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多発性硬化症・視神経脊髄炎に対する免疫療法

Immunotherapy for Multiple Sclerosis and Neuromyelitis Optica

荒浪利昌
Toshimasa Aranami

多発性硬化症（MS）は，中枢神経系に広く病変が多発し，髄鞘抗原特異的T細胞やB細胞が介在する自己免疫疾患であると考えられており，病原性細胞としてTh1細胞とともにTh17細胞が注目されている．一方，視神経と脊髄が冒される視神経脊髄炎（NMO）には，血中にアクアポリン4に対する自己抗体（抗AQP4抗体）が存在し，病態形成に関与すると考えられている．MS治療の中心であるIFN-βは，Th1細胞の働きが亢進している病態では効果が期待されるが，Th17細胞が優位な状況では無効である可能性がある．NMOに対する新たな免疫療法として，抗CD20抗体などによる液性免疫を標的とする治療の有効性が示唆されている．

key words 多発性硬化症，視神経脊髄炎，免疫療法

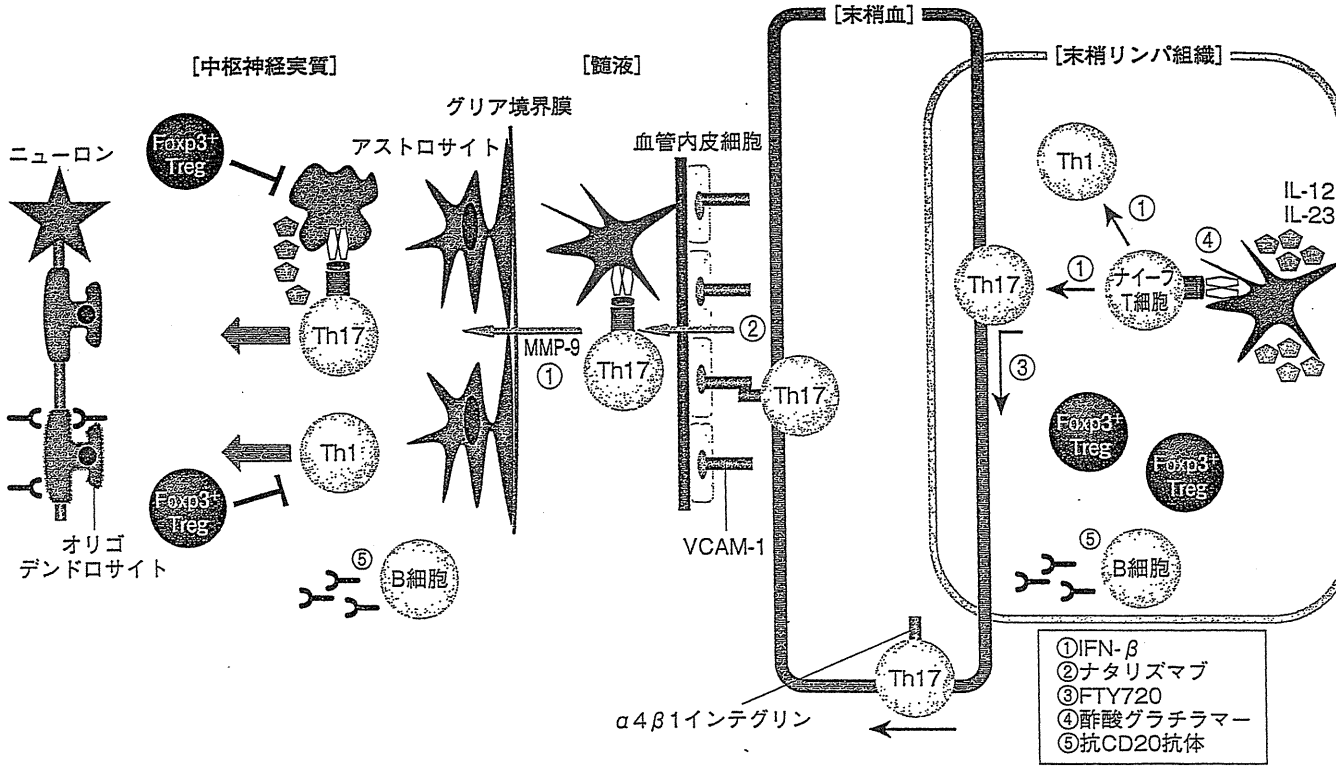
はじめに

多発性硬化症（multiple sclerosis；MS）は，髄鞘抗原特異的なT細胞やB細胞が髄鞘を傷害することにより脱髄病変が生じる中枢神経系の自己免疫疾患であると考えられている．病変は中枢神経系に広く分布し，再発・寛解を繰り返し，視力低下や運動麻痺などの症状が長期にわたる．一方，視神経脊髄炎（neuromyelitis optica；NMO）は，病変が比較的視神経と脊髄に限局している．以前はMSの一病型と考えられていたが，患者末梢血中にアクアポリン4（AQP4）という水チャネル分子に対する自己抗体（抗AQP4抗体）が存在することが発見されて以来[1]，MSとは異なる病態であるとの考えが優勢である．本稿では，MSとNMOの免疫病態およびそれぞれに対する免疫療法の進歩を概説する．

Ⅰ MSおよびNMOの病態

ヘルパーT（Th）細胞は活性化とともに，特定のサイトカイン産生細胞へと分化するが，従来より自己免疫疾患においてはTh1細胞の過剰な働きが関与するとの考えが優勢であった．しかし近年，インターロイキン（IL）-17を産生するT細胞（Th17細胞）が新たなTh細胞分画として提唱され，MSやその動物モデルである実験的自己免疫性脳脊髄炎（experimental autoimmune encephalomyelitis；EAE）への関与が示唆されている[2]．当初，Th17細胞は，Th1細胞とは異なるT細胞分画であり，EAE病態においてTh1細胞よりも高い病原性を有する分画として提唱されたが，近年IL-17を産生しているT細胞が，後にIFN-γ産生細胞へと変化すること，いわゆる可塑性（plasticity）を有することが示唆されている[3]．また，MSの末梢血や髄液においてもIL-17産生細胞の増加が報告されている[4]．その一方で，Th1およびTh17細胞の分化あるいは病原性の獲得を抑制することが期待されたIL-12とIL-23の中和抗体は，乾癬には有効であったが，MSにおいては再発抑制効果を認めなかった[5]．以上から，MS病態におけるTh1細胞およびTh17細胞の重要性については，いまだ結論は得られていない．また，MS病態においては，$CD4^+Foxp3^+$制御性T細胞（Treg）などのTreg機能の減弱も報告されており[6]，病原性細胞とTregのバランスにより，再発寛解型MSの病態が形成される可能性がある．

一方，NMOの免疫病態の特徴の1つは抗AQP4抗体の存在である．AQP4分子は中枢神経系グリア細胞アストロサイトの足突起などに特に豊富に存在している．NMO患者血清はアストロサイトを補体依存性に傷害し，EAEを誘導したマウスにNMO患者IgGを投与したところ，NMOに酷似した病理像が得られ，臨床症状も増悪したことから，抗AQP4抗体はNMO病態マーカーと言うだけでなく，病態形成に深く関わっているという考え方が有力である．当研究部の千原らは，NMOとMS，健常者の末梢血B細胞分画の頻度を比較し，他の2群と比べ，NMOにおいて形質芽細胞（プラズマブラスト）が有意に増加していることを見いだした．

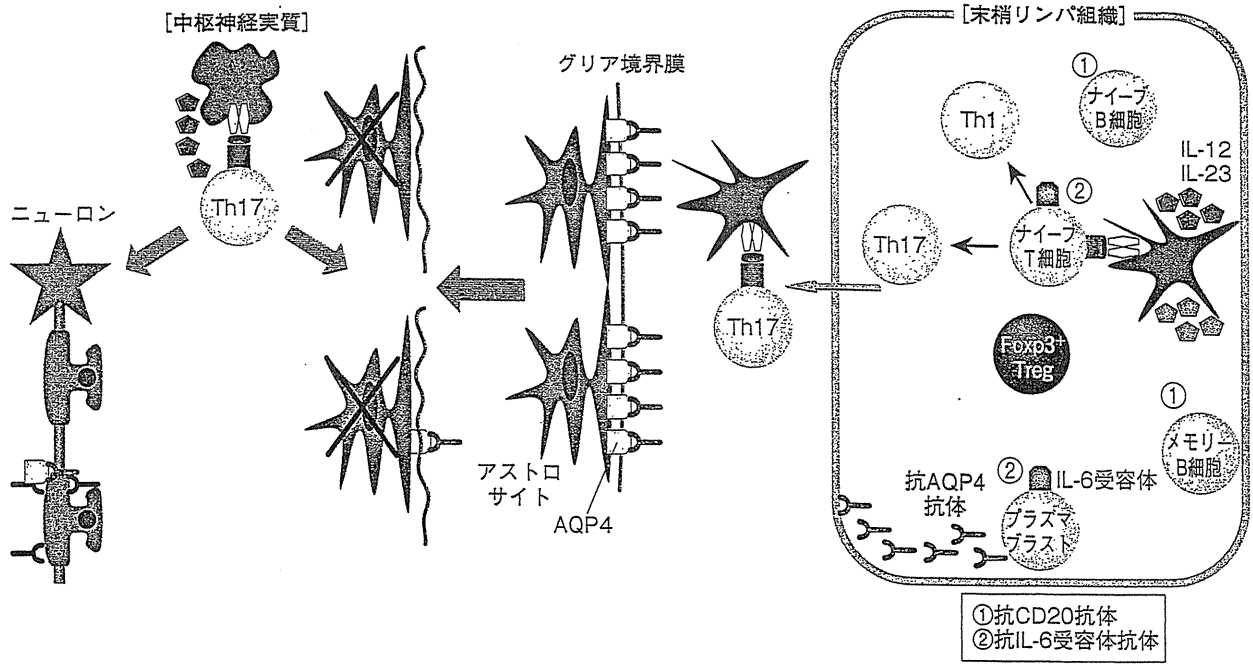


■図1　MS病態と免疫療法
MS病態への関与が示唆される病原性細胞およびTregと免疫療法の想定される作用点(①～⑤)を示す.

さらに，プラズマブラストが末梢血中の主たる抗AQP4抗体産生細胞であり，プラズマブラストの生存および抗AQP4抗体産生にIL-6が重要である可能性を示した[7]．また，脳病変がほとんどなく，脊髄と視神経のみが選択的に冒されるMSが本邦を含むアジア諸国に多く，以前はOSMS (opticospinal MS) と呼ばれていたが，OSMSの30～60％は抗AQP4抗体陽性であることが明らかとなり，その病態形成機構はNMOと類似していると考えられている．そのOSMSの髄液においてはIL-17, IL-8, G-CSF (granulocyte colony-stimulating factor) といったIL-17ファミリーとそれにより誘導されるサイトカインの増加が認められる[8]．また，NMOの髄液ではマウスのTh17細胞分化に重要なサイトカインであるIL-6も増加している．これらの報告から，NMOの病態形成に液性免疫とともにTh17細胞やIL-6が関与している可能性が示唆される．

Ⅱ　MSとNMOの免疫療法

MSおよびNMOの急性増悪時の治療法としては，メチルプレドニゾロンなどのステロイド大量点滴静注療法（パルス療法）や血漿交換療法が用いられる．それとともに，MSおよびNMOの治療においては，再発を予防する治療が長期的な神経機能的予後への影響を考えるうえで最も重要である．IFN-βは再発寛解型MSの再発予防療法として最も広く用いられている．それとともにMS患者の10～50％がIFN-βノンレスポンダー（無効群）であると報告されている．IFN-βの効果発現機構として，Th1細胞やTh17細胞への分化の抑制，あるいは炎症性細胞浸潤に重要なMMP-9 (matrix metalloproteinase-9) の抑制など，様々な効果が指摘されている．近年，MS病態におけるIFN-βの治療効果発現機構について新たな知見があった．Axtellらは，髄鞘抗原特異的Th1細胞で誘導したEAEにはIFN-βが有効であり，逆に髄鞘抗原特異的Th17細胞で誘導したEAEはIFN-β投与により症状の増悪が見られることを報告した．Th1細胞を移入して誘導したEAEでは，IFN-β投与により脾臓でのIFN-γ産生の減少と，抑制性サイトカインIL-10産生の著明な増加が認められた．このようなIL-10産生亢進はIFN-γノックアウトマウスでは観察されなかったことから，IFN-βが有効性を発揮するためにはIFN-γシグナルが必要と考えられ



■図2 NMO病態と免疫療法
NMO病態への関与が示唆される病原性細胞およびTregとステロイド以外の免疫療法の想定される作用点(①～②)を示す.

