fingolimod, as indicated by their reduction in the peripheral blood following fingolimod treatment. It is demonstrated in mice that surface expression levels of S1P1 on B cells in the SLT are controlled by transcription levels and CD69-mediated internalisation of S1P1. Stimulation of B-cell receptors induces not only a cessation of S1P1 transcription, but also an upregulation of CD69. Both of these changes reduce the expression levels of surface S1P1 in the SLT to some extent.[2]

Although we were not able to directly analyse B cells in the SLT of the patients, we speculated that surface S1P1 expression on mBs within the SLT in human may also decrease greatly, following antigen activation and exposure to fingolimod, which would result in these B lymphocytes having a reduced responsiveness to S1P. In fact, the activated mB subpopulations that we isolated from the patients' peripheral blood, in particular $CD38^{high}$ mB, were found to contain a substantial proportion of Ki-67^+ cells (Figure 3(a) and (b)). We confirmed that the proportions of Ki-67^+ cells in the activated $CD38^{int}$ and $CD38^{high}$ mB subpopulations were significantly decreased following fingolimod treatment, suggesting that recently-activated cells were selectively trapped in the SLT following fingolimod treatment. Because activation of autoreactive mBs in the SLT followed by their migration to the CNS could trigger a relapse of RRMS,[35] we assumed that inhibition of activated mB cell egress from the SLT was at least partly involved in the reduced relapses of RRMS after fingolimod treatment.

We also identified a PB subpopulation that is relatively resistant to fingolimod as being $CD138^+$ PBs. The frequency of the $CD138^+$ subpopulation in the total PBs, and that of $CXCR3^+$ cells in $CD138^+$ PBs, was significantly increased by fingolimod treatment. Of note, the $CD138^+CXCR3^+$ PBs are enriched in the CSF of NMO during relapse,[27] and fingolimod could induce exacerbation of NMO, accompanied by the appearance of large brain lesions.[11,12] Although knowledge on the biology of PBs is limited, the percentages of $CCR7^+$ cells are much lower as compared with nBs or mBs, indicating that fingolimod may differentially alter the in vivo migration of PBs and other B cells.

It is of relevance to note that despite reductions of circulating lymphocytes, RRMS patients receiving fingolimod may develop clinical relapses. These relapses are not always mild, but could be serious and accompany huge brain lesions.[7–10] Although the trapping of regulatory lymphocytes in the SLT[8,9] or the enrichment for $CD45RO^-CCR7^-CD8^+$ T cells in the CSF[7] is proposed as a possible mechanism for formation of tumefactive brain lesions, we were very curious to know if the increased proportion of $CD138^+$ PBs over other lymphocytes in the peripheral blood might influence the character of the CNS pathology and induce large demyelinating lesions. In fact, it was recently reported that $CD45^+CD19^+CD138^+$ PBs are relatively enriched in the CSF of fingolimod-treated MS patients,[16] raising the possibility that the dominance of $CD138^+$ PBs in the peripheral blood is preserved or even promoted in the CNS of patients with MS who develop tumefactive brain lesions[7–10] and NMO patients who deteriorate[11,12] after being treated with fingolimod. Therefore, resistance of activated PBs in fingolimod-treated patients with MS or NMO may give us a clue to understanding the individual patients' differences regarding the effectiveness of fingolimod therapy.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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EDITORIAL

How do T cells mediate central nervous system inflammation?

In the history of experimental multiple sclerosis (MS) research, the real breakthrough came around 1970, when experimental autoimmune encephalomyelitis (EAE), a classical animal model of MS, was successfully reproduced in syngeneic rodents, which were transferred with sensitized T cells. In contrast, EAE transfer by immune serum failed without exception. Along with the development of basic immunology and of supportive technology, T cell lines and clones reactive to myelin antigen were established *in vitro*, and the works using these cloned T cells soon gained popularity and flourished in the research community. Currently, the T cells used for EAE transfer are not derived from the *in vitro*-generated T cell clones, but are from T cell receptor transgenic mice lymphocytes. However, characterization of pathogenic T cells has stayed in the mainstream of EAE/MS research for almost three decades.

The basic research of CD4+ T cells, recognizing myelin peptide associated with major histocompatibility complex class II molecules, actually brought us a deeper understanding of central nervous system inflammation and led us to design a reasonable strategy to combat MS. In fact, most current MS drugs, including fligolimod and anti-Vα4 integrin antibody natalizumab, were tested in EAE for their efficacy, and showed efficacy by influencing the functions or behavior of autoreactive T cells *in vivo*. Although potential target molecules in MS are not restricted to those expressed by T cells, but extend to B cell markers and others, it is still believed that basic research of T cell biology will continuously give us correct answers to key questions and provide us new ideas on how to discover a cure for MS.

In this special issue of *Clinical and Experimental Neuroimmunology*, five review articles were contributed by experts, given the theme entitled "New mechanistic insights into the pathogenesis of multiple sclerosis – from a T cell point of view". As known to senior scientists over the age of 40 years, the classical paradigm of Th1/Th2 balance prevailed for several years, until Th17 cells were discovered approximately one decade ago. Reflecting the shorter history of research, there remain many questions about the biology of Th17 cells regarding their identity and plasticity. Furthermore, the presence of Th17 cells in the lesions of MS is still a matter of controversy, as reviewed by Thomas Korn,[1] although the clinical relevance of Th17 pathogenic T cells in neuromyelitis optica (NMO) has substantial support. However, elevation of interleukin-17 mRNA was shown in lesions of MS,[2] and Th17 cell-associated molecule, NR4A2, was also increased in the peripheral blood of MS patients,[3] showing the role of Th17 cells in MS. Therefore, Th17-associated molecules, NR4A2[3] and Tob1,[4] are potential targets of therapy in MS and NMO.

For future perspectives, the authors[5,6] and the Editor recognize that more research is required into human T cells in conditions of health and disease. It will be important to re-analyze the human T cell populations without holding any prevailing dogma. Supporting this, $CCR2^{+}CCR5^{+}$ Th1 cells, which have not been given attention, unexpectedly increased in the cerebrospinal fluid of patients with MS in relapse, and showed pathogenic potentials.[5] Readers of this special issue might share the opinion with us that application of new technologies will bring the next breakthrough within years, and immunology textbooks could require major revision.

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Takashi Yamamura
Department of Immunology, National Institute of Neuroscience, NCNP, Tokyo, Japan

免疫動態

佐藤 和貴郎　山村 隆

はじめに

多発性硬化症(multiple sclerosis；MS)は，病変が脳・脊髄・視神経の様々な部位に(空間的多発)繰り返し(時間的多発)おこることを特徴とする炎症性脱髄性疾患である．臨床的経過から，大きく「再発と寛解を繰り返す病態」と「進行性の病態」の二つに分類できる．免疫学的病態について，「再発期」には獲得免疫系による自己免疫性の炎症性脱髄が中心で，「進行期」は自然免疫系の関与が優位となり，炎症より神経変性が目立つというモデルが提案されている(図1)[1]．このうち前者の「再発期」については近年理解が進み，再発抑制効果をもつ複数の免疫治療薬が臨床現場で用いられるようになった．一方，進行性の病態については未知の部分が多く「神経変性」や「慢性炎症」をキーワードに研究が進行中であるが，再発を抑制し疾患活動性を抑制することの意義は認識されている．疫学的に興味深いことは，従来アジア人に少なかったMSがわずか数十年の間にわが国で確実に増加している点である．昨今の腸内細菌・腸管免疫系の研究の進歩により，食生活を含む環境因子とMSの関連性が明らかとなりつつある．本稿では，MSの代表的な動物モデルである実験的自己免疫性脳脊髄炎(experimental autoimmune encephalomyelitis：EAE)の知見を述べながら，MSの病態に関与する免疫学的機序について概説する．

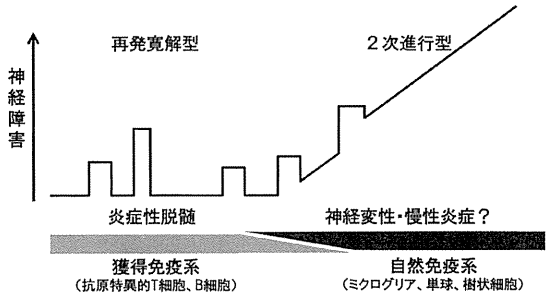

図1　多発性硬化症の経過と免疫系

多発性硬化症の最も一般的な臨床経過を示す．再発寛解型の時期はCD4陽性T細胞を中心とした獲得免疫系の役割が大きいのに対し，進行型では自然免疫系の比重が増加すると考えられている．(Weiner[1]を参考に作図)

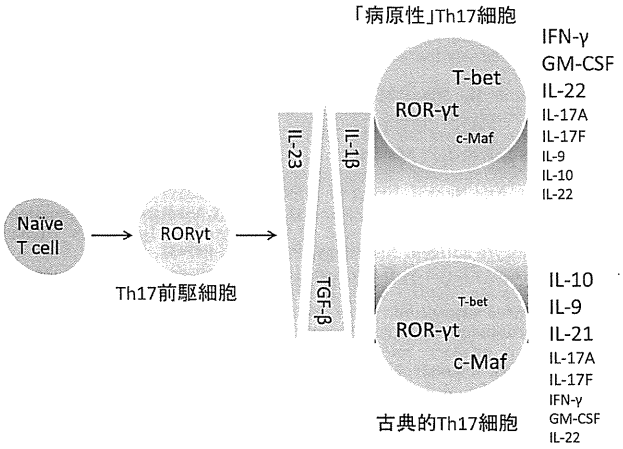

図2　Th17細胞をスペクトラムとして捉える

Th17細胞はRORγtを発現しTh17前駆細胞となったのち，サイトカイン微小環境の影響により，IL-10産生能などを特徴とし免疫系のホメオスターシスに関わる古典的Th17細胞，あるいはIFN-γ等を産生しEAEを惹起する病原性Th17細胞へと分化する．(Petersら[10]を参考に作図)

MS発症の遺伝的背景

MSの一卵性双生児における同胞発症率は約30%とさ

さとう わきろう　(独)国立精神・神経医療研究センター神経研究所/免疫研究部室長
やまむら たかし　同 部長

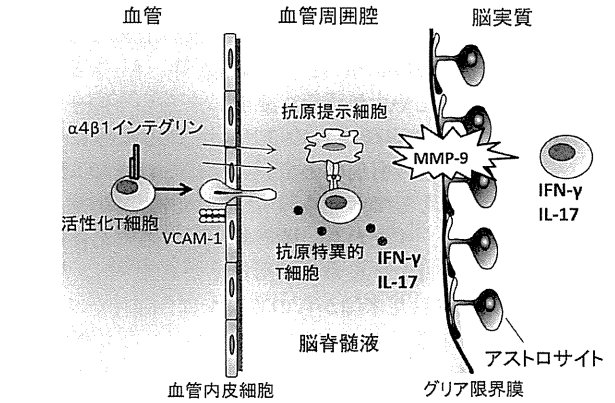

図3　病原性リンパ球が脳内へ侵入し炎症を惹起するモデル図

末梢で活性化されたT細胞はα4β1インテグリンを介し血管内皮細胞に接着し血管周囲腔へ到達する．ここで抗原提示細胞により再度活性化されたのち，IL-17やIFN-γなどの炎症性サイトカインを産生，さらにMMP-9などの作用によりグリア限界膜を越えて脳内に侵入する．

