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

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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 $TNF\text{-}\alpha^+$ $IL\text{-}10^-$ 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

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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 ($[^3H]$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

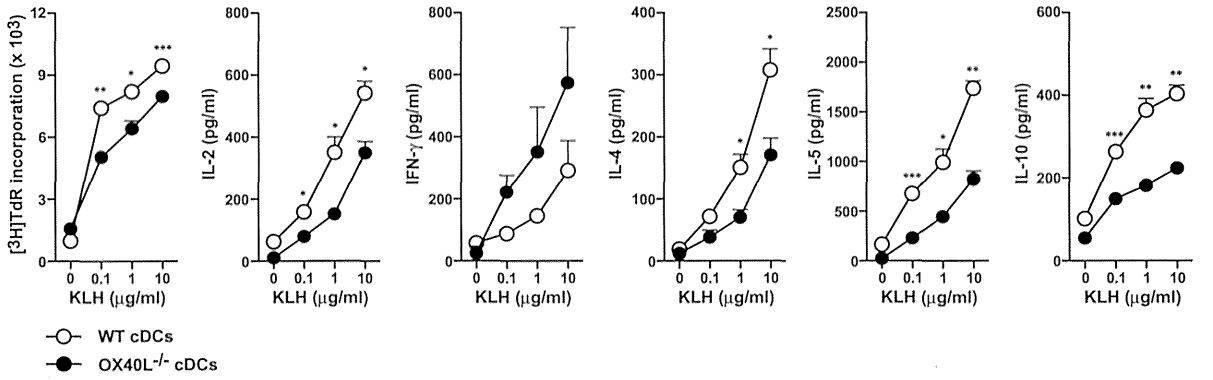

Fig. 1. OX40L is required for optimal Th2 responses by splenic cDCs *in vivo*. BALB/c mouse hind footpads were injected with KLH-pulsed cDCs isolated from the spleen of wild-type BALB/c or OX40L$^{-/-}$ BALB/c mice. LN cells were harvested at day 5 and cultured with indicated doses of KLH. To estimate 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.

