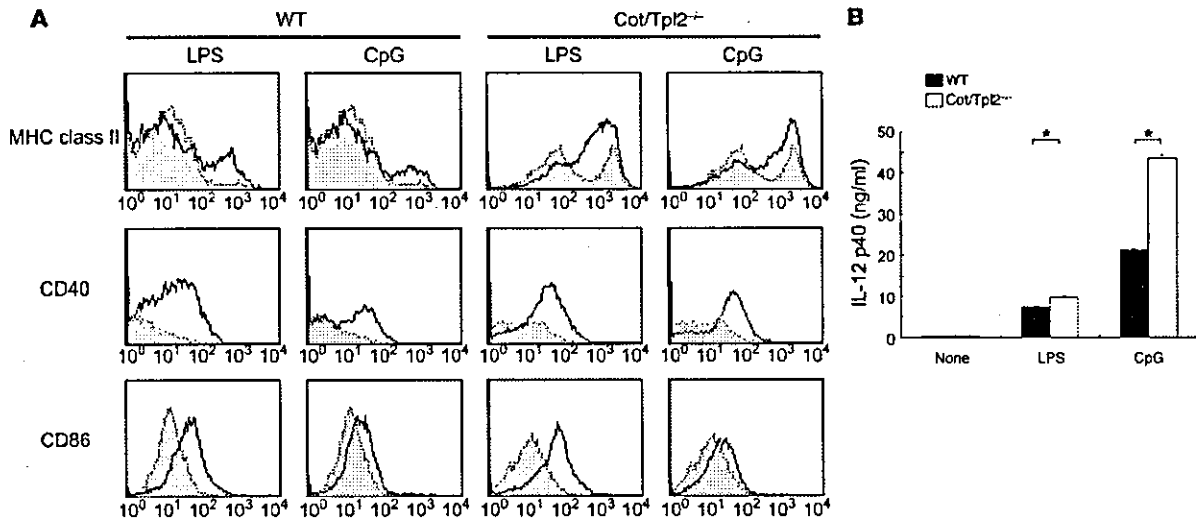

Figure 5

Maturation and cytokine production of DCs. (**A**) On day 5, bone marrow–derived DCs from WT or Cot/Tpl2-/- mice were left unstimulated (shaded histograms) or stimulated with 1 μg/ml LPS or 1 μM CpG-DNA (open histograms) for 24 hours. The DCs were subjected to MHC class II, CD40, and CD86 expression analyses by flow cytometry. (**B**) Immature WT (black bars) and Cot/Tpl2-/- (white bars) DCs (positively selected using magnetized Ab for CD11c) were unstimulated or stimulated with 1 μg/ml LPS or 1 μM CpG-DNA for 24 hours. IL-12 p40 in culture supernatants was measured by ELISA. Data are expressed as mean of wells ± SD and are representative of 3 independent experiments. $^*P < 0.05$.

Effect of Cot/Tpl2 deficiency on the maturation and IL-12 production of DCs. Like macrophages, DCs have been reported to express significant amounts of TLRs (5). In order to assess the roles of Cot/Tpl2 in TLR signals in DCs, we isolated immature DCs from the bone marrow of WT and Cot/Tpl2-/- mice and examined their maturation and cytokine secretion in response to LPS and CpG-DNA. The Cot/Tpl2-/- DCs treated with LPS and CpG-DNA did upregulate the surface expression of MHC class II, CD40, and CD86 (Figure 5A), indicating that Cot/Tpl2 is not necessary in the maturation of DCs. Unlike CD40 and CD86 expression, the MHC class II surface expression on untreated Cot/Tpl2-/- DCs was consistently higher than that on WT counterparts. The Cot/Tpl2-/- DCs produced significantly larger amounts of IL-12 in the presence of either LPS or CpG-DNA. Thus, as in macrophages, Cot/Tpl2 appears to play an inhibitory role in the IL-12 production in DCs (Figure 5B).