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れ，発症には遺伝的背景が関与する．2011年に欧米人MS患者を対象とした疾患感受性遺伝子ゲノムワイド関連解析(GWAS)の結果が報告された[2]．Major histocompatibility complex(MHC)クラスⅠ・Ⅱ分子，インターロイキン(IL)-2受容体，IL-7受容体の関与が再確認され，新たに29の新規感受性遺伝子が示された．その多くが1型糖尿病や炎症性腸疾患，関節リウマチと同様CD4陽性ヘルパーT細胞(helper T cells：Th cells)の機能に関わる遺伝子であった．すなわち獲得免疫系による自己免疫機序の重要性が改めて認識された．ただ，この研究は欧米白人の再発寛解型MS患者を対象にコモンバリアントについて調べたものであり，レアバリアントの関与やMSの多様性に寄与する遺伝子については今後の課題である．またアジアなど異なる地域の患者での検証が待たれる．

再発に関わる病原性T細胞は何か

MS研究はEAE研究とともに発展してきた．EAEはマウスなどの実験動物にミエリン塩基性タンパク(MBP)などの髄鞘抗原をアジュバント(免疫応答の増強剤)とともに接種(免疫)して中枢神経炎症を誘導する動物モデルである(active EAE)．髄鞘抗原に特異的に反応するCD4陽性Th細胞を動物に注射する方法でも神経炎症が誘導されることから(passive EAE)，病原性T細胞はTh細胞であるといえる．MS患者においてもミエリン塩基性タンパクに対し特異的に反応するT細胞が患者末梢血中に検出される．また，MS患者の中枢神経病理では，病変部に特定の限られた種類のT細胞クローンが集簇している．これはT細胞が抗原特異性をもつこと，すなわち特定の抗原を標的とするT細胞が病変部を形成していることを意味し，MS自己免疫説の重要なポイントである．なお，MSの病変部位で認められるT細胞の標的抗原は証明されていない．

Th17細胞の登場と研究の展開

1990年代以降，Th細胞はTh1/Th2細胞に二分されるドグマが支配的で，EAEを誘導するTh細胞はインターフェロンガンマ(IFN-γ)産生性のTh1細胞であると信じられてきたが，矛盾する結果も報告されていた．2003年，Cuaらの論文はこの問題に対し解決への道筋を与えた[3]．Th1細胞の分化に重要なIL-12の欠損マウスでもEAEが誘導されたことから，EAEの誘導にはTh1細胞は必ずしも必要ではないことが示された．しかしIL-12に類似するIL-23の欠損マウスではEAEが発症しないことが示され，IL-23に注目が集まった．解析の結果，IL-23はTh細胞からのIL-17産生を介しIL-6，TNF-α，GM-CSFなどの炎症性サイトカインを誘導することが示された．2006年には，IL-17産生T細胞がナイーブCD4陽性T細胞からIL-6とTGF-βの作用により分化誘導され，特異的な転写因子RORγtが同定され，Th1，Th2とは異なる第三のTh，Th17細胞として認知され，EAE/MSの発症のキーとなる細胞として注目された[4]．髄鞘抗原特異的なTh17細胞とTh1細胞を比較すると，Th17細胞がTh1細胞よりも少ない細胞数でEAEをおこすと報告されたため，Th17細胞の病原性が広く認知された．しかし，意外にもIL-17遺伝子欠損マウスにおけるEAEの改善は軽度であることから，IL-17の作用が決定的ではないことも分かった[5]．

Th17細胞に関する検討が進むとTh17細胞は一つの均一な細胞集団というより，「多様性」と「可塑性」をもつ細胞と捉えられるようになった．Th17細胞と免疫寛容の維持に重要な制御性T細胞(Treg)との関係について，FoxP3陽性細胞がTh17細胞に分化転換して(conversion)病原性細胞となりうること[6]や，IL-17を産生しつつも抑制能を維持しているFoxP3陽性Tregの存在が報告された[7]．一方，一般的にはTh1細胞が産生するIFN-γの産生能を有するTh17細胞の存在も報告されている．マウスを用いた検討でEAEを発症する過程でIL-17A産生性の細胞がIFN-γ産生性の細胞に分化転換し中枢神経に浸潤していた[8]．また，再発期MS患者の末梢血中にはIFN-γとIL-17の両者を同時に産生する細胞(Th1/Th17細胞)が増加しており，同細胞は血液脳関門(blood brain barrier：BBB)モデルを通過する能力が高いとの報告もある[9]．様々な結果にもとづき，KuchrooらはTh17細胞をスペクトラムとして捉えることを提唱している(図2)[10]．そのモデルではpre-Th17細胞の誘導後，TGF-βの作用が持続する場合はIL-10などを産生し，腸管免疫系のホメオスターシスに寄与する細胞(非病原性細胞)になるが，IL-23やIL-1βが作用するとGM-CSFやIFN-γ産生性の病原性タイプに分化しEAE誘導能を獲得するとされる．

Th17細胞の「発見」を契機として新たなThサブセットが次々と報告されている．IL-9産生を特徴とするTh9やIL-22産生が特徴的なTh22などである．興味深いことに，ThサブタイプによりEAEの病変分布が異なるという複数の報告がある．一例をあげると，in vitroで誘導されたTh17，Th1，Th2，Th9細胞をマウスに注射してEAEを誘導したところ，それぞれのEAEの発症様式や病変分布が異なっていた[11]．MSの病変分布には多様性が認められるが，Th細胞のサブタイプとMSの病変分布に何らかの関連がある可能性が考えられる．それと関連して，MSの再発予防薬として頻用されるIFN-βには無効例や悪化例があることが知られ，とくに視神経と脊髄に主病変をもつ視神経脊髄炎(neuromyelitis optica：NMO)では悪化例が

多く使用禁忌となっている．Steinmanらは Th1 細胞で誘導した EAE は IFN-β 投与により軽減するのに対し，Th17 細胞で誘導した EAE は逆に IFN-β 投与により悪化することを示し，IFN-β は Th17 細胞の介在する自己免疫病態には無効ないし増悪因子となる可能性を指摘している[12]．

リンパ球の中枢神経系への移行

病原性リンパ球が脳内へ到達するプロセスは以下のように考えられている．まず，リンパ節で活性化を受けたのち，リンパ節を出て血管内に移行する．次に，脳血管内皮細胞を越えて脳脊髄液中に移動する．その後，脳内へ侵入し神経炎症を惹起するという流れである(図3)．病原性リンパ球の移動をブロックすると EAE/MS の病態を制御することができる．リンパ球が二次リンパ節から移出する際に必要な SIP1 受容体の発現を低下させる薬剤 fingolimod は EAE を軽減し MS の再発を抑制する．T 細胞が脳の血管内皮細胞に接着するのに必要な α4β1 インテグリンに対する阻害抗体 natalizumab も EAE/MS に対し治療効果をもつ．fingolimod および natalizumab は MS の再発予防薬として臨床現場で使用されている．リンパ球が脳内に到達する前に立ちはだかる最後の壁が，アストロサイトの足突起で構成されるグリア限界膜である．ラットの EAE の髄膜血管をライブイメージングにより詳細に観察した研究がある[13]．活性化 T 細胞は抗原特異性に関係なく血管周囲腔へ到達するが，脳実質に到達できたのは抗原特異的 T 細胞に限られていた．脳脊髄液中で抗原提示細胞に遭遇し再度活性化されることが，T 細胞がグリア境界膜を越えるために必要であると結論づけている．なおリンパ球の移動には，ケモカインシステムや，膜を構成する基底膜を変性させるマトリックス・メタロプロテアーゼ 9(MMP-9)などのタンパク分解酵素も重要である．

腸内細菌と腸管免疫系

従来アジア人には少なかった MS がわずか一世代の間にわが国で著増していることから，環境因子の変化が疾患感受性を高めたと考えられる．MS の発症に関与する環境因子として，これまで精神的ストレス，高緯度地域，ビタミン D 不足，喫煙，Epstein-Barr(EB)virus 感染などの関与が報告されてきた．しかし，これらのリスクだけで発症頻度の増加を説明することは困難である．近年，腸管免疫に関する研究が進歩し，MS 病態への関与について検討が始まっている．非吸収性の抗生物質経口投与により全身の免疫系には影響を与えずマウスの腸内細菌の組成を変化させたところ，EAE が軽症化した[14]．また，マウスの腸管の常在菌である segmented filamentous bacteria が Th17 細胞誘導能をもち，この細菌がいないマウスではやはり EAE が軽症化した[15]．自然発症で再発寛解型の脳脊髄炎を示す遺伝子改変マウスを用いた研究で，無菌環境下では脳脊髄炎が発症せず，腸内細菌を定着させると再び自然発症すると報告された[16]．従って，MS において腸管免疫系が発症や経過に影響を与える可能性が示唆される．

自然リンパ球の役割

近年自然免疫と獲得免疫の橋渡しの細胞として，両者の特徴を兼ね備える innate lymphocyte(自然リンパ球：ILCs)に注目が集まっている．Natural killer(NK)細胞の他，mucosal associated invariant T(MAIT 細胞)や iNKT 細胞(invaritant NKT 細胞)が代表的な ILC である．MS 患者の NK 細胞は寛解期には IL-5 を産生し，髄鞘抗原反応性 T 細胞に対し抑制的に働くことが報告されている[17]．MAIT 細胞の T 細胞受容体の α 鎖は Vα7.2 のみ，iNKT 細胞では Vα24 と固定されており，抗原受容体の多様性が乏しい(invariant)特徴がある．MAIT 細胞は腸管に多いことから名づけられた経緯があるが，血中にも数％以上認められる．MAIT 細胞は MS の病変部位で検出されるほか，患者の末梢血中での頻度低下が認められ病態と関連し変化する[18]．EAE に対して抑制的な効果をもつことから MS 病態に対し制御性の機能が示唆される[19]．長らく未知であった抗原がビタミン B2 の代謝物質であり腸内細菌が産生に関与することが最近報告された[20]．iNKT 細胞は糖脂質を抗原として認識し，抗原として同定された α-galactoceramide は強いサイトカイン産生能を誘導するが，改変体である OCH は IFN-γ などの Th1 サイトカインの産生は低く，IL-4 などの Th2 サイトカインを優位に産生し EAE を軽減する効果をもつ[21]．OCH は経口投与での MS 病態の改善効果が期待され，現在筆者の施設で医師主導の臨床試験が進行中である．