た. 一方, Th17介在性EAEでは, IFN-β投与により脾臓でのIL-17産生は低下するが, IL-10産生に変化がなく, 脊髄でIL-17産生細胞がむしろ増加していたことから, IFN-γ産生の亢進がなければIFN-βによるIL-10産生誘導が生じず, 治療効果が見られないと考えられた. さらに, IFN-βノンレスポンダー中には, 治療前の血清中IL-17FとIFN-β濃度がレスポンダー(有効群)と比べて高い一群が存在し, マウスの実験系と同じく, Th17に偏倚(へんい)している状態では, IFN-β治療が無効であることが示唆された[9]. このように, MSの病態にはTh1に偏倚している状態とTh17に偏倚している状態が存在する可能性がある. 前者にはIFN-β治療が有効であるが, 後者ではむしろ病態を悪化させてしまう可能性があり, 検討が必要である.

MSの再発抑制療法としては, その他にも各種免疫抑制剤のほか, MS病態に即した免疫修飾薬が欧米を中心に用いられている. (1) 中枢神経系への免疫細胞の遊走を抑制する薬剤. (2) 自己反応性T細胞による髄鞘抗原の認識を阻害する薬剤. (3) 生体よりB細胞を除去する薬剤, などである.

(1) としては, ナタリズマブやFTY720がある. ナタリズマブはヒト化抗α4インテグリンモノクローナル抗体であり, 免疫系細胞に広く発現するα4β1インテグリンとVCAM-1 (vascular cell adhesion molecule-1) という接着分子の結合を阻害する働きがあり, 中枢神経系への炎症性細胞浸潤を阻害する機能を有する. MSの再発率を有意に低下させ, 欧米で再発寛解型MSの治療薬として使用されている. FTY720は, リンパ節や二次リンパ組織からのリンパ球の流出に必要なシグナルを阻害する新規化合物である. リンパ節からのリンパ球の流出には, スフィンゴシン1リン酸(S1P)がリンパ球上のS1P受容体に結合することが必要だが, FTY720は体内でリン酸化された後, S1P1受容体にアゴニストとして結合する. これによりS1P1受容体の細胞内へのinternalizationを誘導し, 結果としてS1Pに結合できるS1P1受容体数が減少し, リンパ球はS1P不応性となる. その結果, T細胞はリンパ節に留められることになり, 神経組織に浸潤するリンパ球が減少し, 炎症も抑制されると考えられる[10].

(2) としては酢酸グラチラマー(copolymer-1)がある. これは髄鞘タンパク質の1つであるミエリン塩基性タンパク質(myelin basic protein; MBP)と同じ頻度で混合された4種類のアミノ酸のランダムポリマーである. 酢酸グラチラマーはマウスおよびヒトの各種MHCクラスII分子に高いアフィニティーで結合することにより, MBPなどの髄鞘抗原がクラスII分子に結合することを競合的に阻害する.

(3) は抗CD20抗体であり, 生体よりB細胞を除去することで, やはりMSの再発率を有意に低下させることが示され

ている（図1）．

NMOの免疫療法に関して，MSとの違いはIFN-βに対する反応性である．NMO患者へのIFN-β治療において，かえって重症化した例が報告されたため，現在ではNMOと判定されれば，IFN-βは使用しない．IFN-βがNMOに対して無効である原因は不明であるが，IFN-β投与患者において，血清中BAFF（B cell-activating factor belonging to the TNF family）濃度が上昇することが報告されている．BAFFはB細胞生存や分化を促進する因子として重要なため，NMOにおいては抗AQP4抗体産生など液性免疫を刺激して，症状の悪化を引き起こす可能性が考えられる[11]．NMOの再発抑制療法としては，現在，経口ステロイド剤が中心であるが，MS同様，抗CD20抗体によるB細胞除去療法が有効であると報告されている．これは，NMO病態における液性免疫の重要性と符合する結果である．また，当研究部では現在，前記の研究結果に基づき，NMOに対する抗IL-6受容体抗体療法の臨床研究を計画している（図2）．

おわりに

MSにはIFN-βが有効な一群と無効な一群が存在し，後者にはTh17細胞やIFN-βが病態形成に関与している可能性がある．また，NMOにおいてもTh17細胞と液性免疫の重要性が示唆されており，やはりIFN-βは無効である可能性が高い．以上より，MS，NMOの免疫療法においては，個々の患者における病態機序を検討したうえで，治療法を選択することが重要であると考えられる．

PROFILE 荒浪利昌
■ 国立精神・神経医療研究センター 神経研究所
■ E-mail：aranami@ncnp.go.jp
1993年北海道大学医学部卒業．北海道大学大学院免疫科学研究所にて基礎免疫学を学ぶ．卒業後，国立精神・神経センター神経研究所免疫研究部研究員として多発性硬化症の病態解析研究に従事．2006年より同研究部室長．

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Review

総　説

多発性硬化症における αB-crystallinとosteopontinの関与*

荒浪利昌**　山村　隆**

Key Words：multiple sclerosis, osteopontin, αB-crystallin

背　景

多発性硬化症(multiple sclerosis；MS)は中枢神経系にT細胞，B細胞，マクロファージなどのリンパ球浸潤，抗体沈着，補体活性化などを伴う脱髄巣が多発し，視力障害，小脳失調，運動麻痺，感覚障害など多彩な神経症状を呈する慢性疾患である．MSの病因は不明であるが，髄鞘蛋白反応性CD4陽性T細胞が介在する自己免疫疾患であると考えられている．この病原性CD4陽性T細胞としては，IFN-γ産生性Th1細胞や，最近ではIL-17産生性Th17細胞も重要視されている[1]．MSの大部分は急性に発症し，再発と寛解(時間的多発)を繰り返す．再発の誘因として，感染症やストレスなどが想定されているが，詳細なメカニズムは不明である．T細胞が産生するサイトカイン以外にもMS病態形成に関与が示唆されている因子として，αB-crystallin(CRYAB)やosteopontin(OPN)などがある(図1)．そのきっかけとなった研究が，2001年にSteinmanらのグループによって報告された，MS病巣に発現する遺伝子産物の網羅的解析研究であった[2]．MS死後脳病巣とコントロール脳標本から抽出したmRNAよりcDNAライブラリーを合成し，ハイスループットシークエンサーを使用して，expressed sequence Tags(EST)を作製した．これにより，コントロールの脳標本に比べてMS病巣において発現が2.5倍以上の頻度で発現している54個の遺伝子産物を同定した．そのうち最もMSでの発現頻度が高かったものがCRYABであり，OPNがトップ5であった．その後の研究で，これら2つの分子が，MSの再発や寛解の制御に関与する重要なメディエーターであることが示唆されている[3]．

OPN

OPNは別名，SECRETED PHOSPHOPROTEIN 1(SPP1)あるいはEARLY T LYMPHOCYTE ACTIVATION 1(ETA1)とも呼ばれ，その存在は骨基質や細胞外マトリックスに認められるほか，乳汁，胎盤，白血球などの正常組織，および腫瘍組織にも見出される．OPNはさまざまな分子との結合を介して，接着分子，ケモカイン，サイトカイン様に働き，多様な役割を果たすことが報告されている．骨組織においては，骨芽細胞がOPNを産生し，破骨細胞のビトロネクチン受容体と結合することにより骨吸収調節に関与する可能性が報告された[4]．また，CD44に結合し，がん細胞の遊走や転移に関与することが示唆されている[5]．さらに，肝炎動物モデルにおいては，トロンビン切断型OPNがα4β1やα9β1インテグリンへの結合を介して，炎症性細胞浸潤に関与していることが示されている[6]．MSにおいては，T細胞に発現するα4β1インテグリンに対する抗体が高い再発抑制効果があることが証明されており，再発抑制薬として使用されている[7]．抗α4β1インテグリン抗体は，α4β1インテグリン

* Roles of osteopontin and αB-crystallin in the pathology of multiple sclerosis.

** Toshimasa ARANAMI, M.D., Ph.D. & Takashi YAMAMURA, M.D., Ph.D.: 独立行政法人国立精神・神経医療研究センター神経研究所免疫研究部〔〒187-8502 小平市小川東町4-1-1〕; Department of Immunology, National Institute of Neuroscience, NCNP, Kodaira 187-8502, JAPAN

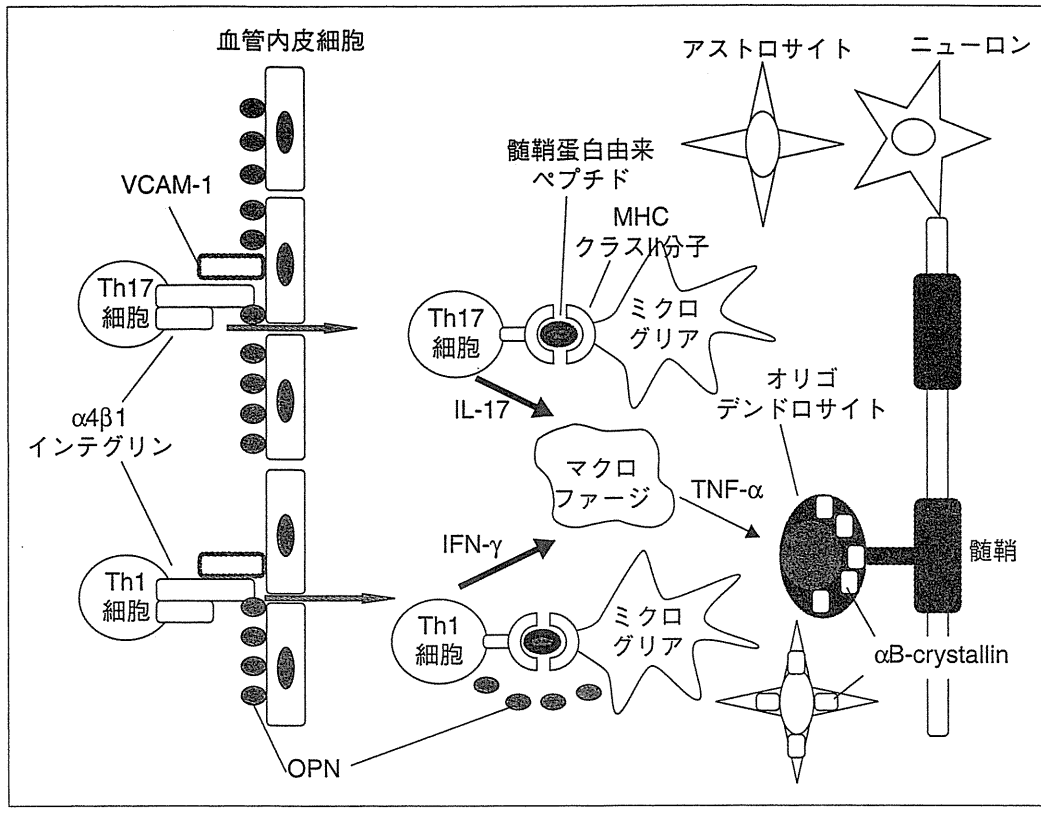

図1　MS病態形成に関与する細胞および分子
活性化T細胞は血管内皮細胞が発現するVCAM-1やOPNとα4β1インテグリンを介して接着し，中枢神経実質に侵入する．ミクログリアやマクロファージなどの抗原提示細胞はクラスII分子に結合した髄鞘蛋白由来ペプチドを細胞表面に提示し，自己反応性Th1およびTh17細胞が活性化され，それぞれIFN-γ, IL-17を産生する．これらのサイトカインは抗原提示細胞を活性化し，TNF-αなどのサイトカイン産生を誘導する．また，抗原提示細胞はOPNを産生し，活性化T細胞の生存を促進する．種々の炎症性サイトカインによってオリゴデンドロサイトは障害され，細胞質にαB-crystallin発現が誘導される．αB-crystallinはアストロサイトにも誘導され，これらの膠細胞のアポトーシスや炎症性サイトカイン産生を抑制する．