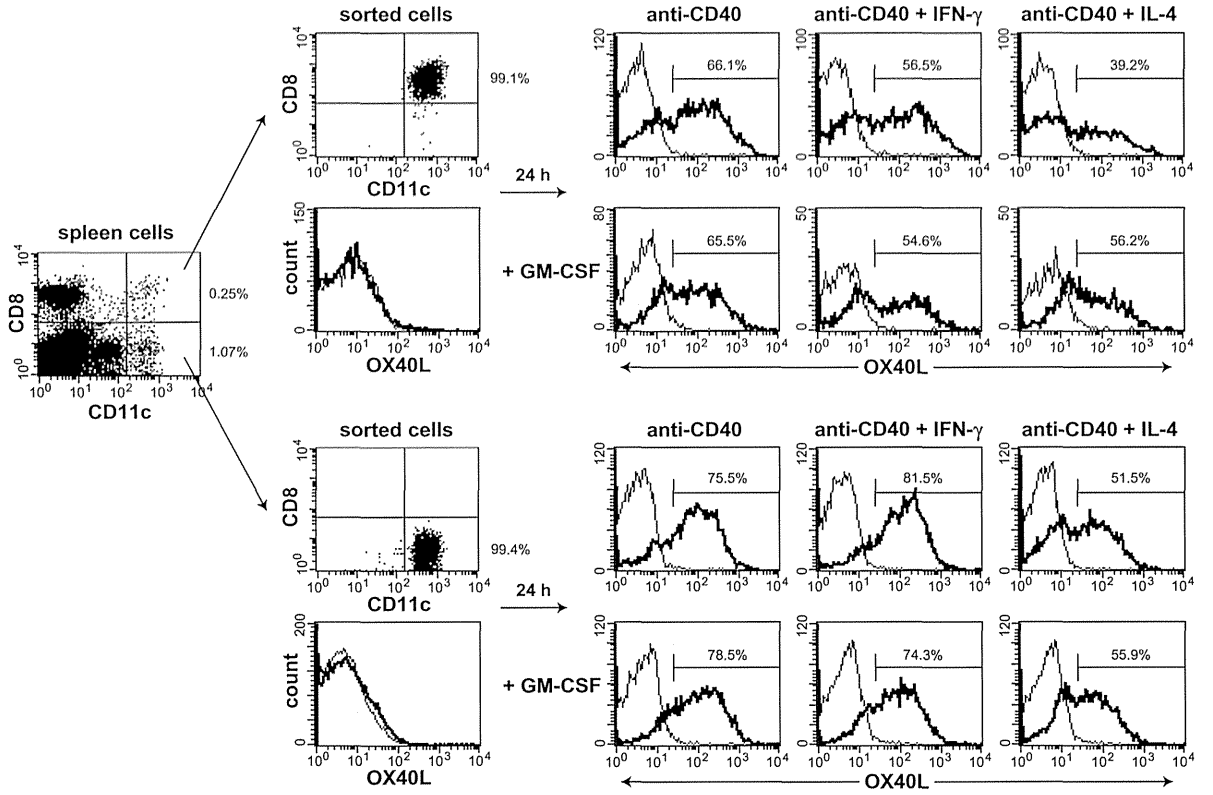

Fig. 2. Expression of OX40L on activated CD8^{-} and CD8^{+} cDCs. Spleen cells were isolated from BALB/c mice and stained with FITC-labeled anti-CD11c, APC-labeled anti-CD8α, and biotinylated anti-OX40L or control IgG followed by PE-labeled streptavidin. CD8^{-}CD11c^{high} and CD8^{+}CD11c^{high} cDCs 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

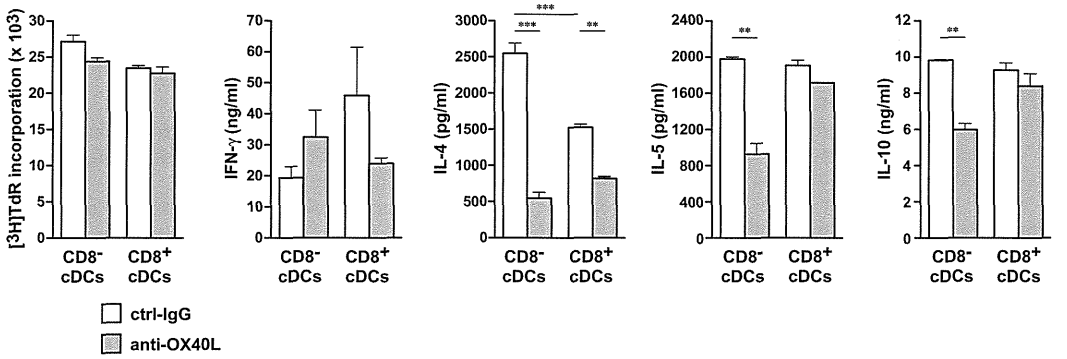

Fig. 3. Effect of anti-OX40L mAb on the development of Th2 responses induced by KLH-pulsed $CD8^{-}$ cDCs *in vivo*. BALB/c mouse hind footpads were injected with KLH-pulsed $CD8^{-}$ or $CD8^{+}$ cDCs. Mice were administered 400 μg of anti-OX40L mAb or control rat IgG (ctrl-IgG) i.p. at days 0, 1, and 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.

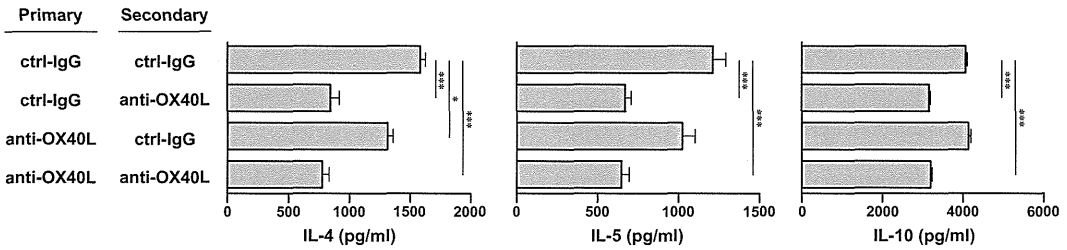

Fig. 4. Effect of anti-OX40L mAb on the development of memory Th2 responses induced by $CD8^{-}$ cDCs *in vivo*. BALB/c mice were immunized first with KLH-pulsed $CD8^{-}$ cDCs at day 0 and boosted with the same KLH-pulsed $CD8^{-}$ cDCs at day 14. Mice were administered 400 μg of anti-OX40L mAb or ctrl-IgG 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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REVIEW ARTICLE