炎症惹起性および炎症制御性分子

2001 年，Steinman らのグループにより再発寛解型 MS の病巣に発現する遺伝子産物の網羅的解析の結果が報告された[22]．もっとも発現の高いグループの中に αB crystallin とオステオポンチン(osteopontin：OPN)が含まれ，病態への関与が検討された．OPN は色々な分子と結合し多様な機能を発揮する炎症惹起性分子であるが，T 細胞上の α4β1 インテグリンに結合すると T 細胞活性化と抗アポトーシス作用を発揮する．再発時 MS 患者髄液での増加がみられ，再発への関与が疑われる．抗 α4 インテグリン抗体 natalizumab の作用機序の一つは OPN による T 細胞活

性化抑制であると考えられる．一方 αB crystallin は寛解状態との関連が注目されている．低分子量熱ショックタンパク質(small heat shock proteins：small HSP)の一つであるが，同タンパクを欠損したマウスでは EAE が重症化し，同タンパクを投与すると臨床症状は軽快した．その分子メカニズムとしては，神経細胞やグリア細胞のアポトーシス抑制および炎症性サイトカイン抑制作用が考えられている[23]．

B 細胞の関与

MS 患者の髄液では，IgG 産生が亢進していること(IgG index の上昇)や髄腔内特異的な抗体の存在(オリゴクローナルバンド：oligoclonal band：OCB)が知られ，B 細胞の関与が示唆されてきた．病理学的にも MS の脳病変が 4 種類に分類されるなかで，T 細胞・マクロファージ浸潤とともに IgG や補体成分の沈着を認める病変パターンが最も一般的であると報告されている[24]．また，B 細胞のマーカーである CD20 に対するモノクローナル抗体 rituximab が再発・寛解型 MS に有効であることが報告されている．rituximab は CD20 陰性の形質芽細胞や形質細胞には作用せず，また髄液 IgG 濃度低下や OCB 消失は伴わなかったことから，効果発現には B 細胞の抗原提示能やサイトカイン産生能の関与が推定されている．一部の 2 次進行型 MS の病理において髄膜リンパ濾胞様構造が認められる[25]．濾胞内には胚中心様構造もあることから，親和性成熟を伴う B 細胞クローン増殖が髄腔内でおこり，進行性の病態に関与している可能性が示唆される．IFN-β は液性免疫を活性化することが知られているが，IFN-β 投与 MS 患者の血清中の BAFF (B cell-activating factor belonging to the TNF family)濃度の上昇が報告されている[26]．BAFF は B 細胞の生存や分化を促進する因子であり，IFN-β で悪化する病態には BAFF を介した B 細胞活性化が関与している可能性がある．

むすび

以上，主として MS の再発病態と Th 細胞の関与に焦点を当て解説した．MS の進行性の病態には未知の部分が多いが，自然免疫系の役割が注目され現在精力的に検討が進められている．また，古くから神経系・内分泌系・免疫系は生体の恒常性維持のためのシステムとして互いに密接に関連することが知られている．例えばステロイドはストレスホルモンであり，リンパ球は神経伝達物質の受容体を発現している．システムバイオロジーの一環として臓器連関が注目されており，今後の展開が期待される．

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OX40 ligand regulates splenic CD8$^-$ dendritic cell-induced Th2 responses *in vivo*

Fumitaka Kamachi [a], Norihiro Harada [a,b], Yoshihiko Usui [a,c], Tamami Sakanishi [d], Naoto Ishii [e], Ko Okumura [a], Sachiko Miyake [a], Hisaya Akiba [a,*]

[a] Department of Immunology, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[b] Department of Respiratory Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[c] Department of Ophthalmology, Tokyo Medical University, 6-7-1 Nishi-Shinjuku-ku, Tokyo 160-0023, Japan
[d] Division of Cell Biology, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[e] Department of Microbiology and Immunology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan

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ABSTRACT

In mice, splenic conventional dendritic cells (cDCs) can be separated, based on their expression of CD8α into CD8$^-$ and CD8$^+$ cDCs. Although previous experiments demonstrated that injection of antigen (Ag)-pulsed CD8$^-$ cDCs into mice induced CD4 T cell differentiation toward Th2 cells, the mechanism involved is unclear. In the current study, we investigated whether OX40 ligand (OX40L) on CD8$^-$ cDCs contributes to the induction of Th2 responses by Ag-pulsed CD8$^-$ cDCs *in vivo*, because OX40–OX40L interactions may play a preferential role in Th2 cell development. When unseparated Ag-pulsed OX40L-deficient cDCs were injected into syngeneic BALB/c mice, Th2 cytokine (IL-4, IL-5, and IL-10) production in lymph node cells was significantly reduced. Splenic cDCs were separated to CD8$^-$ and CD8$^+$ cDCs. OX40L expression was not observed on freshly isolated CD8$^-$ cDCs, but was induced by anti-CD40 mAb stimulation for 24 h. Administration of neutralizing anti-OX40L mAb significantly inhibited IL-4, IL-5, and IL-10 production induced by Ag-pulsed CD8$^-$ cDC injection. Moreover, administration of anti-OX40L mAb with Ag-pulsed CD8$^-$ cDCs during a secondary response also significantly inhibited Th2 cytokine production. Thus, OX40L on CD8$^-$ cDCs physiologically contributes to the development of Th2 cells and secondary Th2 responses induced by Ag-pulsed CD8$^-$ cDCs *in vivo*.

1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells critical for the induction of adaptive immune responses. Conventional DCs (cDCs) are specialized for antigen processing and presentation to T cells and can be subdivided by their surface expression of CD8α and CD4 as CD8$^-$CD4$^+$, CD8$^-$CD4$^-$, and CD8$^+$CD4$^-$ cDCs in the spleen [1–4]. Both CD8$^-$CD4$^+$ and CD8$^-$CD4$^-$ cDCs appear functionally similar and are referred to as CD8$^-$ cDCs [2,3]. In contrast, the physiologic functions of both CD8$^-$ cDCs and CD8$^+$ cDCs markedly differ. *In vivo* experiments demonstrated that injection of antigen-pulsed CD8$^-$ cDCs induced CD4 T cell differentiation toward Th2 responses (high levels of IL-4, IL-5, and IL-10) whereas antigen-pulsed CD8$^+$ cDCs induced Th1 responses (high levels of IFN-γ [5]. The ability of CD8$^+$ cDCs to induce Th1 differentiation is explained by their ability to produce IL-12 efficiently [6,7]. However, the mechanisms of Th2 responses induced by CD8$^-$ cDCs are not understood.

CD4 T cell differentiation might be regulated by cytokines and various costimulatory molecules expressed on CD4 T cells, and their cognate ligands expressed on DCs such as OX40 (CD134) costimulatory molecule, a member of the TNF receptor superfamily, and its ligand, OX40L (CD252) [8,9]. OX40 is preferentially expressed on activated CD4 T cells and OX40L is mainly expressed on antigen-presenting cells, including activated DCs, B cells, and macrophages. Recent studies emphasized the role of OX40L on DCs for Th2 polarization. In humans, schistosomal egg antigen induced monocyte-derived DCs to express OX40L, which contributed to the induction of Th2 responses [10]. IL-3-treated plasmacytoid DCs expressed OX40L and induced Th2 responses by promoting CD4 T cells to secrete IL-4, IL-5, and IL-13. Blockade of OX40L significantly inhibited this ability of IL-3-treated plasmacytoid DCs [11]. Moreover, OX40L expressed on thymic stromal lymphopoietin (TSLP)-activated DCs induced naïve CD4 T cells to differentiate into TNF-α$^+$ IL-10$^-$ inflammatory Th2 cells [12]. In mice, OX40L expression on bone marrow-derived DCs (BMDCs) is upregulated downstream of CD40 signaling and is critical for optimal Th2 priming *in vivo* [13]. In contrast to these studies, the use of agonistic anti-OX40 mAb revealed OX40-mediated costimulation enhanced the

* Corresponding author. Fax: +81 3 3813 0421.
E-mail address: hisaya@juntendo.ac.jp (H. Akiba).

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development of Th1 responses induced by splenic $CD8^-$ cDCs *in vivo* [14]. Thus, the function of OX40L on splenic $CD8^-$ cDCs is still controversial. In this study, we examined the physiological contribution of OX40–OX40L interactions on $CD8^-$ cDCs-induced Th2 responses by using blocking anti-OX40L mAb.

2. Materials and methods

2.1. Animals

Female BALB/c mice were purchased from Charles River Laboratories (Kanagawa, Japan). OX40L-deficient mice were generated as previously described [15] and backcrossed for seven generations with BALB/c mice purchased from Oriental Yeast Co. (Tokyo, Japan). All mice were 6–8 week old at the start of experiments and kept under specific pathogen-free conditions during the experiments. All animal experiments were approved by Juntendo University Animal Experimental Ethics Committee.

2.2. Antibodies and reagents

An anti-mouse OX40L (RM134L) mAb was previously generated in our laboratory [16]. Control rat IgG was purchased from Sigma–Aldrich (St Louis, MO, USA). Purified anti-CD40 (HM40-3), allophycocyanin (APC)-conjugated anti-CD8α (53-6.7), and rat IgG isotype control were purchased from eBioscience (San Diego, CA, USA). Purified anti-CD16/32 (2.4G2) and FITC-conjugated anti-CD11c (HL3), recombinant mouse GM-CSF, IL-4, and IFN-γ were purchased from BD Biosciences (San Jose, CA, USA).

2.3. Preparation and stimulation of splenic DCs

To isolate splenic DCs, spleens from BALB/c or OX40L-deficient mice were digested with 400 U/ml of collagenase (Wako Biochemicals, Tokyo, Japan), further dissociated in Ca^{2+}-free medium in the presence of 5 mM EDTA, and separated into low- and high-density fractions by Optiprep-gradient (Axis-Shield, Oslo, Norway) as described previously [17]. Low-density cells were pulsed overnight with 50 μg/ml of keyhole limpet hemocyanin (KLH) in culture medium supplemented with 20 ng/ml of GM-CSF as described previously [5]. After overnight culture, splenic $CD11c^+$ DCs were isolated by incubation with anti-CD11c-coupled magnetic beads and positive selection by autoMACS column (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD11c^+$ DCs were further separated according to CD8α expression by FACS sorting. $CD11c^+$ cells were incubated with FITC-conjugated anti-CD11c and APC-conjugated anti-CD8α mAbs, and two populations ($CD8^+CD11c^+$ DCs and $CD8^-CD11c^+$ DCs) were sorted by FACSVantage (BD Biosciences). To examine OX40L expression, separated DC populations were incubated with anti-CD40 mAb (10 μg/ml) with IL-4 (20 ng/ml) or IFN-γ (20 ng/ml) in the presence or absence of GM-CSF (20 ng/ml) at 37 °C for 24 h.

2.4. Flow cytometric analysis

Cells were pre-incubated with unlabeled anti-CD16/32 mAb to avoid non-specific binding of Abs to FcγR, incubated with FITC- or APC-labeled mAbs, or biotinylated mAb followed by PE-labeled streptavidin. Stained cells (live cells gated by forward and side scatter profiles and propidium iodide exclusion) were analyzed by FACSCalibur (BD Biosciences), and data were processed by CellQuest (BD Biosciences).