とそのリガンドであるvascular cell adhesion molecule-1(VCAM-1)およびOPNの結合をブロックすることにより，自己反応性T細胞が中枢神経系へ侵入するのをブロックすると考えられている．

MS病態におけるOPNの重要性を明らかにする目的で，動物実験モデルによる解析が行われた[2]．MSの最も代表的な動物実験モデルは，実験的自己免疫性脳脊髄炎(experimental autoimmune encephalomyelitis；EAE)である．これは，髄鞘蛋白由来のペプチドを結核死菌とともに不完全フロイントアジュバントに混ぜ，そのエマルジョンをマウスに免疫すると，約2週間で尾から下肢，体幹と上行する運動麻痺を発症するというものである．EAEを誘導したマウス脊髄におけるOPN蛋白の発現を解析したところ，発症急性期および再発期に，特に脱髄病巣部血管周囲のミクログリアとニューロンに高度な発現が誘導されていることが免疫組織化学染色により見出された．ミクログリアは中枢神経系におけるマクロファージ様の貪食細胞であると考えられている．実際にMS病巣における発現も死後脳の免疫組織化学染色により解析され，大脳白質のMS病巣の微小血管内皮細胞，マクロファージ，星状膠細胞(アストロサイト)やミクログリアにも発現が認められた．また，OPN欠損マウスを作製し，野生型とOPN欠損マウスにEAEを誘導したところ，両方のマウスがEAEを発症したが，野生型マウスでは慢性進行型のEAEが誘導されたのに対して，OPN欠損マウスではピーク時の重症度が軽減されるなど，EAEの軽症化が認められた．このことは，OPNがEAE病態の重症化に関与していることを示唆した．そのメカニズムを明らかにするため，免疫したマウスの所属リ

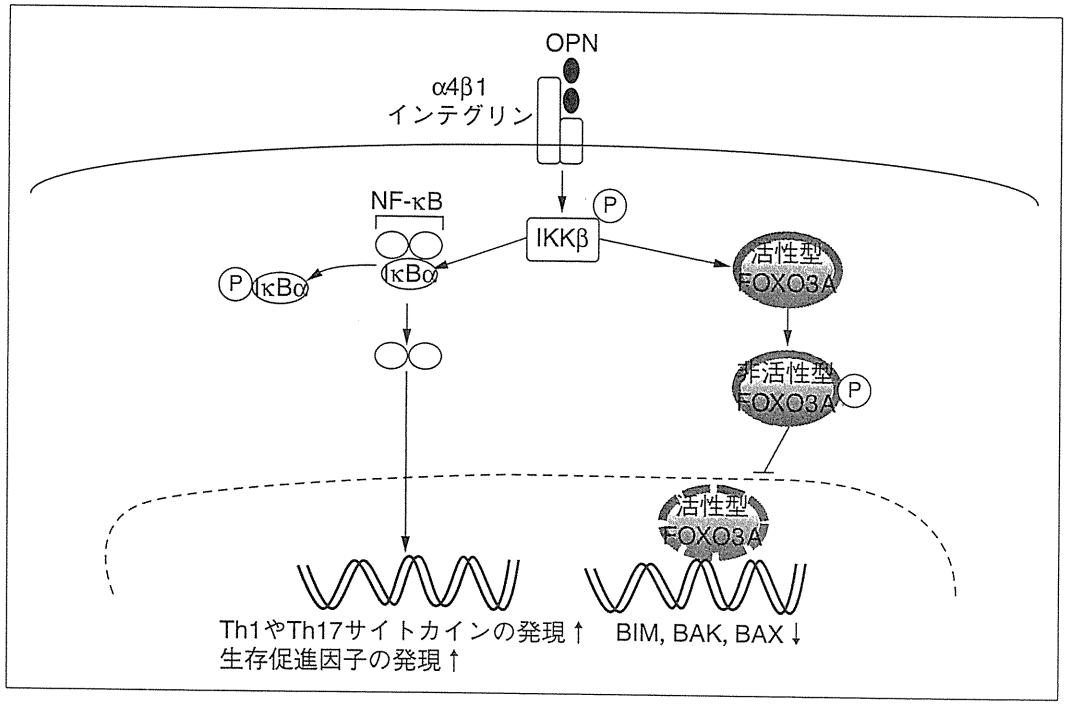

図2　OPNによって誘導される活性化T細胞内シグナル
OPNはIKKβをリン酸化を誘導し，これがIκBαの分解を誘導する．IκBαにマスクされていたNF-κB (p50-RelA)の核内移行シグナルが露出し，核に移行し，Th1およびTh17サイトカイン発現および種々の生存促進遺伝子の発現が上昇する．一方，IKKβは活性型FOXO3Aをリン酸化し不活性型FOXO3Aとする．これによりFOXO3Aは核内に移行出来ず，結果としてアポトーシス蛋白であるBIM, BAK, BAXの発現が低下し，アポトーシスが抑制される．

ンパ節よりT細胞を分離し，試験管内で再度免疫原ペプチドで刺激した．その結果，OPN欠損マウス由来のT細胞では増殖反応およびIFN-γ産生が減弱し，抑制性サイトカインIL-10産生が増強していることがわかった．すなわち，OPNは主としてTh1反応の増強を介して，EAEを増悪させることが判明したのである．その後Shinoharaらは，OPN発現の制御機構を解析し，活性化T細胞においては，OPN発現はT-betによって誘導されることを見出した[8]．T-betはTh1細胞分化に必須の転写因子であることから，自己反応性Th1細胞への分化が起こる際に誘導されるT-betによってOPNが誘導され，Th1反応を増強させるという制御機構が示唆された．

OPN欠損マウスにおけるEAEの結果から，OPNはEAE発症に必須ではないことが示唆されたが，再発や慢性的な炎症の進行における役割が示唆された．実際MS患者血清においても，再発時にOPN増加が認められることがわかった[9]．再発過程におけるOPNの役割を明らかにする目的で，リコンビナントOPNを用いた実験がなされた[10]．

この実験モデルでは，OPN欠損マウスにEAEを誘導すると，EAE発症後早期の軽症化が認められた．その時点でリコンビナントOPNを投与すると，いったん軽症化したマウスが再度重症化(再発)した．そしてその際，OPNによる活性化T細胞の生存促進効果が認められることがわかった．その分子メカニズムとしては，活性化T細胞においてOPNによりNF-κBが活性化され，同時にforkhead box O3A(FOXO3A)核内移行が抑制されていることがわかった(図2)．OPNはinhibitor of NF-κB kinase β(IκB kinase-β；IKKβ)の活性化を介してinhibitor of NF-κBα(IκBα)を分解する．IκBαによりマスクされていたNF-κB (p50-RelA) の核内移行シグナルが露出し，核に移行できるようになる．一方IKKβは，FOXO3Aを非活性型にし，核内への移行を阻害し，アポトーシスを抑制する．さらに，OPNはアポトーシス促進分子であるBim, Bak, Baxの発現を抑制することも明らかとなった．これらの転写因子やアポトーシス関連分子に対する影響により，OPNは活性化T細胞のアポトーシスを抑制する

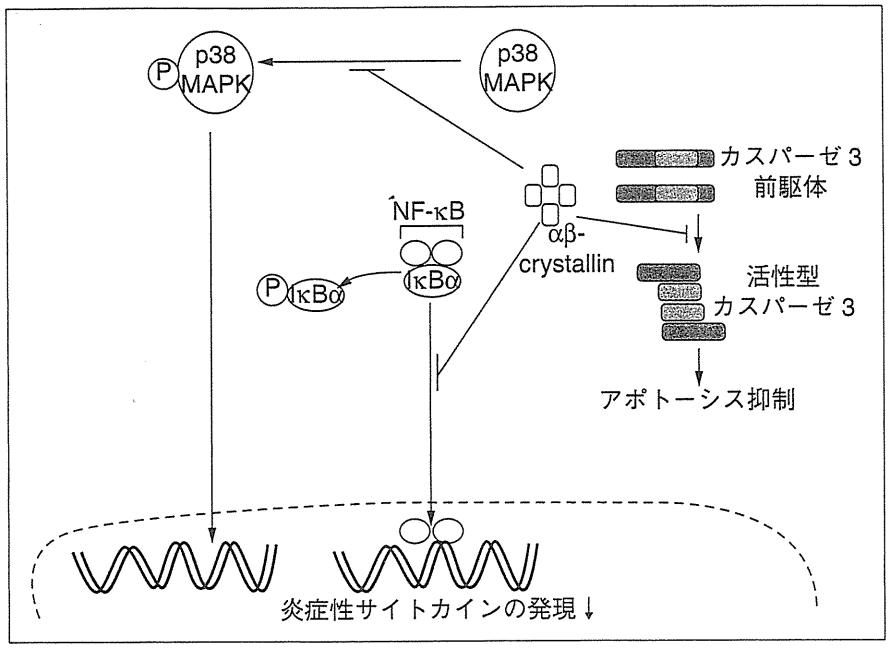

図3　αB-crystallinによって誘導される炎症シグナルの抑制
αB-crytallinはカスパーゼ3前駆体から活性型カスパーゼ3への活性化を抑制し，アストロサイトのアポトーシスを抑制する．またCRYABは，NF-κBの活性化，核内移行を抑制し，炎症を促進するp38MAPキナーゼの活性化を抑制する．これらの働きにより，Th1, Th17反応において誘導されるさまざまな炎症性サイトカイン産生を抑制し，MS病態を寛解へと導く．