Increased Th1 response to OVA immunization in Cot/Tpl2-/- mice. Since IL-12 production from DCs is decisive in determining the nature of the antigen-specific T cell response that follows, we next analyzed the immune responses of Cot/Tpl2-/- mice in vivo after immunization with OVA. Cot/Tpl2-/- and WT mice were immunized in the footpad with OVA mixed with CFA, and adaptive immune responses were analyzed 9 days after immunization. T cells derived from the draining lymph nodes of Cot/Tpl2-/- mice showed enhanced proliferation in vitro in response to antigen stimulation compared with the WT T cells (Figure 6A), suggesting that the specific priming of T cells was enhanced in Cot/Tpl2-/- mice. We purified CD4+ T cells from lymph nodes using magnetic beads conjugated with CD4 Ab. CD4+ T cells from OVA-sensitized Cot/Tpl2-/- mice produced a significantly higher amount of IFN-γ and a lower amount of IL-4 on antigen stimulation than did those from control mice (Figure 6B).

Analysis of antigen-specific Ig responses in the sera showed that the Cot/Tpl2-/- mice consistently produced higher amounts of OVA-specific IgG2a after immunization (Figure 6C). There was no difference of OVA-specific IgG1 and IgE concentrations between the 2 groups of mice. On immunization with OVA mixed with alum, a strong promoter of Th2 responses, OVA-specific IgG1 and IgE responses were decreased in the Cot/Tpl2-/- mice compared with the WT mice (Figure 6D). Moreover, we obtained similar results when LPS or CpG-DNA was used as an adjuvant. In Cot/Tpl2-/- mice, IFN-γ production is enhanced in CD4+ T cells sensitized with OVA mixed with CpG-DNA. In contrast, IL-4 production is decreased in CD4+ T cells sensitized with OVA mixed with LPS or CpG-DNA (Figure 6E).

Increased Th1 response to L. major infection in Cot/Tpl2-/- mice. Finally, we examined the immune response to *L. major*, an infection model in which adequate Th1 development is required for disease control. To test this, we infected WT and Cot/Tpl2-/- mice with *L. major*. We then monitored inflammatory responses to the infection by measuring footpad swelling for 12 weeks and measuring parasite loads in the popliteal lymph nodes after 6 weeks. Cot/Tpl2-/- mice developed lesions that were almost identical in size to those in WT mice (Figure 7A). Consistently, parasite load in Cot/Tpl2-/- mice was indistinguishable from that in WT mice (Figure 7B). CD4+ T cells from *L. major*–infected Cot/Tpl2-/- mice produced a significantly higher amount of IFN-γ and a lower amount of IL-4 than did those from WT mice after stimulation with the soluble *L. major* antigen in vitro (Figure 7C).

Discussion

Properly stimulated TLRs activate various signal transduction pathways including NF-κB and MAPKs. Previous work had shown that Cot/Tpl2 is obligatory for the activation of ERK1/2 in LPS-stimulated macrophages (17, 18). We show here that all of the 3 TLR ligands examined, LPS, synthetic lipopeptide, and CpG-DNA, activate Cot/