2.5. Immunization protocol

KLH-pulsed splenic cDCs were washed in PBS and immunized (3×10^5 cells) into the hind footpad of BALB/c mice. Some groups of mice ($n = 5$–6) were administered 400 μg of anti-OX40L mAb or rat IgG intraperitoneally (i.p.) at days 0, 1, and 3, or daily from days 0 to 3 and days 14–17. Popliteal lymph node (LN) cells were harvested 5 days after primary or secondary immunizations.

2.6. T cell stimulation in vitro

LN cells were isolated and cultured in RPMI1640 medium (containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-mercaptoethanol) at a density of 6×10^5 cells/well in the presence of indicated doses of KLH. To assess proliferative responses, cultures were pulsed with tritiated thymidine ([^{3}H]TdR; 0.5 μCi/well; PerkinElmer, Winter Street Waltham, MA, USA) for the last 6 h of a 48 h or 72 h culture and harvested on a Micro 96 Harvester (Molecular Devices, Sunnyvale, CA, USA). Incorporated radioactivity was measured using a microplate beta counter (Micro β Plus; PerkinElmer). To determine cytokine production, cell-free supernatants were collected at 48 h or 72 h and assayed for IL-2, IL-4, IL5, IL-10, and IFN-γ by ELISA using Ready-SET-Go! kits (eBioscience) according to the manufacturer's instructions.

2.7. Statistical analysis

Statistical analyses were performed by unpaired Student *t*-test or Tukey's multiple comparison test. Results are expressed as mean ± SEM. Values of $P < 0.05$ were considered significant.

3. Results

3.1. OX40L is required for optimal Th2 responses induced by splenic cDCs in vivo

Because a previous report demonstrated KLH-pulsed $CD8^-$ and $CD8^+$ cDCs differentially regulated Th cell development, we followed the same protocol using KLH as an antigen. To clarify the contribution of splenic cDC OX40L on CD4 T cell differentiation, we examined CD4 T cell responses induced by splenic $OX40L^{-/-}$ cDCs. cDCs were purified from spleens of OX40L-deficient or wild-type BALB/c mice without treatment, pulsed with KLH during overnight culture with GM-CSF, to isolate $CD11c^{high}$ $B220^-$ cells (cDC population). $OX40L^{-/-}$ cDCs or WT cDCs (3×10^5) were injected into hind footpads of syngeneic BALB/c mice. LNs were prepared on day 5 and proliferative responses and cytokine production against various doses of KLH were assessed. KLH-specific proliferative responses and IL-2 production were reduced in LN cells from $OX40L^{-/-}$ cDCs-injected mice compared with WT cDCs-injected mice (Fig. 1). Th2 cytokine production (IL-4, IL-5, and IL-10) was also significantly reduced in $OX40L^{-/-}$ cDCs-injected mice compared with WT cDCs-injected mice. In contrast, Th1 type cytokine IFN-γ production was non-significantly increased in $OX40L^{-/-}$ cDCs-injected mice compared with WT cDCs-injected mice.

Similar results were obtained when KLH-pulsed $OX40L^{-/-}$ bone marrow-derived DCs (BMDCs) were injected into hind footpads of BALB/c mice (Supplemental Fig. S1). KLH-specific proliferative responses and IL-2 production were reduced in LN cells from $OX40L^{-/-}$ BMDCs-injected mice compared with WT BMDCs-injected mice. Th2 cytokine production (IL-4, IL-5, and IL-10) was significantly reduced in $OX40L^{-/-}$ BMDCs-injected mice, whereas IFN-γ production was similar between $OX40L^{-/-}$ BMDCs-injected

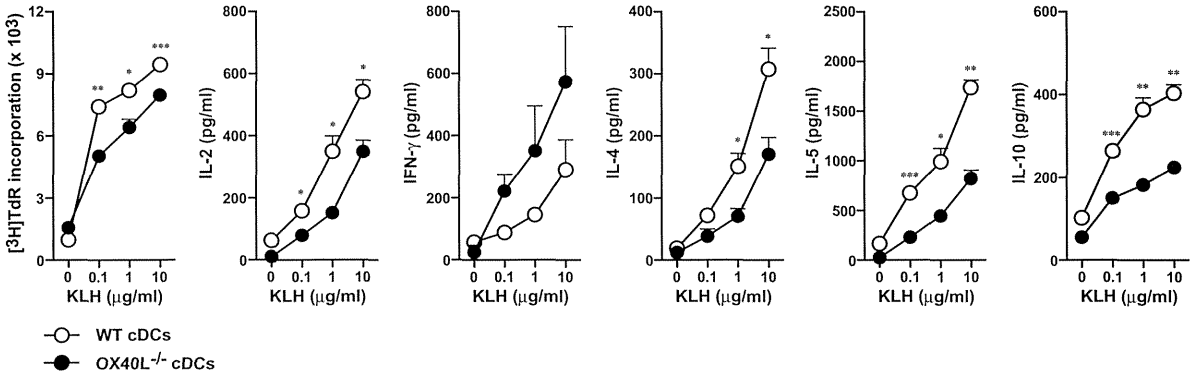

Fig. 1. OX40L is required for optimal Th2 responses by splenic cDCs *in vivo*. BALB/c mouse hind footpads were injected with KLH-pulsed cDCs isolated from the spleen of wild-type BALB/c or OX40L$^{-/-}$ BALB/c mice. LN cells were harvested at day 5 and cultured with indicated doses of KLH. To estimate proliferation, 0.5 μCi ^{3}H-thymidine ([^{3}H]TdR) was added during the last 6 h of a 48 h culture. Production of IFN-γ, IL-2, IL-4, IL-5, and IL-10 in culture supernatants at 48 h was determined by ELISA. Results are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Similar results were obtained in three independent experiments.

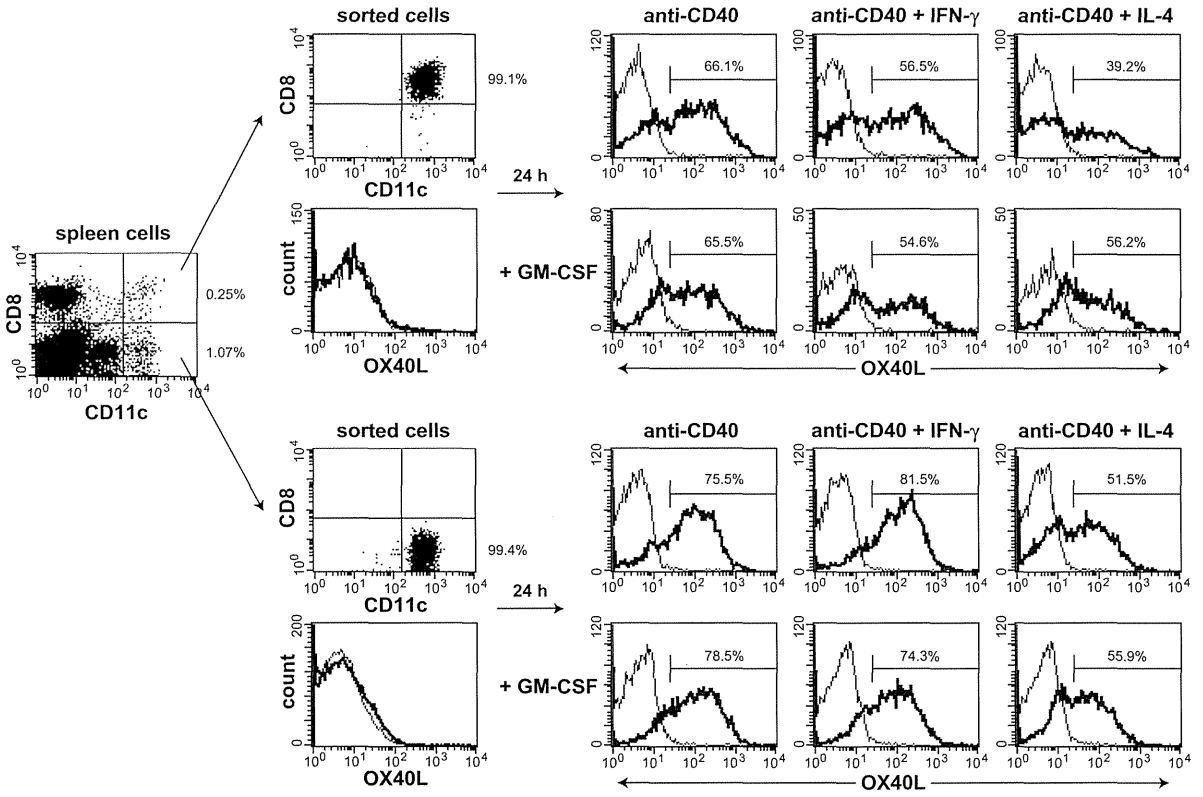

Fig. 2. Expression of OX40L on activated CD8^{-} and CD8^{+} cDCs. Spleen cells were isolated from BALB/c mice and stained with FITC-labeled anti-CD11c, APC-labeled anti-CD8α, and biotinylated anti-OX40L or control IgG followed by PE-labeled streptavidin. CD8^{-}CD11c^{high} and CD8^{+}CD11c^{high} cDCs were isolated from spleens by FACS sorting. Isolated CD8^{-}CD11c^{high} and CD8^{+}CD11c^{high} cDCs were stimulated with anti-CD40 mAb in the presence or absence of GM-CSF, IFN-γ, and IL-4. Cells were harvested at 24 h and stained with anti-OX40L mAb or control rat IgG. Thick lines indicate staining with anti-OX40L mAb and thin lines indicate background staining with control IgG. Data are representative of three experiments.

and WT BMDCs-injected mice. In addition, administration of neutralizing anti-OX40L mAb to WT BMDCs-injected mice significantly reduced Th2 cytokine production similar to OX40L$^{-/-}$ BMDCs-injected mice. Th2 cytokine reduction was also observed in KLH-pulsed WT BMDCs injected with anti-OX40L mAb into IFN-γ-deficient mice (Supplemental Fig. S2). These results indicated a critical role of OX40L in splenic cDCs- and BMDCs-induced Th2 responses *in vivo*. The inhibition of Th2 responses by anti-OX40L treatment was not necessarily a result of a shift to Th1 responses.

3.2. Expression of OX40L on splenic cDCs

The expression of OX40L on two major subsets of splenic cDCs was assessed by flow cytometry. Splenic cDCs were separated

based on CD8α and CD11c expression, into $CD8^-CD11c^{high}$ cDCs ($CD8^-$ cDCs) and $CD8^+CD11c^{high}$ cDCs ($CD8^+$ cDCs), and stimulated with agonistic anti-CD40 with or without cytokines (GM-CSF, IFN-γ, or IL-4) for 24 h (Fig. 2). While OX40L expression was not observed on freshly isolated $CD8^-$ or $CD8^+$ cDCs, it was induced by anti-CD40 mAb stimulation. Addition of IL-4 reduced OX40L expression on anti-CD40-stimulated $CD8^-$ and $CD8^+$ cDCs, whereas OX40L expression was not affected by the addition of GM-CSF or IFN-γ.