ことがわかった．以上より，OPNはサイトカイン産生，アポトーシス抑制など，自己反応性T細胞の病原性の増強を介して，MS再発病態の増悪に関与していると考えられる．

αB-crystallin（CRYAB）

Crystallin蛋白は，水晶体の可溶性蛋白質の90％を占め，α, β, γの3種類のcrystallin蛋白が含まれる．CRYABは，分子シャペロンであるsmall heat shock proteinファミリーに属し，水晶体，心臓，骨格筋などに発現が認められる[11]．van Noortらは，すべての髄鞘蛋白に対するヒト末梢血T細胞の増殖反応を解析した．その結果，CRYABがMS患者および健常者T細胞両方の増殖反応を誘導することを見出し，CRYABがMSの標的抗原の一つである可能性を指摘した[12]．免疫組織化学染色により，CRYABが活動性MS病巣の稀突起膠細胞（オリゴデンドロサイト）および星状膠細胞の細胞質に発現が認められた[13]．さらに，先に述べたMS病巣における遺伝子産物の網羅的解析において，最も高頻度に認められる遺伝子産物であることがわかり，CRYABのMS病態における重要性が高まった．そこで，SteinmanらはCRYABのMS病態形成における重要性を調べる目的で，CRYAB欠損マウスを作製し，EAEを誘導した．その結果，野生型マウスに比べ，CRYAB欠損マウスではEAEが重症化した[14]．特に，臨床症状のピークおよび慢性時の症状の増悪が認められた．病理学的解析により，CRYAB欠損マウスでは，炎症性細胞浸潤と脱髄の増強が認められた．このような病理学的変化とアポトーシスの関連を調べる目的で，カスパーゼ3の免疫組織化学染色を行ったところ，CRYAB欠損マウスにおいては膠細胞でのカスパーゼ3の発現増強が認められた．また，CRYAB欠損マウスではこれに相応して，アポトーシス検出方法の一つであるTUNELL染色陽性膠細胞の頻度が高いことがわかった．このことは，CRYABがEAEにおいてアポトーシスを抑制することにより病態を抑制する働きがあることを示唆する．試験管内の実験系においては，まず免疫系細胞におけるCRYAB欠損の影響が調べられた．EAEを誘導したマウスの所属リンパ節T細胞を免疫原ペプチドで再刺激すると，CRYAB欠損マウス由来T細

胞は野生型に比べて，p38MAPキナーゼの活性化の亢進と大量のIL-2, IFN-γ, IL-17産生および高い増殖反応を示した．また，マクロファージをLPSで刺激する実験においては，CRYAB欠損マウス由来のマクロファージでは，野生型に比べて，p38MAPキナーゼの活性化の亢進とより大量の炎症性サイトカイン(IL-1, IL-6, IL-12 p40)産生が認められた．次に，星状膠細胞のアポトーシスおよび炎症性サイトカイン刺激に対する反応が調べられた．TNF-αによって星状膠細胞を刺激する系において，CRYAB欠損マウス由来の星状膠細胞では，IκBαの発現低下とNF-κBの核内移行の促進が認められた．そして，再度TUNNEL陽性細胞の増加が認められた．以上より，CRYABは免疫系細胞(T細胞やマクロファージ)において，p38MAPキナーゼの活性化と炎症性サイトカイン産生を抑制するだけでなく，膠細胞に対してはアポトーシスを抑制するとともに，炎症に伴うNF-κBの活性化の抑制を介して炎症性サイトカイン産生を抑制し，EAEの症状を抑制する働きがあることが示唆される(図3)．さらに，再発寛解型MSの脳脊髄液中には，ほかの中枢神経疾患に比べて高濃度の抗CRYAB抗体が存在することが判明した．以上のような，EAE病態に対するCRYABの制御性機能をより明らかにする目的で，EAEを発症したマウスにリコンビナントCRYABを投与した．その結果，EAEの臨床症状は軽快し，自己反応性T細胞の増殖反応およびTh1, Th17サイトカイン産生は減弱した．したがって，CRYABは，EAE病態において寛解を誘導する働きがあると考えられる．MSにおいては中枢神経系での炎症に対して，CRYABが本来抑制的に働くはずが，それに対する自己抗体産生が誘導されることにより，CRYABの制御性機能が阻害されていると予想されている．最近，CRYABがマイクログリアを刺激し，TNF-α, CCL5, IL-13といったさまざまなサイトカイン，ケモカイン産生を誘導することが示された[15]．これらのCRYABの機能は，免疫学的監視機能として炎症を収束させる方向に働くのではないかと考えられている．

結　語

MS病態では，Th17細胞，Th1細胞以外にもB細胞，マクロファージ，CD25陽性制御性T細胞，NKT細胞やNK細胞など，さまざまな免疫系細胞のバランスの変化によって，再発と寛解の病態が形成されることが示唆されている[16]．OPNとCRYABは，これら種々の免疫系細胞に働き，OPNは活性化T細胞の生存を維持し，NF-κBシグナルの増強を介して炎症性サイトカイン産生を増加させ再発を誘導するのに対して，CRYABはNF-κBシグナルを抑制し，膠細胞のアポトーシスを抑制することにより寛解を誘導することで，それぞれMS病態形成に重要な役割を果たしていると考えられる．

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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

In vitro differentiation of lineage-negative bone marrow cells into microglia-like cells

Daisuke Noto,[1] Kazuya Takahashi,[1,2] Sachiko Miyake[3] and Masahito Yamada[1]

[1]Department of Neurology and Neurobiology of Aging, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

[2]Department of Neurology, National Hospital Organization Iou National Hospital, Kanazawa, Japan

[3]Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan

Keywords: astrocyte, GFP mice, primary mixed glial culture, TREM2

Abstract

Microglia are believed to be the only resident immune cells in the CNS, originating from hematopoietic-derived myeloid cells and invading the CNS during development. However, the detailed mechanisms of differentiation and transformation of microglial cells are not fully understood. Here, we demonstrate that murine microglial cells show two morphological forms *in vitro*, namely, small round cells expressing CD11b, Iba1, triggering receptor expressing on myeloid cells-2 (TREM2), and weakly expressing major histocompatibility complex class II and large flat cells expressing only CD11b and Iba1. Moreover, lineage-negative bone marrow (LN) cells cultured with primary mixed glial culture cells could differentiate into only the small round microglia-like cells, despite the absence of CCR2 and Gr-1 expression. Addition of macrophage colony stimulating factor (M-CSF) to LN cell culture allowed the proliferation and expression of TREM2 in LN cells, and the addition of neutralizing anti-M-CSF antibodies suppressed the proliferation of LN cells despite the expression of TREM2. When LN cells were cultured with M-CSF, the number of small round cells in the culture was considerably low, indicating that the small round morphology of the immature cells is not maintained in the presence of only M-CSF. On the other hand, when LN cells were grown in the presence of astrocytes, the small round cells were maintained at a concentration of approximately 30% of the total population. Therefore, cell–cell contact with glial cells, especially astrocytes, may be necessary to maintain the small round shape of the immature cells expressing TREM2.

Introduction

Microglia are believed to be the only resident immune cells in the CNS; they develop from hematopoietic-derived myeloid cells and invade the CNS during development (Ling & Wong, 1993). Microglial cells are recognized to play an important role not only in neuroinflammatory and neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease, but also in neuroprotective and anti-neuroinflammatory processes (Akiyama & McGeer, 2004; Sanders & De Keyser, 2007; Takahashi *et al.*, 2007). In a recent study, it has been reported that physiological microglial phagocytosis induced the efficient removal of apoptotic cells and cellular debris without inflammatory processes; this process is expected to be a novel, attractive target for protection from neuroinflammation or neurodegeneration (Takahashi *et al.*, 2005, 2007; Neumann & Takahashi, 2007). The detailed mechanisms underlying the differentiation and transformation of microglial cells, however, are not fully understood. Even in adulthood, hematopoietic-derived cells develop into resident perivascular macrophages and microglia (Hickey & Kimura, 1988; Priller *et al.*, 2001; Simard & Rivest, 2004). Although the exact cellular subtype of myeloid precursors that develop into microglia is unknown, it has been reported that only Ly-6C^{high} CCR2+ monocytes can invade and differentiate into perivascular microglia (Mildner *et al.*, 2007).

The bone marrow produces new blood cells, including all cell types of the myeloid lineage, some of which may differentiate into microglia. Lineage-negative bone marrow (LN) cells are defined by the absence of surface markers, such as CD3, CD4, CD5, CD8α, CD11b/MAC-1α, B220, Gr-1 and TER-119, and are considered to comprise many hematopoietic precursors, including microglia precursors. Thus, LN cells might represent microglial precursors and may serve as a natural vehicle for CNS cells in gene therapy.

In this study, we show that murine microglial cells are present *in vitro* in two morphological forms, namely, as small round cells expressing CD11b, Iba1, triggering receptor expressing on myeloid cells-2 (TREM2), and weakly expressing major histocompatibility complex (MHC) class II and as large flat cells expressing only CD11b and Iba1. We found that LN cells could differentiate into the small round-type but not the large flat-type microglia-like cells. Moreover, we concluded that not only macrophage colony-stimulating factor (M-CSF) but also cell–cell contacts with astrocytes play an important role in microglial differentiation.

Correspondence: Dr K. Takahashi, [2]Department of Neurology, as above.
E-mail: ktakaha@ioudom.hosp.go.jp

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Materials and methods

Isolation of LN cells from adult green fluorescence protein (GFP) mice bone marrow

Bone marrow cells were collected from 8- to 10-week-old C57BL/6 mice (Charles River, Japan) or GFP transgenic mice on a C57BL/6 mice background that were kindly provided by Dr Masaru Okabe (Osaka University, Japan) by flushing out the femora and tibiae of the hind limb under deep anesthesia by the diethyl ether. Erythrocyte removal was performed by lysis with the Mouse Erythrocyte Lysing Kit (R&D, Minneapolis, MN, USA). For eliminating lineage marker-positive cells via negative selection, bone marrow cells were incubated at 4°C for 30 min with eight types of rat monoclonal antibodies against mice lineage markers [CD3, CD4, CD5, CD8α, CD11b/MAC-1α, B220, Gr-1 and TER-119 (R&D)]. The cells were then washed and incubated with immunomagnetic beads (Invitrogen, Tokyo, Japan) at 4°C for 30 min. Finally, LN cells were collected by the removal of lineage marker-positive bone marrow cells by using a magnet stand that attracted the lineage marker-positive cells attached to the antibodies.

All experiments were approved by the Ethics Committee of Kanazawa University, and performed in accordance with the guidelines of the local animal care and use committee of Kanazawa University.

Primary mixed glial cell culture

Microglial cells were prepared from the brains of postnatal days 3–5 (P3–P5) C57BL/6 mice under deep anesthesia by the diethyl ether, as previously described (Takahashi *et al.*, 2005). Briefly, meninges were removed mechanically, and the cells were dissociated by trituration and cultured in basal medium Eagle (Invitrogen), 10% fetal calf serum (Invitrogen), 1% glucose (Sigma, Tokyo, Japan), 1% L-glutamine (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) for 14 days to form a confluent glial monolayer. LN cells obtained from GFP mice were added onto the confluent glial monolayer.

Treatment of LN cell culture with mixed glial cell culture supernatant, neutralizing antibodies or cytokines

LN cells were cultured with the mixed glial cell culture supernatant obtained from the Day 14 primary mixed glial culture. LN cells were also cultured with a culture medium containing M-CSF (10 ng/mL; Peprotech, Rocky Hill, NJ, USA), tumor necrosis factor-α (TNF-α; 10 ng/mL; Peprotech) or vascular endothelial growth factor (VEGF; 50 ng/mL; Peprotech) for 7 days. For the neutralizing assay, anti-M-CSF (2 μg/mL; R&D), anti-TNF-α (2 μg/mL; R&D) or anti-VEGF (1 μg/mL; R&D) antibodies were added in the LN cell culture for 7 days.