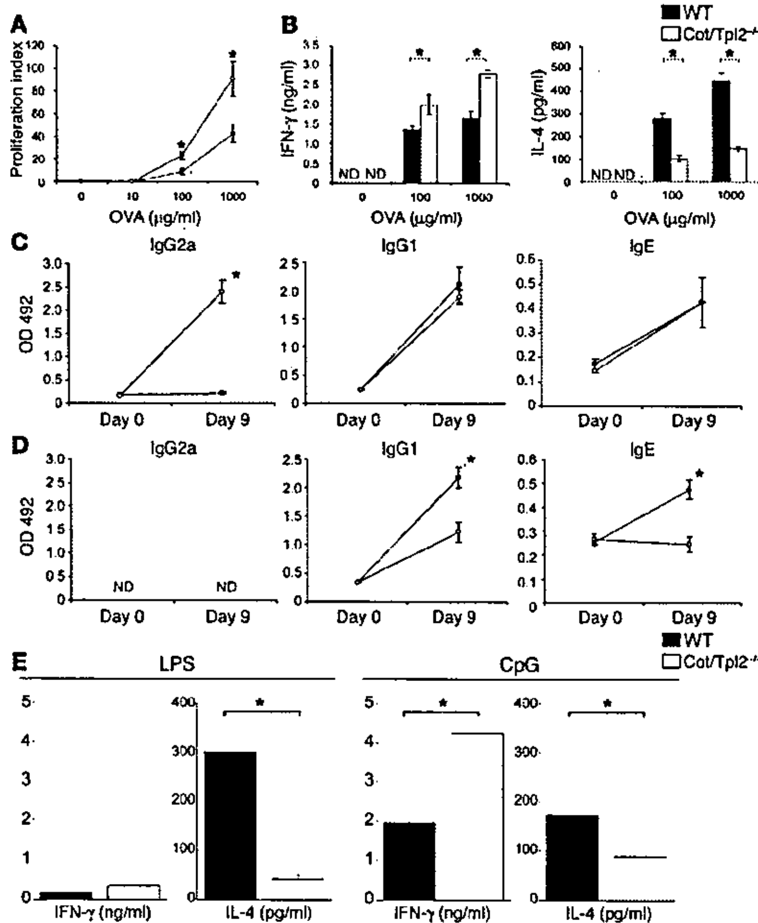

Figure 6
Antigen-specific Th1 cell responses were enhanced in Cot/Tpl2$^{-/-}$ mice. (**A**) Lymph node cells were collected from WT (filled circles, $n = 4$) or Cot/Tpl2$^{-/-}$ mice (open circles, $n = 4$) on day 9 after immunization with OVA in CFA. Proliferation of antigen-specific T cells was analyzed by cell proliferation assay using cell culture supernatants in different concentrations of OVA for 72 hours. (**B**) CD4$^+$ T cells from lymph nodes of WT (black bars, $n = 4$) or Cot/Tpl2$^{-/-}$ mice (white bars, $n = 4$) immunized with OVA in CFA were cultured in different concentrations of OVA with APCs for 72 hours. Cytokines in supernatants of antigen-stimulated pooled cells were determined by specific ELISA. (**C** and **D**) Serum was collected from WT (filled circles, $n = 5$) and Cot/Tpl2$^{-/-}$ mice (open circles, $n = 5$) before (day 0) and after (day 9) immunization with OVA plus CFA (**C**) or OVA plus alum (**D**). OVA-specific IgG2a, OVA-specific IgG1, and OVA-specific IgE were analyzed. The *y* axis indicates the OD value at 492 nm. (**E**) CD4$^+$ T cells from lymph nodes of WT (black bars, $n = 3$) or Cot/Tpl2$^{-/-}$ mice (white bars, $n = 3$) immunized with OVA plus LPS or OVA plus CpG-DNA were cultured in the presence of OVA (1 mg/ml) with APCs for 72 hours. Cytokines in supernatants of antigen-stimulated pooled cells were determined by specific ELISA. Data are expressed as mean of wells ± SD and are representative of 3 independent experiments. $^*P < 0.05$.

Tpl2, which indicates that Cot/Tpl2 is widely involved in TLR signaling. Interestingly, however, unlike LPS or lipopeptide, CpG-DNA still activated ERKs in Cot/Tpl2-deficient macrophages, which suggests that there is an alternative Cot/Tpl2-independent ERK activation mechanism in TLR9 signaling. The uniqueness of the CpG-DNA/TLR9 pathway has also been described in various other reports (23–25) and may partly be explained by the observation that TLR9 locates intracellularly while most other TLRs, including TLR4, locate on the cell surface (26). Thus, to gain access to the signaling compartment of the immune cells, a process referred to as endosomal maturation is required for CpG-DNA, but not for LPS (27).

The alternative pathway of ERK activation by CpG-DNA is sensitive to pretreatment with specific MEK1/2 inhibitors, which indicates that MEK1/2 is involved, probably directly upstream from ERKs (Figure 3C). ERK activation by CpG-DNA was also efficiently inhibited by staurosporine pretreatment. Since staurosporine was originally recognized as a PKC inhibitor, and 2 isoforms of PKC (ε and δ) were reported to be involved in LPS-induced signaling (28, 29), we then tested specific inhibitor peptides for PKCε and PKCδ. However, neither inhibitor peptide inhibited CpG-DNA-induced ERK phosphorylation in Cot/Tpl2$^{-/-}$ macrophages (data not shown). Thus, the precise mechanisms of Cot/Tpl2-independent ERK activation remain ambiguous.