3.3. Effect of anti-OX40L mAb on the development of Th2 responses induced by KLH-pulsed $CD8^-$ cDCs in vivo

We next examined whether KLH-pulsed $CD8^-$ cDCs could induce Th2 responses compared with KLH-pulsed $CD8^+$ cDCs, and whether OX40L contributes to $CD8^-$ cDCs-induced Th2 responses. BALB/c mice were injected into the hind footpads with KLH-pulsed $CD8^-$ or $CD8^+$ cDCs, and treated with anti-OX40L mAb or control IgG at days 0, 1, and 3. LN cells were isolated at day 5 and KLH-specific proliferative responses and cytokine production were assessed. Consistent with previous reports, IL-4 production by LN cells from $CD8^-$ cDCs-injected mice was significantly higher than in $CD8^+$ cDCs-injected mice (Fig. 3). In contrast, IFN-γ production in $CD8^+$ cDCs-injected mice was non-significantly increased compared with the $CD8^-$ cDCs-injected mice. Proliferative responses and other Th2 cytokine production (IL-5 and IL-10) were similar between $CD8^-$ cDCs-injected and $CD8^+$ cDCs-injected mice. Anti-OX40L mAb administration strongly inhibited IL-4, IL-5, and IL-10 production induced by $CD8^-$ cDCs injection, while IFN-γ was slightly increased. Thus, OX40L has an important role in the development of Th2 responses induced by KLH-pulsed $CD8^-$ cDCs *in vivo*. Furthermore, administration of anti-OX40L mAb reduced IL-4 production induced by $CD8^+$ cDCs injection. Therefore, OX40L may also regulate IL-4 production induced by KLH-pulsed $CD8^+$ cDCs.

3.4. Effect of anti-OX40L mAb in secondary Th2 responses induced by KLH-pulsed $CD8^-$ cDCs in vivo

The OX40–OX40L pathway is crucial for recall responses when memory T cells are reactivated [18]. Therefore, we further examined the role of OX40L in secondary Th2 responses induced by KLH-pulsed $CD8^-$ cDCs *in vivo*. BALB/c mice were immunized first into the hind footpads with KLH-pulsed $CD8^-$ cDCs at day 0 and then under the same conditions with KLH-pulsed $CD8^-$ cDCs at day 14. Some groups of mice were treated with anti-OX40L mAb or control IgG daily from days 0 to 3 in the primary phase and days 14–17 in the secondary phase. LN cells were isolated at day 19 and the KLH-specific Th2 cytokine production was assessed. Anti-OX40L mAb administration during the primary phase only, reduced IL-4 and IL-5 production compared with control IgG (Fig. 4). In addition, anti-OX40L mAb administration in the secondary phase strongly inhibited IL-4, IL-5, and IL-10 production compared with control IgG. The inhibitory effect of anti-OX40 mAb

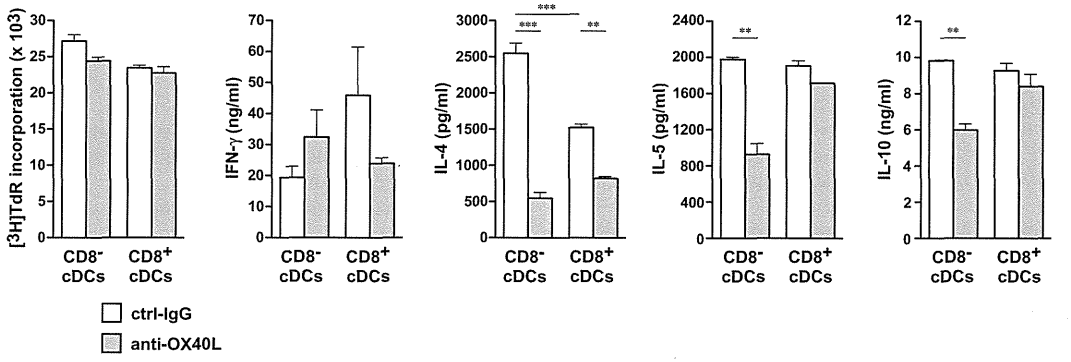

Fig. 3. Effect of anti-OX40L mAb on the development of Th2 responses induced by KLH-pulsed $CD8^-$ cDCs *in vivo*. BALB/c mouse hind footpads were injected with KLH-pulsed $CD8^-$ or $CD8^+$ cDCs. Mice were administered 400 μg of anti-OX40L mAb or control rat IgG (ctrl-IgG) i.p. at days 0, 1, and 3. LN cells were harvested at day 5 and cultured with 20 μg/ml of KLH. To estimate proliferation, 0.5 μCi [^{3}H]TdR was added during the last 6 h of a 72 h culture. Production of IFN-γ, IL-4, IL-5, and IL-10 in the culture supernatants at 72 h was determined by ELISA. Results are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Similar results were obtained in three independent experiments.

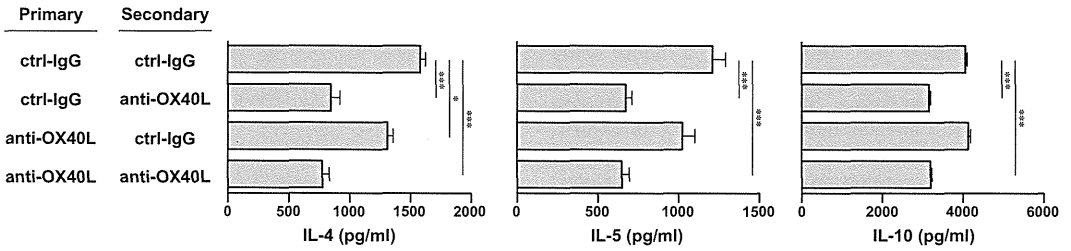

Fig. 4. Effect of anti-OX40L mAb on the development of memory Th2 responses induced by $CD8^-$ cDCs *in vivo*. BALB/c mice were immunized first with KLH-pulsed $CD8^-$ cDCs at day 0 and boosted with the same KLH-pulsed $CD8^-$ cDCs at day 14. Mice were administered 400 μg of anti-OX40L mAb or ctrl-IgG i.p. daily from days 0 to 3 and days 14–17. LN cells were harvested at day 19 and cultured with 10 μg/ml of KLH. To estimate proliferation, 0.5 μCi [^{3}H]TdR was added during the last 6 h of a 72 h culture. Production of IFN-γ, IL-4, IL-5, and IL-10 in culture supernatants at 72 h was determined by ELISA. Results are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Similar results were obtained in three independent experiments.

treatment in the secondary phase was comparable to mice treated with anti-OX40 mAb in both primary and secondary phases. Thus, OX40L might have an important role in both primary and secondary Th2 responses induced by KLH-pulsed $CD8^-$ cDCs *in vivo*.

4. Discussion

The current study investigated the physiological role of splenic $CD8^-$ cDC OX40L to regulate CD4 T cell Th2 differentiation *in vivo*. When antigen KLH-pulsed OX40L-deficient cDCs were injected into BALB/c mice, LN Th2 cytokine production (IL-4, IL-5, and IL-10) was significantly reduced. Splenic cDCs were separated into $CD8^-$ and $CD8^+$ cDCs. A previous study demonstrated that although injection of KLH-pulsed $CD8^-$ cDCs induced CD4 T cell differentiation toward Th2 responses, KLH-pulsed $CD8^+$ cDCs promoted Th1 responses [5]. Consistently, our results indicated that $CD8^-$ cDCs markedly induced IL-4 production and $CD8^+$ cDCs tended to induce IFN-γ production. Administration of neutralizing anti-OX40L mAb significantly inhibited IL-4, IL-5, and IL-10 production induced by KLH-pulsed $CD8^-$ cDCs. Moreover, treatment of anti-OX40L mAb with KLH-pulsed $CD8^-$ cDCs during a secondary response also significantly inhibited Th2 cytokine production. Thus, OX40L contributes to both the development of Th2 cells and secondary Th2 responses induced by KLH-pulsed $CD8^-$ cDCs *in vivo*. However, these findings are inconsistent with a previous report where administration of anti-OX40 mAb enhanced the development of Th1 cells secreting high levels of IFN-γ, but no IL-4 and IL-5, induced by KLH-pulsed $CD8^-$ cDCs *in vivo* [14]. The reason for this discrepancy is not clear, but it may be attributable to differences in experimental conditions. The previous study isolated splenic cDCs from mice treated with FMS-like tyrosine kinase 3 ligand (Flt3L) on 11 days, whereas mice were untreated in our study. Flt3 is a crucial factor in humans and mice to promote the development of cDCs *in vivo* and *in vitro*. However, a bias toward the generation of $CD8^+$ cDCs in the spleen was observed in mice treated with Flt3L [19,20]. The previous study also examined the effect of exogenous OX40 costimulation using agonistic anti-OX40 mAb, suggesting such an effect is not mediated by endogenous OX40–OX40L interactions between CD4 T cells and cDCs. Our results suggest that physiological OX40–OX40L interactions participate in CD4 T cell-$CD8^-$ cDCs interactions, and that OX40L on $CD8^-$ cDCs might contribute to the induction of Th2 responses *in vivo*.

In humans, TSLP-activated DCs can promote the differentiation of naïve CD4 T cells into a Th2 phenotype and the expansion of CD4 Th2 memory cells in an unique manner dependent on OX40L in the absence of IL-12 [12]. TSLP, an IL-7-like cytokine, is produced mainly by damaged epithelial cells and is a key molecule that links epithelial cells and DCs at the interface of allergic inflammation by participating in the programming of DC-mediated Th2 polarization [21–24]. TSLP activates STAT1, STAT3, STAT4, STAT5, and STAT6, whereas the contributions of individual STAT proteins to the activation of DCs is unclear [25]. Most recently, a mouse study demonstrated that DC-specific deletion of STAT5 was critical for TSLP-mediated Th2 differentiation, but not Th1 differentiation [26]. Loss of STAT5 in DCs affected upregulation of OX40L expression in response to TSLP. However, DC subsets in $Stat5^{-/-}$ chimeric mouse spleens had a higher proportion of $CD8^+$ cDCs and a reduced frequency of $CD4^+$ $CD8^-$ cDCs compared with $Stat5^{+/+}$ chimeras, suggesting STAT5 signaling regulates a balanced production of these splenic DC subsets *in vivo* [27]. Thus, STAT5 may be required for OX40L-dependent Th2 cell differentiation induced by KLH-pulsed $CD8^-$ cDCs. To confirm this, further studies are required using STAT5-specific deleted $CD8^-$ cDCs. In this study, we demonstrated that KLH-pulsed $OX40L^{-/-}$ BMDCs injected into hind footpads of BALB/c mice significantly reduced Th2 cytokine production (IL-4, IL-5, and IL-10) in LN cells compared with WT BMDCs-injected mice. Consistent with these observations, it was reported that OX40L expression by GM-CSF-induced BMDCs is required for optimal induction of primary and memory Th2 responses *in vivo* [13]. GM-CSF can activate STAT5, and GM-CSF-activated STAT5 inhibits the transcription of *Irf8* [27], which encodes interferon regulatory factor 8 (IRF8). IRF8 is required for IL-12 production [25], an essential cytokine required for the induction of Th1 responses [28]. Therefore, OX40L-dependent Th2 responses induced by KLH-pulsed $CD8^-$ cDCs might depend on the absence of IL-12, as IL-12 has a dominant effect over OX40L in Th cell differentiation [12]. Indeed, we observed that $CD8^+$ cDCs produced high amounts of IL-12p40 after stimulation with agonistic anti-CD40 mAb, whereas IL12p40 production on $CD8^-$ cDCs was markedly lower (unpublished observation). Taken together, these findings suggest that the development of Th2 responses by KLH-pulsed $CD8^-$ cDCs requires two conditions: the expression of OX40L and the absence of IL-12.