Immunohistochemistry

Mixed glial cell cultures with or without GFP-positive (GFP+) LN cells were fixed in 4% paraformaldehyde for 1 h, blocked by Protein Block (Dako, Denmark) for 2 h, and then immunostained with monoclonal rat antibodies directed against CD11b (Serotec, Oxford, UK) and a secondary fluorescence rhodamine-conjugated antibody directed against rat IgG (1 : 200; Millipore, Billerica, MA, USA). To identify the cell type, cells were double-labeled with a purified polyclonal sheep antibody directed against TREM2 (1 : 50, R&D), monoclonal rabbit antibodies directed against Iba1 (Wako, Kanagawa, Japan), and glial fibrillary acidic protein (GFAP; Dako) and monoclonal mouse antibodies directed against IAb (BD Pharmingen, Tokyo, Japan), followed by a secondary fluorescein isothiocyanate (FITC)-conjugated antibody directed against mouse IgG. Images were collected by fluorescence microscopy with a 20 × objective (Olympus, Tokyo, Japan). A confocal microscope with a 40 × objective (Zeiss, Jena, Germany) was used to obtain *Z*-stack images, and series of optical sections (512 × 512 pixels, pixel size: 440 nm) were collected at intervals of 380 nm. Images were analysed using the Zeiss LSM Image browser (Zeiss).

To quantify the number of cells, 10 fields under the 20 × objective were randomly selected and photographed by fluorescence microscopy using a digital camera (Olympus) for each experiment. Total cells, GFP-positive cells and positively immunostained cells were classified and counted according to their morphology.

Isolation of microglia and splenic macrophages

Microglial cells and GFP+ LN cells-derived microglial cells in primary mixed glial culture were obtained by shaking the flasks at 0.25 *g* for 2 h. Adult CNS microglia and splenic macrophages were obtained from GFP mice. The cortex and spinal cord of GFP mice were isolated and homogenized. Homogenates were incubated with 0.3 Wunsch units/mL Liberase Blendzyme 3 (Roche, Tokyo, Japan) and 0.1 mg/mL DNaseI (Roche) in RPMI 1640 medium at 37°C for 30 min. Microglia were separated through a density gradient. The cells were suspended in 27% Percoll (GE Healthcare, Tokyo, Japan) and overlaid with a 72% gradient. The density gradient was centrifuged at 1500 *g* for 30 min at 4°C. Myelin collected in the 27% Percoll layer was removed. The majority of microglia were found in the interface of the 27 and 72% Percoll layers. Cells were obtained from this interface and washed from the Percoll with phosphate-buffered saline. The spleen was isolated and cut into small fragments. Cells were incubated with 0.3 Wunsch units/mL Liberase Blendzyme 3 (Roche) and 0.1 mg/mL DNaseI (Roche) in RPMI 1640 medium at 37°C for 45 min. Erythrocyte removal was performed using ACK solution.

Flow cytometry analysis of LN cells, microglia and splenic macrophages

For flow cytometry analysis, cells were first incubated for Fc-receptor blockade by CD16/CD32 antibody (BD Pharmingen) and then stained with phycoerythrin (PE)-conjugated anti-IAb antibody and anti-CD45 antibody (BD Pharmingen), PerCP-Cy5.5-conjugated anti-CD11b antibody (BD Pharmingen), APC-conjugated anti-TREM2 antibody (R&D) and anti-F4/80 antibody (eBioscience, San Diego, CA, USA), or purified anti-CCR2 antibody (Abcam, Tokyo, Japan) followed by rhodamine-conjugated anti-Goat IgG (Millipore). Analysis was performed with a FACSCalibur flow cytometer (BD Biosciences, Tokyo, Japan).

Proliferation assay

Microglial cells and GFP+ LN cells-derived microglia-like cells in primary mixed glial culture, adult CNS microglia and splenic macrophages were cultured with various concentrations of M-CSF (0–100 ng/mL), and cultures were pulsed with 1 mCi of [^{3}H]TdR (MP Biomedicals, Tokyo, Japan) for the last 16 h of the incubation. Cell incorporation of [^{3}H]TdR was counted with a Topcount (Perkin Elmer, Boston, MA, USA). The mean cpm of triplicate cultures was calculated.

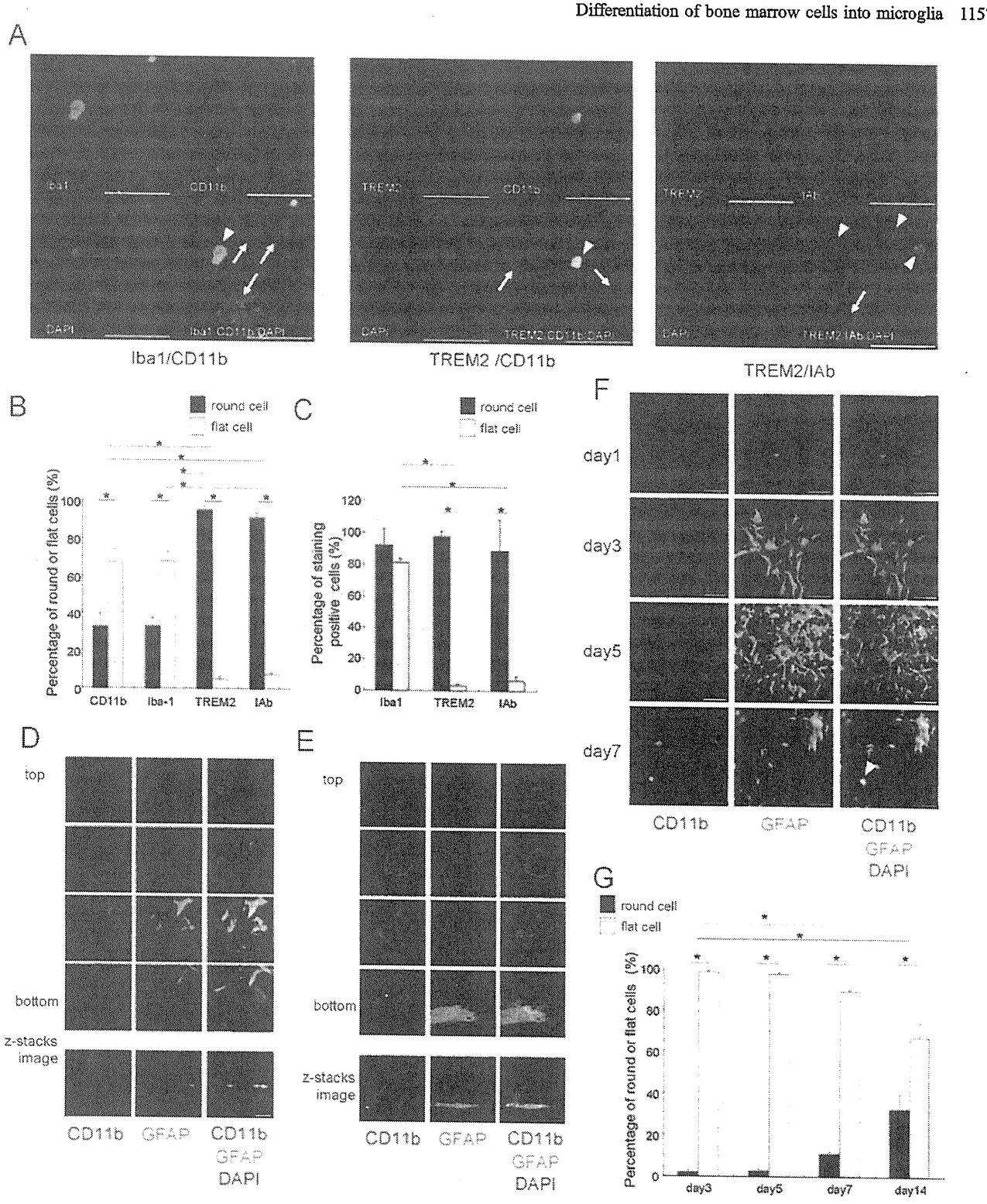

FIG. 1. Morphology of microglia in primary mixed glial culture. (A) Immunocytology of primary mixed glial culture stained with anti-Iba1, anti-triggering receptor expressing on myeloid cells-2 (TREM2) anti-IAb, anti-CD11b and 4′,6-diamidino-2-phenylindole (DAPI). Arrowheads indicate small round cells. Arrows indicate large flat cells. Scale bar: 50 μm. (B) The percentage of round cells or flat cells among staining-positive cells was quantified by microscopic analysis. Data are presented as mean ± standard deviation (SD). (C) The percentage of Iba-1-, TREM2- or IAb-positive cells among CD11b-positive (CD11b+) round or flat cells was quantified by microscopic analysis. Data are presented as mean ± SD. (D) *Z*-stack immunofluorescence confocal microscopy of primary mixed glial culture stained with anti-CD11b (red), anti-glial fibrillary acidic protein (GFAP; green) and DAPI (blue). Scale bar: 10 μm. (E) *Z*-stack immunofluorescence confocal microscopy of primary mixed glial culture stained with anti-CD11b (red), anti-GFAP (green) and DAPI (blue). Scale bar: 10 μm. (F) Immunocytology of primary mixed glial culture. Cultures were fixed at Days 1, 3, 5 and 7 of the culture and stained with primary anti-CD11b and anti-GFAP antibodies followed by rhodamine- or FITC-conjugated secondary antibody and DAPI. Arrowheads indicate small round cells. Scale bar: 50 μm. (G) The percentage of CD11b+ round or flat cells was quantified by microscopic analysis. Data are presented as mean ± SD; $*P \leq 0.05$. Data are representative of three independent experiments.

Stimulation by anti-TREM2 antibody

Small round cells were added to culture dishes coated with the anti-TREM2 monoclonal antibody (R&D) or control antibody, and centrifuged at 400 *g* for 5 min. After 60 min, the cells were fixed at a final concentration of 4% formaldehyde for 10 min at 37°C. Following centrifugation, the supernatant was removed, and the cells were resuspended in 90% ice-cold methanol and incubated for 30 min at 4°C. Cells were washed and stained with Alexa 647-conjugated anti-phospho-ERK1/2 antibody (Cell Signaling Technology, Tokyo, Japan) for 30 min. Analysis was performed using a FACSCalibur flow cytometer.

Bio-Plex cytokine assay system

Culture supernatant samples were analysed simultaneously for 17 different cytokines and chemokines (IL-1b, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-γ, TNF-α, IL-15, IL-18, FGF-basic, LIF, M-CSF, MIG, MIP-2, PDGF-BB and VEGF) using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Briefly, 50 μL of each sample and standard (Bio-Rad) was added to 50 μL of antibody-conjugated beads (Bio-Rad) in a 96-well filter plate (Millipore). After 30-min incubation, the plate was washed, and 25 μL of a biotinylated antibody solution (Bio-Rad) was added to each well, followed by 30-min incubation again. The plate was washed, and 50 μL of streptavidin-conjugated PE (Bio-Rad) was added to each well and incubated for 10 min. After a final wash, the contents of each well were resuspended in 125 μL of assay buffer (Bio-Rad) and analysed using a Bio-Plex Array Reader (Bio-Rad). The lower detection limit for each cytokine or chemokine was 2 pg/mL.

Statistical analysis

Data are presented as mean ± SD of at least three independent experiments. Data were analysed by the Mann–Whitney *U*-test to determine significant differences.

Results

Characterization of microglia in the primary mixed glial culture

To characterize microglial cells in the mixed glial culture, we stained several microglia markers such as Iba1, CD11b, TREM2 and IAb (MHC class II molecule of C57/BL6 mice) on the cultured microglial cells (Fig. 1A). CD11b-positive (CD11b+) cells in the primary mixed glial culture showed two major morphological forms, namely, small round-shaped cells (32.8 ± 6.9% SD of CD11b+ cells) expressing Iba1, TREM2 and IAb (Iba1+ cells, 91.9 ± 10.2% SD; TREM2+ cells, 97.5 ± 3.5% SD; IAb+ cells; 88.9 ± 19.2% SD), and large flat cells (67.2 ± 6.9% SD of CD11b+ cells) expressing only Iba1 (Iba1+ cells, 81.1 ± 2.6% SD; TREM2+ cells, 3.3 ± 1.1% SD; IAb+ cells, 6.4 ± 2.8% SD; Iba1 vs. TREM2, $P = 0.0495$; Iba1 vs. IAb, $P = 0.0495$; Fig. 1B and C). *Z*-sectioned scans by confocal microscopy revealed that small, spherically shaped cells lay above the astrocytes, and large flat cells lay under the astrocytes (Fig. 1D). Moreover, the spherical cells appeared to have extended processes toward the astrocytes (Fig. 1E).