In the Cot/Tpl2$^{-/-}$ macrophages used in our study, decreased TNF-α secretion in response to LPS is consistently accompanied by lower levels of TNF-α mRNA. This result is apparently inconsistent with the previous report, in which LPS-induced TNF-α mRNA levels were not decreased in Cot/Tpl2$^{-/-}$ macrophages (17). The reason for this discrepancy is currently unknown. However, in support of our data, several groups reported that inhibition of the MEK/ERK pathway using a specific MEK inhibitor (U0126 or PD98059) resulted in decreased TNF-α mRNA induction by LPS (30, 31), which indicates that the decreased MEK/ERK activity leads to lower levels of TNF-α transcripts.

Another intriguing observation in our study is the enhanced IL-12 production in the Cot/Tpl2-deficient macrophages and DCs. Because of its critical role in the immune system (3), IL-12 production needs to be tightly regulated. Among factors proposed to inhibit IL-12 production, IL-10 is supposed to be a major negative regulator, because IL-10 gene-deficient mice showed spontaneous onsets of autoimmune disease-like phenotypes associated with increased IL-12 production (32). However, changes in IL-10 production are not likely to be involved in the enhanced IL-12 induction in CpG-DNA-stimulated Cot/Tpl2$^{-/-}$ macrophages or DCs, for the following reasons. First, IL-12 mRNA increased rather rapidly after CpG-DNA stimulation (Figure 4C), indicating that IL-12

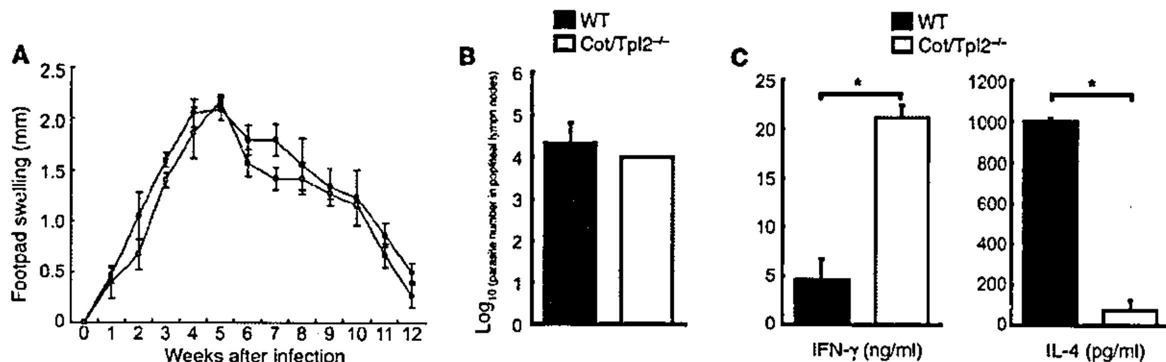

Figure 7

Increased Th1 response to *L. major* infection in Cot/Tpl2$^{-/-}$ mice. (**A**) The footpad swelling of age-matched WT (filled circles, $n = 5$) and Cot/Tpl2$^{-/-}$ (open circles, $n = 5$) mice infected with *L. major* was monitored on a weekly basis. (**B**) The number of *L. major* amastigotes in the popliteal lymph nodes of infected WT (black bars, $n = 4$) and Cot/Tpl2$^{-/-}$ mice (white bars, $n = 4$) was examined 6 weeks after infection (see Methods). (**C**) CD4^{+} T cells were isolated from the lymph nodes of *L. major*–infected WT (black bars, $n = 4$) or Cot/Tpl2$^{-/-}$ mice (white bars, $n = 4$) after 6 weeks of infection and cultured (2×10^5 per well in 200 μl of culture medium) with soluble *L. major* antigen (30 μg/ml) for 3 days. Cytokine production in the culture supernatants was examined by each cytokine-specific ELISA. Data are expressed as mean of wells ± SD and are representative of 3 independent experiments. $^{*}P < 0.05$.

induction was likely to be directly activated by the treatment. Second, IL-10 levels induced by CpG-DNA are higher in Cot/Tpl2$^{-/-}$ macrophages than in normal controls (Figure 4A). The mechanisms of increased IL-10 in Cot/Tpl2$^{-/-}$ macrophages are not clear. It is possible that increased IL-12 drives IL-10 production through IFN-γ as a negative regulator of inflammation.