However, whether OX40 signaling on CD4 T cells directly induces Th2 differentiation is still unclear. It is well known that OX40 can bind to TNF receptor-associated factor (TRAF) 2, TRAF3, and TRAF5. However, these molecules also can bind to other TNF receptor family molecules. On a transcriptional basis, it was determined that OX40L expressed by TSLP-DCs induced the expression of GATA-3 in CD4 T cells, supporting their critical role in Th2 polarization [12]. Another study indicated that OX40 enhanced TCR-induced calcium influx, leading to the enhanced nuclear accumulation of NFATc1 and NFATc2, that likely regulates the production of cytokines [29]. More studies are required to determine how OX40 signaling promotes Th2 differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.060.

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Chondroitin 6-*O*-sulfate ameliorates experimental autoimmune encephalomyelitis

Katsuichi Miyamoto[1,2], Noriko Tanaka[2], Kota Moriguchi[3], Rino Ueno[2], Kenji Kadomatsu[4], Hiroshi Kitagawa[5], and Susumu Kusunoki[2]

[2]Department of Neurology, Kinki University School of Medicine, Osaka-Sayama, Japan; [3]Division of Neurology, Department of Internal Medicine 3, National Defense Medical College, Tokorozawa, Japan; [4]Department of Biochemistry, Nagoya University School of Medicine, Nagoya, Japan; and [5]Department of Biochemistry, Kobe Pharmaceutical University, Kobe, Japan

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Chondroitin sulfate proteoglycans (CSPGs) are the main component of the extracellular matrix in the central nervous system (CNS) and influence neuroplasticity. Although CSPG is considered an inhibitory factor for nerve repair in spinal cord injury, it is unclear whether CSPG influences the pathogenetic mechanisms of neuroimmunological diseases. We induced experimental autoimmune encephalomyelitis (EAE) in chondroitin 6-*O*-sulfate transferase 1-deficient (C6st1$^{-/-}$) mice. C6ST1 is the enzyme that transfers sulfate residues to position 6 of *N*-acetylgalactosamine in the sugar chain of CSPG. The phenotypes of EAE in C6st1$^{-/-}$ mice were more severe than those in wild-type (WT) mice were. In adoptive-transfer EAE, in which antigen-reactive T cells from WT mice were transferred to C6st1$^{-/-}$ and WT mice, phenotypes were significantly more severe in C6st1$^{-/-}$ than in WT mice. The recall response of antigen-reactive T cells was not significantly different among the groups. Furthermore, the number of pathogenic T cells within the CNS was also not considerably different. When EAE was induced in C6ST1 transgenic mice with C6ST1 overexpression, the mice showed considerably milder symptoms compared with those in WT mice. In conclusion, the presence of sulfate at position 6 of *N*-acetylgalactosamine of CSPG may influence the effecter phase of EAE to prevent the progression of pathogenesis. Thus, modification of the carbohydrate residue of CSPG may be a novel therapeutic strategy for neuroimmunological diseases such as multiple sclerosis.

Keywords: central nervous system / chondroitin sulfate / experimental autoimmune encephalomyelitis / multiple sclerosis / myelin oligodendrocyte glycoprotein / proteoglycan

Introduction

Chondroitin sulfate proteoglycans (CSPGs) are a family of glycosaminoglycans consisting of glucuronic acid, *N*-acetylgalactosamine and sulfate. CSPG is the main constituent of the extracellular matrix in the central nervous system (CNS) and influences neuroplasticity (Rolls et al. 2006). For example, CSPG levels are up-regulated after CNS injury (McKeon et al. 1999; Asher et al. 2001; Jones et al. 2002) and during the course of chronic neurodegenerative disorders such as multiple sclerosis (Sobel and Ahmed 2001) and Alzheimer's disease (Inoue 2001). CSPG attenuates regeneration in the CNS (Properzi et al. 2003; Silver and Miller 2004) by inhibiting neuronal growth (Bradbury et al. 2002; Grimpe and Silver 2002) and regulating the activation of immune components (Fitch and Silver 1997).

For instance, CSPG inhibits axonal regeneration in spinal cord injury models and, conversely, CSPG degradation by the enzyme chondroitinase ABC promotes repair (Bradbury et al. 2002; Chau et al. 2004; Huang et al. 2006). In general, inhibition of non-systemic neuronal projection protects the neuronal network. These reports suggest that CS moieties of CSPGs are responsible for PNN formation and control of the critical period plasticity. However, importance of sulfation pattern of CS chains in the plasticity has been overlooked in these previous studies due to the exclusive use of ChABC that degrades all CS chains, irrespective of their sulfation status. Sulfation profiles of CS chains change dramatically during brain development (Mikami and Kitagawa 2013). Although the role of CSPG in autoimmune disease has not been analyzed in detail, some reports have indicated that CSPG promotes recovery in CNS immunopathologies such as experimental autoimmune encephalomyelitis (EAE). For example, administration of CSPG alleviates the clinical symptoms of EAE and reduces microglia activation and the number of infiltrating T cells (Zhou et al. 2010). Furthermore, the deletion of protein tyrosine phosphatase receptor type Z as CSPG in the CNS delays recovery from paralysis associated with EAE. These results indicate that CSPG has a role in CNS recovery and might therefore be helpful in overcoming inflammation-induced neurodegenerative conditions (Harroch et al. 2002).

Chondroitin 6-sulfotransferase (C6ST) catalyzes the transfer of sulfate to position 6 of the *N*-acetylgalactosamine residue of chondroitin. Sulfation that occurs mainly at the C-6 position of the internal GalNAc residue produces chondroitin 6-sulfate (C6S), whereas the C-4 sulfated form is called (C4S).

C6S is the dominant form of chondroitin in the fetal period; however, the percentage of chondroitin 4-sulfate increases

[1]To whom correspondence should be addressed: Tel: +81-723-66-0221; Fax: +81-723-68-4846; e-mail: miyamoto@med.kindai.ac.jp

during development. Sulfation patterns vary depending on organ, age, cell progression and other factors. For instance, distinctive increases in C6S are seen in the spleen, lung, bone marrow and eye, and C6S plays a role in the maintenance of naive T lymphocytes in the spleen of young mice (Uchimura et al. 2002). Molecular cloning of human C6ST has revealed two orthologous genes, *C6ST1* (Fukuta et al. 1998; Uchimura et al. 1998) and *C6ST2* (Kitagawa et al. 2000). Expression of both isoforms in the brain is evident during development but negligible in adulthood. C6ST1 is one of the sulfotransferases involved in the biosynthesis of sulfated glycosaminoglycans; however, in vivo functional analysis of C6ST1-deficient mice shows no apparent abnormality in brain development (Uchimura et al. 2002). In the present study, we analyzed the role of CSPG in EAE using C6ST1-deficient mice.

Materials and methods

Mice

Wild-type (WT) C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). C6ST1$^{-/-}$ mice were originally obtained from Dr. Kadomatsu (Nagoya, Japan) and have been described previously. The mice had been backcrossed to the C57BL/6 background for more than five generations.

Mice were rendered deficient in the *C6st1*$^{-/-}$ gene via embryonic stem cell technology. C6st1$^{-/-}$ mice were born at approximately the expected frequency and were viable through adulthood. Brain development was normal in C6st1$^{-/-}$ mice. Further analysis revealed that the number of CD62L^{+}CD44^{low} T lymphocytes corresponding to naive T lymphocytes in the spleen of C6st1$^{-/-}$ mice was significantly decreased, whereas that in other secondary lymphoid organs was unchanged (Uchimura et al. 2002). Genotyping of C6ST1$^{-/-}$ mice was performed with polymerase chain reaction as described elsewhere.

C6ST1 transgenic mice (C6ST1^{tg}) were obtained from Dr. Kitagawa (Miyata et al. 2012). Full-length human C6ST1 complementary DNA was amplified with reverse transcription polymerase chain reaction using a human placenta complementary DNA library and cloned into the EcoRI site of a pCAG vector, which drives transgene expression using a chicken β-actin promoter and cytomegalovirus enhancer (Niwa et al. 1991). Plasmid DNA was injected into C57BL6 embryos, which were placed into pseudopregnant females to produce transgenic offspring. Transgenic mice were identified with Southern blot using tail DNA and were mated with C57BL6 WT mice. Mice were kept under specific pathogen-free conditions in an environmentally controlled, clean room. All experiments were conducted according to institutional ethics guidelines for animal experiments and safety guidelines for gene manipulation experiments. These mice were maintained under specific pathogen-free conditions. All mice for experiments were 8–12 weeks old.

Peptides

Myelin oligodendrocyte glycoprotein (MOG_{35-55}) (single-letter amino acid code, MEVGWYRSPFSRVVHLYRNGK) was synthesized by Tore Research Institute (Tokyo, Japan). The peptides were of >90% purity, as determined by high-performance liquid chromatography (HPLC).

Induction and assessment of EAE

Mice were injected subcutaneously in both flanks with 200 μL of inoculum containing 100 μg MOG_{35-55} and 0.5 mg *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) in incomplete Freund's adjuvant. Pertussis toxin (200 ng; List Biological Laboratories Inc., Campbell, CA) was injected intravenously on days 0 and 2 after immunization. For EAE induction in the adoptive-transfer model, recipient mice were injected intravenously with encephalitogenic cells (prepared as in Preparation of cells for EAE induction in the adoptive-transfer model section) and 200 ng of pertussis toxin. Immunized mice were examined daily and scored as follows: 0, no clinical signs; 1, limp tail; 2, partial hind leg paralysis; 3, total hind leg or partial hind and front leg paralysis; 4, total hind leg and partial front leg paralysis; 5, moribund or dead. Mice were examined daily in a blind fashion for signs of EAE.