To investigate the time point at which both the forms of the microglial cells appeared in the primary mixed glial culture, we performed a kinetic study. At Day 1 after the culture of mixed glial cells, CD11b+ cells showed a small amoeboid shape (Fig. 1F). At Day 3, the CD11b+ cells assumed a larger flat shape. Small round CD11b+ cells were very few in number until Day 5 (Day 3, 1.9 ± 1.1% SD; Day 5, 2.7 ± 0.9% SD), but increased greatly after Day 7 (Day 7, 10.9 ± 1.1% SD vs. Day 3, $P = 0.0495$; Day 14, 32.8 ± 6.9% SD vs. Day 3, $P = 0.0495$; Fig. 1F and G).

Characteristics of LN cells

LN cells were isolated by negative selection using magnet beads. The purity of LN cells after negative selection by flow cytometry was consistently above 90% on several examinations (Fig. 2A). LN cells expressed neither the microglia marker TREM2 nor CD11b, which is one of the lineage markers for negative selection (Fig. 2B). No expression of CCR2 or Gr-1 (lineage markers for negative selection) was detected on the LN cells, which have previously been described as

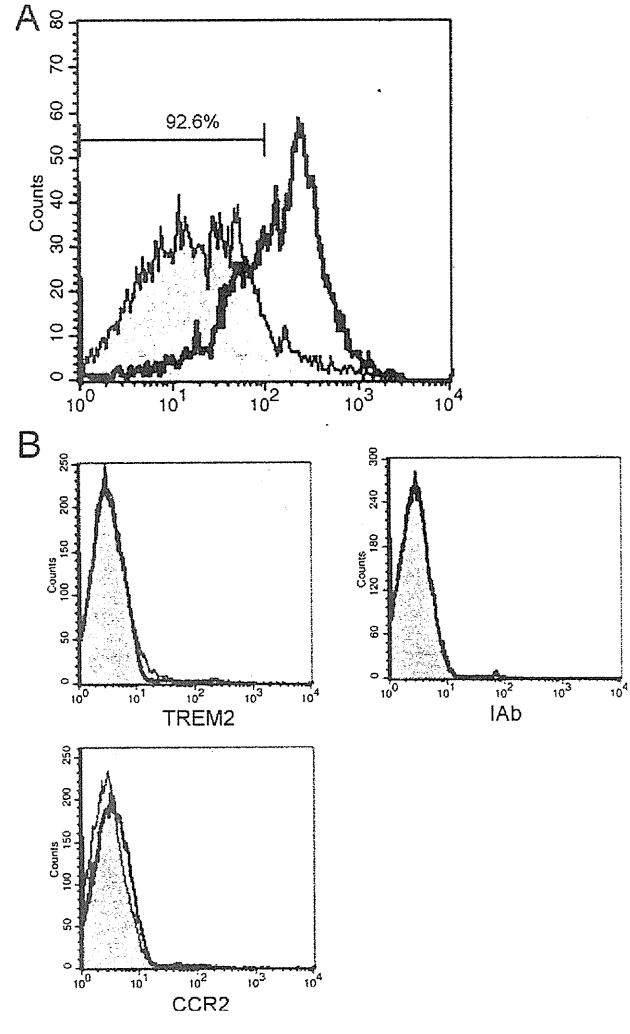

FIG. 2. Flow cytometry analysis of LN cells. (A) Flow cytometry analysis of LN cells (filled histogram) and bone marrow cells (open histogram) stained with rat monoclonal antibodies against mice lineage markers (CD3, CD4, CD5, CD8α, CD11b/MAC-1α, B220, Gr-1 and TER-119). Numbers above the lines indicate the percentage of LN cells. (B) Flow cytometry analysis of LN cells. Filled histograms, staining with antibodies to markers below plots; open histograms, isotype-matched control antibody. Data are representative of three independent experiments. TREM2, triggering receptor expressing on myeloid cells-2.

markers of microglial precursors (Fig. 2B). IAb, which is an MHC class II antigen, was negative on the surface of the LN cells.

Differentiation of LN cells into microglia-like cells

LN cells obtained from GFP mice were co-cultured with primary mixed glial cells for 2 weeks. Two weeks after the co-culture, the cells were stained by anti-TREM2, anti-Iba1, anti-MHC class II and anti-CD11b antibodies, followed by rhodamine- or Cy3-conjugated secondary antibodies (Fig. 3A). GFP+ cells showed two major morphologies, i.e. small and round cells with bright nuclei (27.7 ± 7.9% SD in GFP+ cells) and flat cells with dark nuclei (69.9 ± 6.9% SD in GFP+ cells), similar to the microglia in the primary mixed glial cells seen in Fig. 1. Most of the small round cells were TREM2-, Iba1-, CD11b- and IAb-positive (TREM2+ cells, 92.7 ± 1.9% SD; Iba1+ cells, 93.6 ± 1.6% SD; CD11b+ cells, 96.5 ± 3.1% SD; IAb+ cells, 84.6 ± 13.4% SD; Fig. 3B), and were spherically shaped along the *z*-axis (Fig. 3C). On the other hand, flat cells expressed none of the following: CD11b, Iba1, TREM2 or IAb (TREM2+ cells, 6.5 ± 3.9% SD; Iba1+ cells, 3.5 ± 1.0% SD; CD11b+ cells, 9.3 ± 3.7% SD; IAb+ cells, 3.9 ± 1.9% SD). Because TREM2/DAP12 signaling is known to induce extracellular signaling-regulated kinases (ERK) phosphorylation in immature dendritic

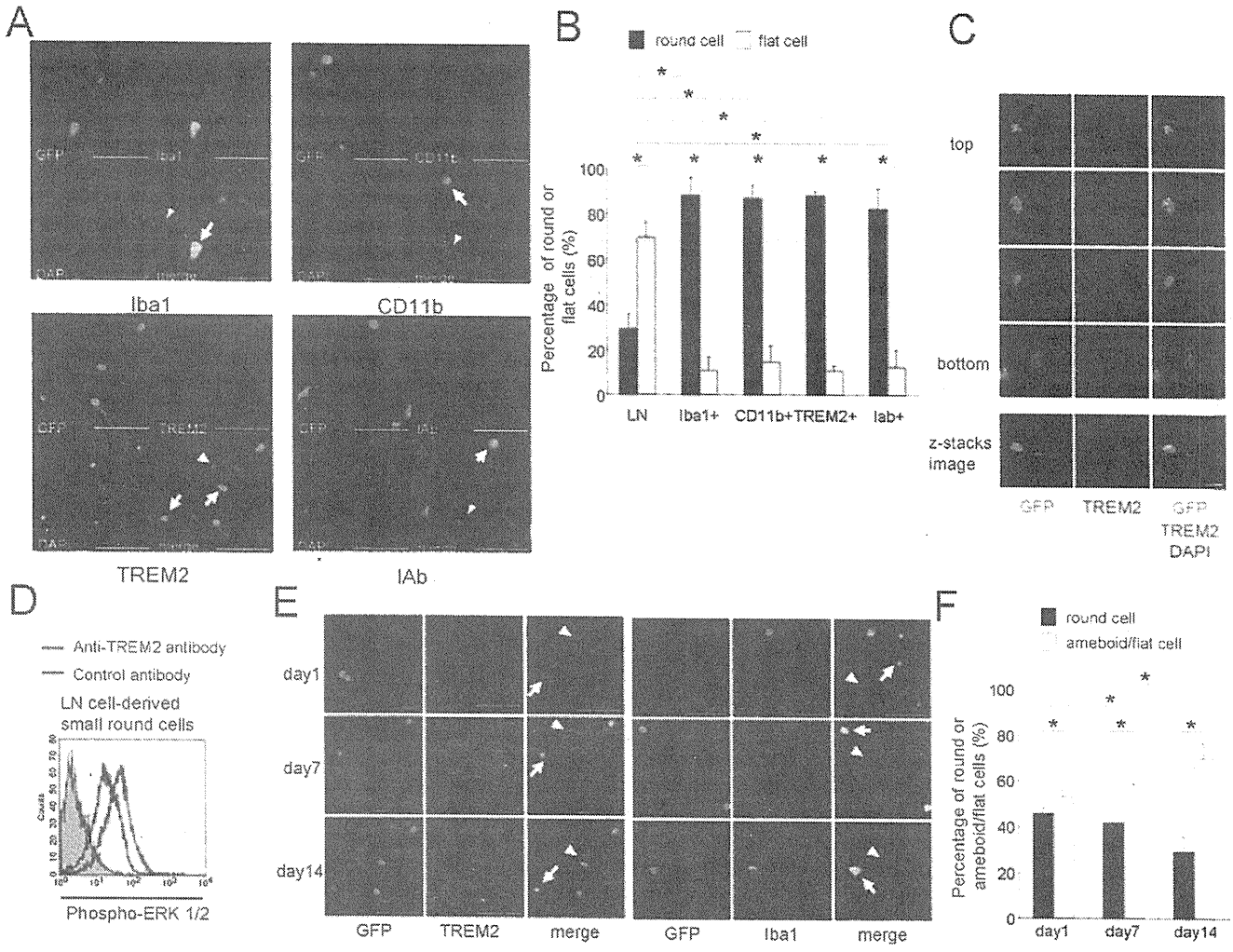

FIG. 3. Lineage-negative bone marrow (LN) cells co-cultured with primary mixed glial culture. (A) Immunocytology of LN cells derived from green fluorescence protein (GFP) mice co-cultured with primary mixed glial culture. Cultures were stained with primary anti-Iba1, anti-triggering receptor expressing on myeloid cells-2 (TREM2), anti-IAb and anti-CD11b antibodies followed by rhodamine- or Cy3-conjugated secondary antibody and 4′,6-diamidino-2-phenylindole (DAPI). Arrows indicate double-positive cells. White arrowheads indicate GFP-negative rhodamine- or Cy3-positive cells. Red arrowheads indicate GFP-positive (GFP+) rhodamine- or Cy3-negative cells. Scale bar: 50 μm. (B) Percentage of round or flat cells among GFP+ cells or double-positive cells. Data are presented as mean ± SD. (C) *Z*-stack immunofluorescence confocal microscopy of LN cells derived from GFP mice co-cultured with primary mixed glial culture stained with anti-TREM2 (red) and DAPI (blue). GFP and TREM2 double-positive cells show a spherical shape. Scale bar: 10 μm. (D) Phosphorylation of ERK after cross-linking stimulation of LN cell-derived small round cells by flow cytometry. Red line histograms, stimulated with anti-TREM2 antibody; blue line histograms, stimulated with control antibody; filled histograms, stained with isotype-matched control antibody. Data are representative of three independent experiments. (E) Immunocytology of LN cells derived from GFP mice co-cultured with primary mixed glial culture. Cells were fixed at Days 1, 7 and 14 after co-culture and stained with primary anti-TREM2 or anti-Iba1 antibodies followed by rhodamine-conjugated secondary antibody and DAPI. Arrows indicate GFP and TREM2/Iba1 double-positive cells. Arrowheads indicate GFP+ and rhodamine-negative cells. Scale bar: 50 μm. (F) The percentage of round or amoeboid/flat cells among GFP+ cells was quantified by microscopic analysis. The proportion of small round and amoeboid/flat cells of GFP+ cells showed no changes at Days 1 and 7, but the number of small round cells reduced at Day 14. Data are presented as mean ± SD; $*P \leq 0.05$. Data are representative of three independent experiments.

cells, we analysed the phosphorylation of ERK using flow cytometry after cross-linking stimulation of LN cell-derived small round cells in order to determine whether the TREM2 on small round cells was functional. Stimulation of TREM2 of the LN cell-derived small round cells induced phosphorylation of ERK as demonstrated by a specific antibody recognizing the phosphorylated form of ERK (Fig. 3D).