Previous reports indicated that MAPKs are critically involved in the regulation of IL-12 production in APCs. For example, activation of p38 MAPK has been related to enhanced IL-12 production, mainly at the transcriptional level (20). In contrast, Häcker et al. reported that inhibition of the MEK/ERK pathway by PD98059 or U0126 results in decreased TNF-α production and enhanced IL-12 production from APCs (33). In the current study, the mechanism of increased IL-12 production in Cot/Tpl2$^{-/-}$ APCs clearly did not involve ERK, since ERK phosphorylation remained intact in these cells; this indicates that Cot/Tpl2 deficiency increased IL-12 production by CpG-DNA without inhibiting ERK activation. Consistently, pretreatment with U0126 did not increase IL-12 production in WT nor Cot/Tpl2$^{-/-}$ macrophages in response to CpG-DNA stimulation (Figure 4B). Together, our data indicated that CpG-DNA–induced ERK activity is not essential in regulating IL-12 production in primarily cultured macrophages.

On the other hand, correlation between mRNA and protein levels of IL-12 is consistent with a previous report indicating that IL-12 expression is primarily regulated at the transcriptional level in monocytic cells (20). Our EMSA results indicated that the induction of the negative regulators of IL-12 p40 transcription (c-Maf and GAP-12) by either LPS or CpG-DNA is significantly inhibited in Cot/Tpl2$^{-/-}$ macrophages (Figure 4D). In contrast, DNA binding of a positive regulator, NF-κB, was similar between WT and Cot/Tpl2$^{-/-}$ macrophages. The 2 other reported positive regulators of the IL-12 p40 gene, Ets and C/EBPβ, did not appear to be activated by LPS signals (22). GAP-12, whose molecular identity remains ambiguous, is a protein that binds to the suppressor site named GA-12 in the IL-12 p40 transcriptional regulatory region (21). Overexpression of c-Maf, another negative regulator, selectively inhibits transcriptional activation of both IL-12 p40 and IL-12 p35 genes (22). Thus the possibility may exist that GAP-12 and c-Maf are identical. However, this possibility was denied in a recent report, which showed that overexpression of c-Maf decreased protein binding of the GA-12 site in the IL-12 p40 gene (22). Thus, Cot/Tpl2 seems to be essential for the induction of 2 negative regulators of IL-12 p40 mRNA transcription.

It has been well established that DCs play essential roles in dictating this Th1/Th2 balance. PAMP-stimulated DCs express higher levels of MHC class II and costimulatory molecules and secrete significant amounts of IL-12, thus strongly inducing T cell differentiation toward a Th1 phenotype. Among the TLR ligands, CpG-DNA is one of the most potent inducers of IL-12. In this study, both LPS and CpG-DNA induced significantly higher levels of IL-12 secretion in Cot/Tpl2$^{-/-}$ DCs while increasing MHC class II and costimulatory molecule expression. It is of note that, unlike expression of costimulatory molecules such as CD86 and CD40, expression of MHC class II in Cot-Tpl2$^{-/-}$ DCs is moderately enhanced before PAMP stimulation (Figure 5A). The molecular mechanism of the increased MHC class II expression in Cot-Tpl2$^{-/-}$ DCs is currently unknown. Increased MHC class II expression may contribute to the Th1/Th2 balance of CD4^{+} T cells by affecting the intensity of antigen presentation (34, 35). DiMolfetto et al. (36) reported that, in mice expressing reduced levels of MHC class II but similar levels of costimulatory molecules on DCs, T cell response to antigen stimulation is skewed to Th1 type. Thus, increased MHC class II expression is not likely to be responsible for the skewed Th1 response in the Cot/Tpl2$^{-/-}$ condition. Taken together, these findings indicate that Cot/Tpl2 is not essential for the expression of costimulatory molecules but is specifically involved in IL-12 production in TLR-mediated signals.