Preparation of cells for EAE induction in the adoptive-transfer model

For preparation of MOG-specific cells to induce EAE in the adoptive-transfer model, mice were immunized with MOG/complete Freund's adjuvant following the protocol used to induce EAE. Draining lymph nodes (LNs) were collected 10 days later, and a single-cell suspension was prepared. The cells were stimulated with 30 μg/mL MOG_{35-55} in 24-well flat-bottomed plates (5×10^6 cells/well) in T-cell medium (RPMI media enriched with 10% fetal bovine serum, 2 mM L-glutamine, 5×10^{-5} M 2-ME, nonessential amino acids, sodium pyruvate and penicillin/streptomycin). Recombinant mouse interleukin (IL)-12 was added at 20 ng/mL. Four days after initiation of the cultures, cells were harvested, and CD4^{+} cells were selected using a column (R&D, Minneapolis, MN). Ten million CD4^{+} cells were injected intravenously into recipient mice as described above for EAE induction.

Establishment of a MOG_{35-55}-specific T-cell line

A MOG-specific T-cell line was established using cells for adoptive-transfer EAE as described above. The cells were cultured in T-cell medium with 10 ng/mL of IL-2. Half of the medium was replaced every few days. Stimulation with 30 μg/mL MOG_{35-55} was performed every 10 days with irradiated (30 Gy) splenocytes as antigen-presenting cells.

MOG_{35-55}-specific T-cell proliferation assay

For proliferation assays, mice were immunized with peptide/CFA as described above, but the mice were not treated with pertussis toxin. A single-cell suspension was prepared from the draining LNs 10 days after immunization. Cells were cultured in Dulbecco's modified Eagle medium (Gibco, Grand Islands, NY) supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/mL of penicillin and streptomycin and 1% autologous mouse serum and seeded onto 96-well flat-bottomed plates (1×10^6 cells/well). The cells were restimulated with peptide for 72 h at 37°C in humidified air with 5% CO_2. To measure cellular proliferation, [^{3}H]-thymidine was added (1 mCi/well) and uptake of the radioisotope during the final 18 h of culture was counted with a beta-1205 counter (Pharmacia, Uppsala, Sweden). To evaluate the proliferative

responses of LN cells to the peptide, we determined the delta (Δ)c.p.m. value for cells in each well by subtracting the background c.p.m. and then used the mean of these values to represent each mouse.

Detection of cytokines

In parallel, LN cells from immunized mice were cultured with peptide concentrations of 0, 1, 10 and 100 μg/mL. Supernatants from the cultures were harvested 48 h postactivation and tested for the presence of various cytokines. The concentrations of interferon gamma (IFN-g), IL-2, IL-4 and IL-10 in the supernatants were measured with sandwich enzyme-linked immunosorbent assay according to the manufacturer guideline (BD Biosciences, San Jose, CA). Limits of detection for IFN-g, IL-2, IL-4 and IL-10 were 195, 25, 12.5, and 50 pg/mL, respectively.

Analysis of infiltrating cells isolated from the CNS

Wt and C6ST1$^{-/-}$ mice were anesthetized with diethyl ether on day 9 after EAE induction. After perfusion with phosphate-buffered saline, the brain and spinal cord were removed and homogenized. After washing with phosphate-buffered saline, mononuclear cells were isolated using Percoll gradient (Amersham Biosciences, Piscataway, NJ) and counted (Miyamoto et al. 2006). The cells were stained with phycoerythrin-labeled anti-CD4 antibody (BD Biosciences) and analyzed with flow cytometry using a BD FACSCalibur device. As a control, naive mice were also analyzed for the infiltration of cells into the CNS using the same method.

Pathological analysis

The brain and spinal cord were removed on day 14 after the induction of EAE. Ten-micrometer-thick frozen sections were fixed with acetone and stained with hematoxylin and eosin or Luxol fast blue.

HPLC analysis

Soluble CSPG fractions from brain were prepared as described previously (Kitagawa et al. 1997). In brief, brains from WT or C6st1$^{-/-}$ mice, at pre- or postimmunization EAE, were homogenized in ice-cold PBS containing EDTA and phenylmethylsulfonyl fluoride. After centrifugation, supernatant fluids were concentrated, and then washed with Tris–HCl buffer containing sodium acetate. The protein concentration of the proteoglycan fractions was determined using the BCA protein assay kit. These fractions were first digested using chondroitin ABC lyase and evaporated to dryness.

The digests were derivatized with 2-aminobenzamide according to the manufacturer's instructions (SIGNAL™ labeling kit, Oxford GlycoSystems). The labeled disaccharides were analyzed by on an amine-bound silica PA03 column. The HPLC was performed in an LC-10AS system (Shimadzu Co., Kyoto, Japan).

Statistics

Non-parametric Mann–Whitney *U* tests were used to calculate significant levels for all measurements. Values of $P < 0.05$ were considered statistically significant.

Results

Exacerbation of EAE in C6ST1$^{-/-}$ mice

To examine the role of C6ST1 in the development of EAE, we first performed active immunization to establish EAE. The clinical symptoms of EAE in C6ST1$^{-/-}$ mice were more severe than those in WT mice were. Statistically, a significant difference in EAE maximum score and cumulative score was found between C6ST1$^{-/-}$ and WT mice (Figure 1, Table I). Histological comparison of the thoracic regions of the spinal cord demonstrated exacerbated monocyte infiltration and demyelination in C6ST1$^{-/-}$ mice compared with WT mice (not shown). We next performed adoptive-transfer EAE. When we transferred MOG-reactive T cells from WT mice to C6ST1$^{-/-}$ or WT mice, the severity of EAE in the C6ST1$^{-/-}$ mice was significantly higher than that in WT mice (Figure 2; Table I). These results indicate that a lack of C6S does not affect the induction phase of EAE but exhibits effects during the effector phase of EAE.

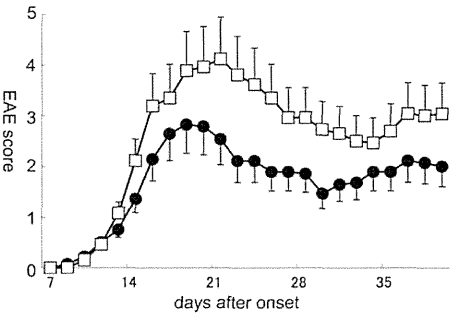

Fig. 1. Exacerbation of experimental autoimmune encephalomyelitis (EAE) in C6ST1$^{-/-}$ mice. EAE was induced in C6ST1$^{-/-}$ (open squares) or WT (closed circles) mice via immunization with myelinoligodendrocyte glycoprotein (MOG_{35-55}) in complete Freund's adjuvant (CFA) as described in Materials and methods section. Statistical analysis is shown in Table IA. Two independent experiments are expressed as the mean EAE score.

Table I. Statistical analysis of clinical EAE scores

Mouse	Incidence	Max score	Cumulative score
A			
C6ST1$^{-/-}$	24/26 (92.3%)	3.08 ± 0.25*	30.6 ± 3.4*
WT	20/28 (71.4%)	2.25 ± 0.32	20.2 ± 3.3
Recipient mouse	Incidence	Max score	Cumulative score
B			
C6ST1$^{-/-}$	8/10 (80.0%)	2.15 ± 0.48	13.4 ± 3.4*
WT	6/11 (54.5%)	1.00 ± 0.33	4.0 ± 1.7

(A) Active-immunization EAE. Each mouse was immunized with MOG_{35-55} peptide for the induction of EAE. The data are the same as in Figure 1.
(B) Adoptive-transfer EAE. One million encephalitogenic CD4$^+$ T cells from WT mice were injected intravenously into WT or C6ST1$^{-/-}$ mice (recipient). The data are the same as in Figure 4.
Mean ± SEM of the following parameters is shown: maximum score of EAE (max score), incidence of paralyzed mice among sensitized rats (Incidence), summation of the clinical scores from days 0 to 35 (Cumulative score). The statistical significance of the difference was determined using ANOVA, *$P < 0.05$ vs. WT mice.

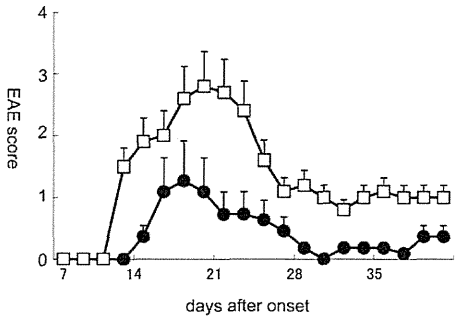

Fig. 2. Chondroitin 6-sulfotransferase plays a role in the effector phase of EAE. Encephalitogenic T cells were prepared by immunizing WT mice and culturing their LN cells in the presence of MOG and interleukin-12 for 4 days. One million $CD4^+$ cells were injected into the tail vein of WT (closed circles) or $C6ST1^{-/-}$ mice (open squares). EAE clinical scores were assessed as described in Table IB. These data are shown as mean clinical scores ± standard error of the mean (SEM).

Spinal cord from $C6ST1^{-/-}$ mice shows severe cell infiltration in comparison with it from WT (Figure 3). This shows that the presence of C6ST inhibits the infiltration of pathogenic lymphocyte in EAE.

C6ST1 has no effect on the recall response of MOG-specific T cells

To determine the mechanisms of C6S in T-cell activation, we examined the proliferative response and cytokine production of draining LN cells in vitro. $C6ST1^{-/-}$ or WT mice were immunized with MOG_{35-55}. Ten days after immunization, draining LN cells were collected and cultured with MOG_{35-55} peptide. As shown in Figure 4, no significant difference was found in the proliferative responses of MOG-reactive T cells in $C6ST1^{-/-}$ and WT mice. We next examined the levels of cytokines in the culture supernatant using enzyme-linked immunosorbent assay. Levels of IFN-g, IL-4, IL-10 and IL-17 in the culture supernatants of LN cells obtained from $C6ST1^{-/-}$ and WT mice were similar (data not shown).

Lack of C6S has no effect on the infiltration of pathogenic T cells into the CNS

To analyze the mechanism of C6S on the infiltration of the inflammatory cells into the CNS, we isolated mononuclear cells in CNS samples obtained from each mouse on day 9 of active-immunization EAE. This period occurs just before the onset of EAE in WT mice; none of the mice exhibited EAE symptoms at that time. As shown in Figure 5, the $CD4^+$ cell number in the CNS samples from $C6ST1^{-/-}$ mice was not significantly different from that in the samples from WT mice (14.3 ± 2.8 vs. 13.3 ± 2.6, as mean ± standard error of the mean, $\times 10^5$). This result suggests that C6S has no effect on the migration of pathogenic T cells into the CNS.

Expression level of C6S in CNS during EAE

To confirm whether C6S is up-regulated in CNS, HPLC analysis was performed. Figure 6 shows that C-unit CS (C6S) did not detected in brains from $C6st1^{-/-}$ mice. In WT mice, increased expression of C-unit CS did not observed in brains from EAE immunized mice in comparison with brains from naive mice.