In the kinetic study, GFP+ LN cells were immunostained Days 1, 7 and 14 after co-culturing with primary mixed glial cells with anti-TREM2 and anti-Iba1 antibodies followed by secondary antibodies. At Day 1, LN cells had already differentiated into two morphological groups – small round cells expressing Iba1 and TREM2, and flat amoeboid cells (Fig. 3E). Interestingly, the ratio of LN cell-derived small round and flat cells remained identical at Days 1 and 7, but reduced at Day 14 (Day 1, 45.7 ± 2.5% SD; Day 7, 41.7 ± 1.9% SD vs. Day 1, $P = 0.0495$; Day 14, 29.5 ± 6.4% SD vs. Day 1, $P = 0.0495$; Fig. 3F).

Analysis of surface cell markers and proliferative capacity of LN cell-derived small round cells

In many publications, not only the expression of CD11b, Iba1 and F4/80, but also low expressions of CD45 and MHC class II have been used as microglial markers. Among them, low expressions of CD45 and MHC class II are one of the most important resting microglial markers. We measured the quantitative expression of CD45 and MHC class II on brain microglia, cultured microglia and LN cell-derived small round cells. As shown in Fig. 4A, LN cell-derived small round cells showed low expressions of CD45 and MHC class II, which was identical to results for cultured and brain microglia as compared with those for spleen-derived macrophages. LN cell-derived small round cells were also F4/80 positive, which is known to be another microglial marker.

Because microglia can continue to proliferate and differentiate to macrophages, we investigated the proliferative capacity of brain microglia and LN cell-derived small round cells with various concentrations of M-CSF, and compared this capacity to that of splenic macrophages. LN cell-derived small round cells showed increased incorporation of [^{3}H] thymidine similar to that by brain microglia, but [^{3}H] thymidine incorporation by splenic macrophages did not increase in a low concentration of M-CSF (1 ng/mL; LN cell-derived small round cells vs. splenic macrophages, $P = 0.0495$; brain microglia vs. splenic macrophages, $P = 0.0495$; Fig. 4B).

These results indicate that small round cells and microglia are similar in terms of their cell surface molecules and the proliferative capacity.

Proliferation and differentiation of LN cells in the presence of M-CSF

To examine the humoral factors necessary for the differentiation of LN cells into microglia, we cultured LN cells with the supernatant of the primary mixed glial culture for 7 days. Most of the LN cells cultured with this supernatant (LN-Sup cells) were flat in shape and expressed CD11b, Iba1, TREM2 and IAb (Fig. 5A).

Next, we measured the concentrations of cytokines in the supernatant of the primary mixed glial culture using the Bio-Plex Cytokine Assay System. Among the assayed cytokines, the concen-

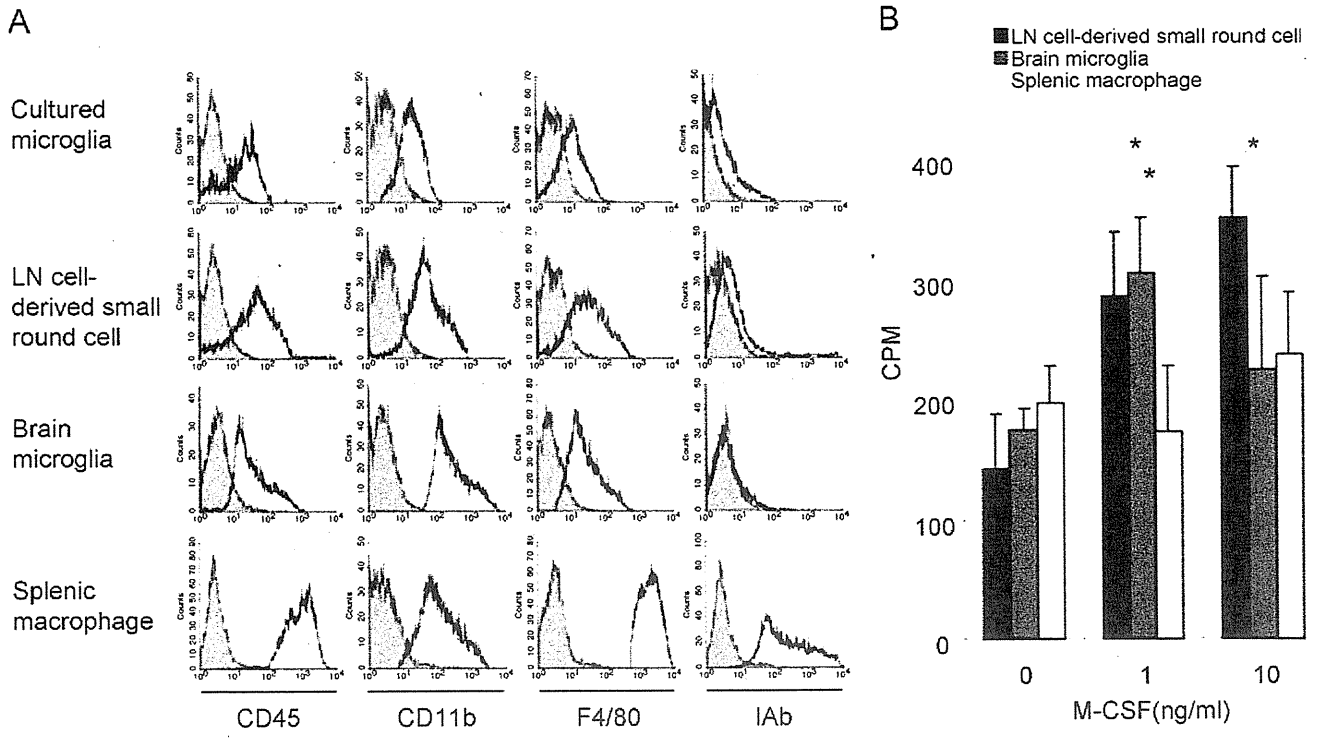

FIG. 4. Surface cell markers and proliferative capacity of lineage-negative bone marrow (LN) cell-derived small round cells. (A) Flow cytometry analysis of cultured microglia, LN cell-derived small round cells, brain microglia and splenic macrophages. Open histograms, staining with antibodies to markers below plots; filled histograms, isotype-matched control antibody. Data are representative of three independent experiments. (B) Proliferative capacity of cultured microglia, LN cell-derived small round cells, brain microglia and splenic macrophages. Proliferation was measured by thymidine incorporation. Data are presented as mean ± SD; *$P \leq 0.05$. Data are representative of three independent experiments. M-CSF, macrophage colony stimulating factor.

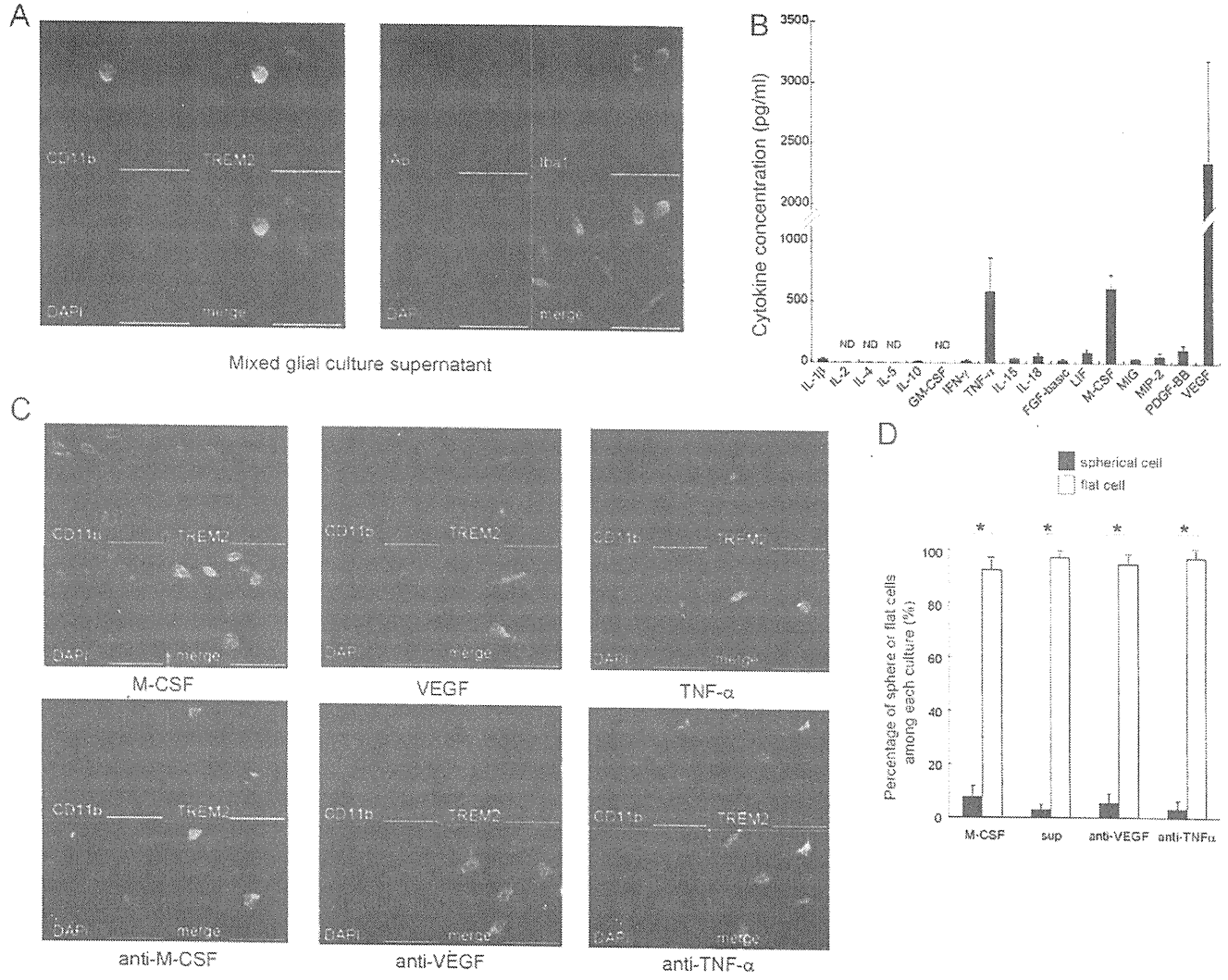

FIG. 5. Proliferation and expression of triggering receptor expressing on myeloid cells-2 (TREM2) on LN cells in the presence of macrophage colony stimulating factor (M-CSF). (A) Immunocytology of LN cells cultured with supernatant of primary mixed glial culture. Cultures were stained with anti-Iba1, anti-TREM2, anti-IAb, anti-CD11b and 4′,6-diamidino-2-phenylindole (DAPI). Scale bar: 50 μm. (B) Analysis of concentration of cytokines [IL-1b, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-γ, tumor necrosis factor-α (TNF-α), IL-15, IL-18, FGF-basic, LIF, M-CSF, MIG, MIP-2, PDGF-BB and vascular endothelial growth factor (VEGF)] in the supernatant of primary mixed glial culture by using the Bio-Plex Cytokine Assay System. ND; not detectable. Data are presented as mean ± SD of 12 independent experiments. (C) Immunocytology of LN cells cultured with M-CSF-, VEGF- or TNF-α-containing medium, and anti-MCSF, anti-VEGF or anti TNF-α antibody-containing supernatant of mixed glial culture. The cells were stained with anti-TREM2 antibodies, CD11b antibodies and DAPI. LN cells cultured with M-CSF showed no change in their morphology or surface markers as compared with LN cells cultured with the supernatant of primary mixed glial culture; however, the addition of anti-M-CSF antibodies remarkably reduced the cell number. Scale bar: 50 μm. (D) The percentage of spherical or flat cells was quantified by confocal microscopy. Data are presented as mean ± SD; $^*P \leq 0.05$. Data are representative of three independent experiments.