Consistently, immunization of Cot/Tpl2$^{-/-}$ mice with OVA and CFA led to effective proliferation of antigen-specific T cells and increased IFN-γ production. Since Ig class switching is regulated by cytokines from T cells (37, 38), we analyzed the profiles of antigen-specific Ig production in Cot/Tpl2$^{-/-}$ mice. Cot/Tpl2$^{-/-}$ mice

exhibited increased levels of OVA-specific IgG2a production as compared with WT mice. On the other hand, immunization of Cot/Tpl2$^{-/-}$ mice with OVA plus alum, a strong inducer of Th2-type immune responses, resulted in less IgG1 and IgE production than in the control mice. These results are consistent with the in vitro data of high IL-12 production and suggest that systemic responses to exogenous antigen are polarized toward Th1 type in Cot/Tpl2$^{-/-}$ mice. Notably, a quite recent report has revealed that Cot/Tpl2 is essential in CD40-mediated ERK activation in B cells (19). This ERK activation is decisive in IgE induction by CD40. Thus, defective ERK activation in B cells might also be responsible for the changes of antigen-specific Ig subtypes that we observed in OVA-immunized Cot/Tpl2$^{-/-}$ mice. It should also be noted that, besides IL-12, other cytokines produced by DCs, such as IL-18 and IL-23 (39, 40), contribute to the preferential Th1/Th2 T cell responses. Thus, further study is warranted to analyze these cytokines in Cot/Tpl2$^{-/-}$ mice.

In this study, we also tested the relevance of skewed Th1 response of Cot/Tpl2$^{-/-}$ mice using *L. major*, an infection model in which adequate Th1 development is required for disease control. Consistent with our data using OVA, we confirmed that Cot/Tpl2$^{-/-}$ mice had enhanced Th1 responses against *L. major* challenge in CD4$^+$ T cells (Figure 7C). It has been reported that mice lacking MyD88 were more susceptible to *L. major* infection than control mice (41, 42). In these reports, a major parasite molecule, lipophosphoglycan, activated innate immune response via TLR2. It is also presumed that TLR9 is involved in the recognition of *L. major*, since protozoal DNA is largely nonmethylated and contains CpG immunostimulatory sequence (43). We also confirmed that HEK 293 cells transiently transfected with mouse TLR9 were responsive to the soluble *L. major* antigen (K. Sugimoto et al., unpublished observation), which indicates that a molecule in *L. major*, most likely *L. major* DNA, is recognized through TLR9. Inflammatory responses and parasite elimination, however, were not strongly altered in the Cot/Tpl2$^{-/-}$ mice. Since the strain we used, C57BL/6, is genetically resistant to *L. major* infection, a further increase in Th1 response might not be necessary for the protozoan elimination in this mouse strain. Another possibility is that decreased TNF-α secretion by macrophages impaired the clearance of *L. major* in Cot/Tpl2$^{-/-}$ mice (Figure 4A). Recent studies using gene-knockout technology have revealed that TNF-α is essentially involved in parasite elimination and resolution of inflammatory response against *L. major* infection (44, 45).

In summary, the activity of Cot/Tpl2 in macrophages and DCs appears to be decisive in determining IL-12 production and Th1/Th2 balance both in vitro and in vivo. Since CpG-DNA–based adjuvant does not trigger the severe granulomatous reactions often seen with CFA, CpG-DNA's adjuvant property may be used beneficially in humans in the future. Simultaneous inhibitory operation of Cot/Tpl2 during CpG-DNA–guided vaccination may enhance Th1 response while further decreasing side effects caused by TNF-α. Thus, Cot/Tpl2 may be considered as a potential target for immune therapy of diseases in which Th1/Th2 balance plays an essential role, such as allergy, infectious diseases, and cancer.