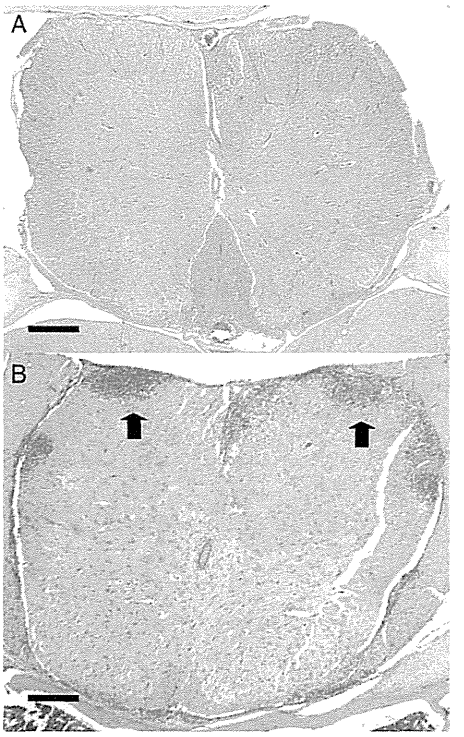

Fig. 3. Histopathological findings of spinal cord. Spinal cord from $C6ST1^{-/-}$ mice (**B**) shows severe cell infiltration (arrow) in comparison with it from WT (**A**).

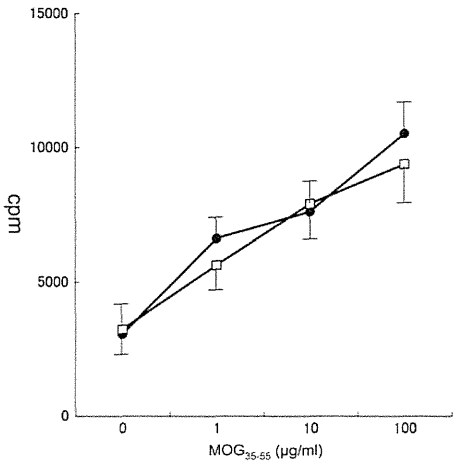

Fig. 4. Comparison of MOG_{35-55}-specific T-cell response in $C6ST1^{-/-}$ and WT mice. Popliteal and inguinal LN cells from $C6ST1^{-/-}$ (open squares) or WT (closed circles) mice were incubated in the presence of MOG_{35-55} for 48 h. Proliferative response was determined by the uptake of $[^3H]$ thymidine. Representative data of two independent experiments are shown ($n = 12$ for each group). Error bars represent SEM.

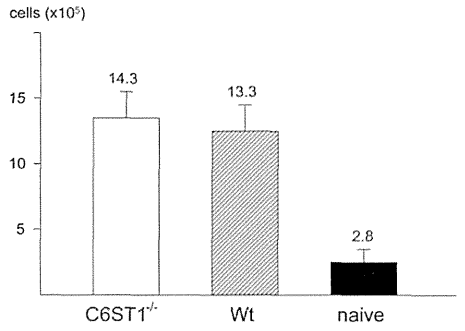

Fig. 5. Analysis of infiltrating cells isolated from the CNS. Infiltrating cells in the CNS from each mouse were collected as described in Materials and methods section. The infiltrating $CD4^+$ T cells were analyzed with flow cytometry. The data are presented as mean ± SEM ($\times 10^5$), WT (13.3 ± 2.6, $n = 3$), $C6ST1^{-/-}$ (14.3 ± 2.8, $n = 3$) and naive mice (1.4 ± 0.3, $n = 3$). $^*P < 0.05$ by Mann–Whitney *U* test.

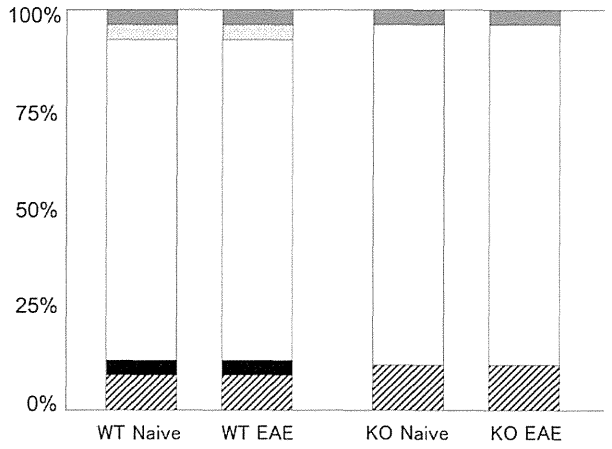

Fig. 6. C6S is not up-regulated during the development EAE. To confirm whether C-unit CS (C6S, closed square) is up-regulated in the CNS during the development EAE, HPLC analysis was performed. The fractionation and analysis of oligosaccharides were carried out by HPLC as described in Materials and methods section. This shows that the expression level of C6S is no change during the development EAE.

Amelioration of EAE in C6ST1^{tg} mice

Next, we analyzed whether overexpression of C6ST reduces the progression of EAE. EAE was induced in $C6ST1^{tg}$ mice overexpressing C6ST1. The maximum score of $C6ST1^{tg}$ mice was significantly lower than that of WT mice. The cumulative score of $C6ST1^{tg}$ mice was also significantly lower than that of WT mice. In contrast, the score of $C6ST1^{-/-}$ mice was significantly higher than that of WT mice (Figure 7; Table II). These results suggested that C6ST1 ameliorates the pathogenesis of EAE.

Discussion

We have previously shown that the onset of EAE is delayed in mice lacking complex gangliosides owing to the lack of

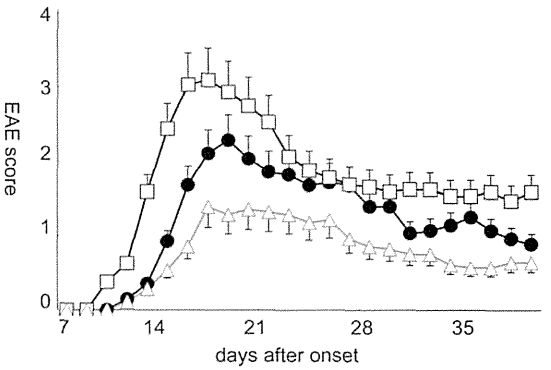

Fig. 7. Overexpression of chondroitin 6-sulfotransferase ameliorates EAE. EAE was induced in $C6ST1^{-/-}$ (open squares), $C6ST1^{tg}$ (open triangle) or WT (closed circles) mice via immunization with MOG_{35-55} in CFA as described in Materials and methods section. Statistical analysis is shown in Table II. Two independent experiments are shown as the mean clinical score ± SEM.

Table II. Statistical analysis of clinical EAE scores

Mouse	Incidence	Max score	Cumulative score
$C6ST1^{-/-}$	15/16 (93.8%)	3.66 ± 0.28*	41.0 ± 4.1*
C6ST1-Tg	11/14 (78.6%)	1.96 ± 0.36*#	18.0 ± 4.5#
WT	12/14 (85.7%)	3.00 ± 0.38	28.5 ± 4.6

Active-immunization EAE. Each mouse was immunized with MOG_{35-55} peptide for the induction of EAE. The data are the same as in Figure 7. Mean ± SEM of the following parameters is shown: maximum score of EAE (max score), incidence of paralyzed mice among sensitized rats (incidence), summation of the clinical scores from days 0 to 35 (cumulative score). The statistical significance of the difference was determined using ANOVA, $^*P < 0.05$ vs. WT mice, $^{\#}P < 0.001$ vs. $C6ST1^{-/-}$ mice.

GM2/GD2 synthase. The carbohydrate portion of complex gangliosides may be involved in the migration of activated T lymphocytes across the blood–brain barrier (Miyamoto et al. 2008). Thus, modification of the carbohydrate portion of the glycoconjugates may affect the pathogenetic mechanisms of EAE. In the current study, we demonstrated that CSPG is involved in the pathogenesis of EAE. Active-immunization and adoptive-transfer experiments showed that lack of C6ST1 exacerbated the disease severity of EAE in the effector phase. No difference was found in the recall response of lymphocytes to MOG_{35-55} in C6ST1-deficient mice compared with WT mice. Furthermore, the lack of C6ST1 did not affect the passage of activated T lymphocytes across the blood–brain barrier in EAE. Moreover, overexpression of C6ST reduced the pathogenesis of EAE. The results suggest that C6S could have a neuroprotective effect in the effector phase of EAE. Because C-unit CS (C6S) does not increase during EAE, C6S may be acting protectively regardless of the immunization.

Recently, CSPG has been implicated in several intriguing biological phenomena, such as the regulation of growth factor functions (Deepa et al. 2002), cell division (Sugahara and Mikami 2007) and neuritogenesis (Li et al. 2007). In addition, a missense mutation of C6ST1 has been demonstrated to

abolish C6ST activity almost completely and cause severe human chondrodysplasia with major involvement of the spine (Thiele et al. 2004; van Roij et al. 2008).

In a spinal cord injury model, CSPG inhibits re-innervation, and a CSPG-degrading enzyme promotes axonal regeneration (Kawano et al. 2005). Chondroitinase ABC treatment up-regulates regeneration-associated proteins in injured neurons and promotes the regeneration of both ascending sensory projections and descending corticospinal tract axons (Bradbury et al. 2002). Treatment with transforming growth factor-beta1 upregulates keratan sulfate and CSPG biosynthesis (Yin et al. 2009), and it promotes the expression of C6ST1 (Properzi et al. 2005) in microglia after brain injury. CSPG3, or neurocan, is up-regulated in the periventricular white matter and cortex in the effector phase of EAE. CSPG3 is indicative of cell recruitment for repair processes and is confirmed by the presence of thin myelin sheaths in the plaques (Sajad et al. 2011). Thus, CSPG3 may play a crucial role in CNS regeneration in EAE.

Phosphacan is a CSPG that accelerates nerve recovery and has crucial roles in the maintenance of oligodendrocytes (Ranjan and Hudson. 1996). Studies have shown that the administration of CSPG causes severe clinical symptoms in EAE (Rolls et al. 2006). Zhou et al. demonstrates that a disaccharide fraction of 6-sulfated C units reduces EAE, while 4-sulfated CS-A may actually make things worse (Zhou et al. 2010). The variable results of these experiments indicate that a consensus has not yet been reached on the therapeutic effect of CSPG. In our study, C6S displayed a neuroprotective effect in EAE. Modification of sulfation pattern to increase C6S levels in the CNS is used as a treatment for EAE. C6S may inhibit the spread of pathogenic T cells in the CNS. Although CSPG prevents nerve regeneration in the recovery phase of the nerve injury model, CSPG may prevent disease progression in the acute phase of EAE. Thus, modification of the carbohydrate structure of CSPG could be used as a new therapeutic method for neuroimmunological diseases such as multiple sclerosis.

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Conflict of interest

None declared.

Abbreviations

C6ST, chondroitin 6-sulfotransferase; CSPG, chondroitin sulfate proteoglycan; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; HPLC, high-performance liquid chromatography; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein

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