trations of M-CSF, VEGF and TNF-α were remarkably high (614.2 ± 121.5 SD pg/mL of M-CSF, 2349.4 ± 845.9 SD pg/mL of VEGF and 585.5 ± 278.2 SD pg/mL of TNF-α) compared with those of the other cytokines (Fig. 5B). IL-2, IL-4, IL-5 and GM-CSF were not detected, and the concentrations of IL-1β, IL-10, IFN-γ, IL-15, FGF-basic and MIG were very low (24.8 ± 10.7 SD pg/mL of IL-1β, 12.4 ± 6.9 SD pg/mL of IL-10, 18.0 ± 13.0 SD pg/mL of IFN-γ, 34.4 ± 12.5 SD pg/mL of IL-15 and 21.6 ± 11.9 SD pg/mL of FGF-basic). The concentrations of IL-18, LIF, MIP-2 and PDGF-BB were at an intermediate level (61.9 ± 23.3 SD pg/mL of IL-18, 92.5 ± 23.8 SD pg/mL of LIF, 34.7 ± 11.6 SD pg/mL of MIG, 57.0 ± 31.6 SD pg/mL of MIP-2 and 115.7 ± 36.1 SD pg/mL of PDGF-BB).

To investigate the effect of each cytokine on the differentiation of LN cells, we cultured LN cells with M-CSF, VEGF or TNF-α for 7 days. All the LN cells cultured with M-CSF (LN–MCSF cells) were CD11b- and TREM2-positive (Fig. 5C). Morphologically, most LN–MCSF cells showed flat shapes on confocal microscopy (Fig. 5D). The number of LN cells cultured with TNF-α (LN–TNF cells) or with VEGF (LN–VEGF cells) were very few, although these cells expressed CD11b and TREM2 (Fig. 5C).

Next, LN cells were cultured with the mixed glial culture supernatant containing neutralizing antibodies of M-CSF, TNF-α or VEGF. Addition of the anti-VEGF antibody or anti-TNF-α antibody resulted in no change in the morphology or surface markers as compared with the culture containing the control antibodies; however,

the addition of anti-M-CSF antibodies remarkably reduced the cell number despite the expression of TREM2 (Fig. 5C).

Cell-to-cell contact between microglia and astrocytes

In order to reveal the role of cell-to-cell contact between microglia and other glial cells, especially astrocytes, in the differentiation of LN cells to microglia, we performed immunostaining of the mixed glial culture with LN cells by anti-GFAP antibody followed by secondary rhodamine-conjugated antibody against rabbit-IgG. Analysis by confocal microscopy revealed that LN cell-derived small round cells were positioned above the GFAP-positive astrocytes (Fig. 6). On the other hand, large flat cells lay immediately beneath the GFAP-positive astrocytes.

Discussion

Microglia, the immune cells of the CNS, exist in three distinct forms – amoeboid, ramified and reactive microglia. Ramified microglia are present in the brain parenchyma and constitute approximately 10–20% of the total population of glial cells in an adult (Vaughan & Peters, 1974; Banati, 2003). Ramified microglia are small round cells comprising branching processes and are considered to be functionally inactive. Further, they are known to express TREM2 *in vivo* (Schmid *et al.*, 2002; Sessa *et al.*, 2004), but not MHC class II (Santambrogio *et al.*, 2001; Servet-Delprat *et al.*, 2002). Recently, HSP60 has been identified as the ligand of TREM2, and its interaction with microglia has been demonstrated to stimulate microglial phagocytosis (Stefano *et al.*, 2009). This result indicates that TREM2 might play an important role in physiological phagocytosis as one of the microglia-specific functions. TREM2 expression on small round cells in primary mixed glial culture might indicate that small round cells have greater mobility as microglia than do large flat cells in patrolling the brain environment in order to identify HSP60-expressing cells. Moreover, as shown in Fig. 4A, small round cells weakly expressed MHC class II molecules similar to brain microglia. These results support our hypothesis that small round microglia *in vitro* differentiated to a greater extent than did large flat cells. Consistent with the results of the current study, previous studies have reported two different morphological shapes of microglia in primary mixed glial culture cells derived from mice (Saura *et al.*, 2003) and rats (Tanaka *et al.*, 1999; Kuwabara *et al.*, 2003). In another study, the kinetic analysis of rat primary mixed glial culture demonstrated that 'amoeboid' or 'round' cells appeared during the early stages of the culture, and that the majority of ramified microglia were formed after the complete formation of the astrocyte monolayer (Tanaka *et al.*, 1999). We demonstrated that LN cells directly differentiated into small round microglia-like cells without the intermediary formation of large flat precursors. Although Ly-6C^{high} Gr-1+CCR2+ monocytes in the peripheral blood have been reported to be the precursors of adult murine microglia (Mildner *et al.*, 2007; Getts *et al.*, 2008), the LN cells in our study did not express CCR2 or Gr-1 (the anti-Gr-1 antibodies used in this study also reacted with Ly-6C). The role of CCR2, a crucial chemokine receptor for the chemotactic attraction of monocytes or macrophages during CNS inflammation, has been investigated in animal models of multiple sclerosis, such as experimental autoimmune encephalomyelitis (Fife *et al.*, 2000; Izikson *et al.*, 2000). However, the physiological development and functions of microglia are independent of CCR2 expression (Mildner *et al.*, 2007). Because the absence of CCR2 expression is highly related to the Ly-6C^{high} subpopulation (Mildner *et al.*, 2007), Ly-6C may play a more important role than CCR2 in microglial differentiation. It is unclear why the LN cells differentiated into small round microglia-like cells despite the lack of Ly-6C and Gr-1 expression, but we hypothesize that the blood–brain barrier plays a key role in the differentiation of LN cells. Indeed, Ly-6C is known to regulate endothelial adhesion and the homing of CD8+T cells by activating integrin-dependent adhesion pathways (Hänninen *et al.*, 1997).

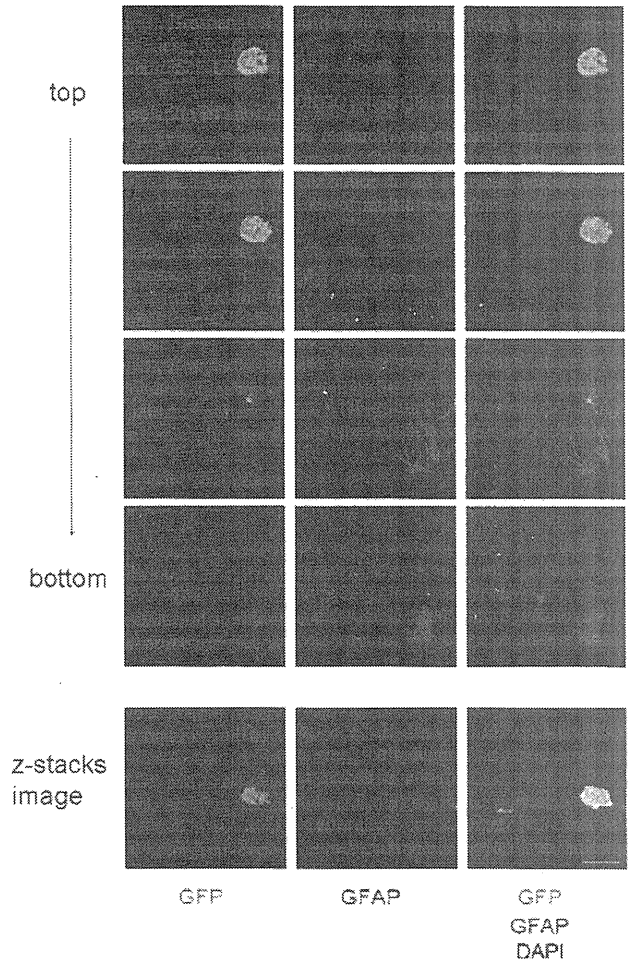

FIG. 6. *Z*-stack immunofluorescence confocal microscopy of LN cells derived from green fluorescence protein (GFP) mice co-cultured with primary mixed glial culture stained with anti-glial fibrillary acidic protein (GFAP; red) and 4′,6-diamidino-2-phenylindole (DAPI; blue). Scale bar: 10 μm.

On the other hand, the LN cells in the current study did not differentiate into flat cells as the precursors of small round microglia. Since they were first described by del Rio-Hortega (1932), the developmental origin of microglia has not been completely elucidated. It is widely hypothesized that microglia are derived from myeloid lineage precursors and/or hematopoietic precursors during CNS development. The fact that the formation of microglia occurs before the onset of vascularization in the developing brain (Wang *et al.*, 1996) suggests that hematopoietic stem cells, which act as precursors to microglia during development, may be more primitive than precursor LN cells. Therefore, it is likely that the LN cells adopt a different pathway to differentiate into small round microglia-like cells.

The supernatant of the primary mixed glial cultures derived from mice contains many cytokines, such as M-CSF, VEGF and TNF-α. Our study showed that M-CSF plays an important role in the

differentiation and proliferation of LN cells. TREM2 is a unique molecule that is only expressed on microglia, osteoclasts and immature dendritic cells (Colonna, 2003), while other microglia-specific markers such as Iba1 and CD11b are also expressed on macrophages and monocytes. Because TREM2 was not expressed on the mature dendritic cells or the activated microglia (macrophages), it is likely that TREM2 expression may be restricted to the immature stages of myeloid lineage cells. In our study, M-CSF induced the proliferation of LN cells, and the expression of not only CD11b and Iba1 but also TREM2 on LN cells; this indicates that M-CSF determines the differentiation of LN cells into the myeloid lineage, but not into fully differentiated cells. Although the addition of anti-M-CSF antibodies appears not to suppress the expression of TREM2 on the LN cells, these TREM2+ cells may have originated from more mature precursors that are independent of M-CSF. These immature LN cells would eventually be fully differentiated under physiological conditions. When LN cells were cultured with M-CSF, the number of small round cells in the culture was considerably low, indicating that the small round morphology of the immature cells is not maintained in the presence of only M-CSF. On the other hand, when LN cells were grown in the presence of other glial cells (especially astrocytes), the small round cells were maintained at a concentration of approximately 30% of the total population. Therefore, cell–cell contact with glial cells, especially astrocytes, may be necessary to maintain the small round shape of immature cells expressing TREM2.

Acknowledgements

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Abbreviations

ERK, extracellular signaling-regulated kinases; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; GFP, green fluorescence protein; LN cells, lineage-negative bone marrow cells; M-CSF, macrophage colony stimulating factor; MHC, major histocompatibility complex; PE, phycoerythrin; TNF-α, tumor necrosis factor-α; TREM2, triggering receptor expressing on myeloid cells-2; VEGF, vascular endothelial growth factor.

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