Methods

Generation of Cot/Tpl2$^{-/-}$ mice. A targeting construct was made to replace the 3' half of coding exon 2 and the 5' half of exon 3, encoding the ATP-binding domain, with a neo-resistance gene cassette. CCE embryonic stem cells were transfected and selected as previously described (46). Homologous recombinants were verified by Southern hybridization, and the resulting Cot/Tpl2$^{+/-}$ embryonic stem cells were microinjected into embryonic day 3.5 C57BL/6J blastocysts and transferred to MCH pseudopregnant foster mothers to generate chimeras that were mated with BDF_1 mice for germ-line transmission. Mice with mutant alleles were backcrossed with C57BL/6 mice for more than 6 generations. Genotyping was performed by Southern hybridization and PCR using primers in the neo gene (5'-CCGCTTGGGTGGAGAGGCTAT-3' and 5'-TG-GTGGTC-GAATGGGCAGGTA-3') and in the replaced Cot/Tpl2 gene (5'-AGAACA-CAAAGAGAATGAATG-3' and 5'-GCACCACAAAGAGTAGAAAAT-3'). After heterozygous mating, Cot/Tpl2$^{-/-}$ mice were born at the expected mendelian frequency, with life expectancy no different from that of WT littermates. RT-PCR (primers in the targeted region: 5'-TTCCCTGGCT-GTCATCTGTCA-3' and 5'-GCACGCCATTCTTTTCTTTGT-3') and Western blot analysis (Ab against the nontargeted C-terminal region) confirmed that Cot/Tpl2 mRNA and protein were absent at the level of detection. All experiments were done in accordance with institutional guidelines of the Nagoya University Graduate School of Medicine.

Reagents and Ab's. LPS from *Escherichia coli* (serotype B6:055), synthetic lipopeptide (palmitoyl-Cys[(RS)-2,3-di(palmitoyloxy)-propyl]-Ala-Gly-OH), and synthetic CpG-DNA (5'-TCCATGACGTTCCTGATGCT-3') were obtained from Sigma-Aldrich, Bachem AG, and Rikaken Co., respectively. A polyclonal anti-Cot/Tpl2 Ab (M-20), a polyclonal anti-JNK1 Ab, a polyclonal anti-p38 Ab, a polyclonal anti–c-Maf Ab (M-153), and a monoclonal anti–histone H1 Ab (AE-4) were obtained from Santa Cruz Biotechnology Inc. A polyclonal anti-ERK1/2 Ab, a phosphospecific anti-ERK1/2 polyclonal Ab, and a phosphospecific anti-p38 MAPK polyclonal Ab were purchased from New England Biolabs Inc. Anti-mouse CD3 complex, anti–mouse CD45R/B220, and isotype control rat anti-mouse IgG2a Ab's were purchased from eBioscience. Monoclonal Ab against MHC class II was obtained from VMRD Inc. Rabbit complement was purchased from ICN Pharmaceuticals Inc. Phycoerythrin-conjugated anti-mouse CD80 (B7/BB1), anti-mouse CD86 (B7-2), and anti-I-A^b and FITC-conjugated anti-CD11c were purchased from BD Biosciences – Pharmingen. GST-MEK1 was purchased from Upstate Biotechnology Inc. U0126 and staurosporine were purchased from Calbiochem-Novabiochem Corp. and Sigma-Aldrich, respectively.

Cell preparation and culture. Thioglycollate-elicited peritoneal macrophages were isolated and cultured as previously described (17). Briefly, mice were injected intraperitoneally with 2.0 ml thioglycollate broth (Sigma-Aldrich). Four days later, their peritoneal cavities were washed with 5 ml of cold PBS. Cell pellets were washed and cultured at 1 × 10^6 cells/ml for 2 hours. The dishes were washed twice with medium to remove nonadherent cells. At least 95% of the remaining adherent cells were macrophages according to Giemsa stains. To culture DCs, the bone marrow was isolated from the femurs and tibiae and depleted of erythrocytes. After washing, T cells, B cells, and MHC class II-positive cells were killed with a cocktail of Ab's (anti-mouse CD3 complex, anti-mouse CD45R/B220, and anti-MHC class II) and rabbit complement for 60 minutes at 37°C. Cells (1 × 10^6) were cultured in RPMI 1640 supplemented with 5% FBS, 10 mM HEPES, 10 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml GM-CSF. Media were replaced every 2 days. The phenotype of immature DCs on day 5 was routinely greater than 60% CD11c$^+$ and greater than 95% CD11b$^+$ without expression of CD8α. For ELISA, immature DCs were positively selected using magnetized Ab against CD11c (N418; Miltenyi Biotec Inc.). The resulting population was consistently greater than 85% CD11c$^+$. The viability was greater than 90% according to trypan blue staining. On day 5, immature DCs, defined as CD11c$^+$ cells, were stimulated with LPS or CpG-DNA for 24 hours. CD4$^+$ T cells were purified from the draining popliteal lymph nodes by positive