Chemokine receptors on T cells in multiple sclerosis

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CCR2; MMP-9; multiple sclerosis; osteopontin; Th17 cells

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Abstract

Multiple sclerosis (MS) is a chronic demyelinating autoimmune disease of the central nervous system (CNS) that is usually characterized by alternating periods of relapse and remission. The involvement of CD4+ helper T cells, especially the Th1 and Th17 subsets, during MS relapse is well established. However, recent reports suggest that there is plasticity and functional diversity of Th17 cells in CNS autoimmunity. Therefore, the overall picture of "encephalitogenic" T cells is difficult to draw. The chemokine system is fundamental for T cell trafficking, and plays essential roles in normal physiology and autoimmunity. Each Th subset expresses characteristic chemokine receptors that are critical for homing to inflammation sites. Chemokine receptor expression profiles on T cells in the cerebrospinal fluid (CSF) of MS patients reflect certain aspects of the pathology of MS relapse. Mounting evidence suggests that Th1- and Th17-related chemokines, and chemokine receptors mediate MS pathology. The scope of the present review was to discuss recent findings related to chemokine receptor expression and pathogenic Th cells in MS. This review focuses in particular on CCR2+CCR5+CCR6− Th1 cells, a newly identified Th cell subset that we recently showed is enriched in the CSF of relapsing MS patients. Measuring multiple chemokine receptor expression levels could show unique T cell subsets involved in the pathogenesis of various autoimmune diseases. (Clin. Exp. Neuroimmunol. doi: 10.1111/cen3.12130, June, 2014)

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS). A complete understanding of MS pathogenesis has not yet been attained; however, it is well established that autoimmune mechanisms serve a central role. Approximately 80% of MS patients initially develop a clinical pattern of relapses followed by remissions, termed relapsing–remitting MS (RRMS).[1] For decades, the mechanisms of MS relapse have been extensively studied using the experimental autoimmune encephalomyelitis (EAE) animal model of human demyelinating CNS diseases, which has aided in the development of Federal Drug Administration-approved drugs for treating RRMS. Examining the mechanisms of the action of approved drugs has contributed to our understanding of the pathogenesis of relapse.[2]

Multiple sclerosis relapse can be conceptually divided into two stages. T cell activation in the peripheral lymphoid organs occurs in the first stage, and trafficking of activated T cells to the CNS occurs in the second stage. Such autoreactive T cells in MS are usually termed "pathogenic" or "encephalitogenic" T cells. In the first stage, T cells reacting to myelin antigens, such as the myelin basic protein (MBP), might become activated after microbial infection or exposure to other inflammatory stimuli. Activated T cells undergo phenotypic transformations that are characterized by changes of Th cell subtype and expression profiles of chemokine receptors. The activated T cells might then migrate into the CNS by crossing the blood–brain barrier (BBB). The importance of inflammatory cytokines, such as interferon-γ (IFN-γ) or mediators, such as osteopontin (OPN), is also well documented. The effectiveness of drugs that suppress MS relapse provides proof-of-concept

to support this two-stage model. One good example is a humanized anti-α4 integrin antibody, natalizumab, one of the most potent drugs for RRMS.[3] Recently, a live cell imaging technique was developed using a rodent EAE model to observe *in vivo* trafficking of lymphocytes at the BBB during the course of EAE, which should provide insights into the mechanisms of T cell migration.[4]

IFN-γ-producing Th1 cells have been long thought to be a critical Th subtype of pathogenic T cells. However, after the discovery of interleukin (IL)-17-producing Thelper cells (Th17 cells), the pathogenicity of Th17 cells has been extensively studied. A close relationship exists between Th subsets and the chemokine system, which can orchestrate T cell migration to specific tissues in both physiological and inflammatory situations. Namely, each Th cell subset has a tendency to express (or not express) specific chemokine receptors. The present review summarizes recent findings regarding the roles of pathogenic T cells in MS and their chemokine receptor expression patterns, with a special emphasis on the novel, potentially pathogenic CCR2+CCR5+ CCR6− Th1 cell subset identified by our group.[5]

Th1 and Th17 cells as encephalitogenic T cells

Before the discovery of Th17 cells a decade ago, a model of Th1/Th2 balance was proposed to explain the pathology of autoimmune and allergy diseases. MS was thought to be a representative Th1 disease with Th1 predominance. In fact, treating MS patients with IFN-γ worsened the disease, providing direct evidence of the pathogenic role of IFN-γ in MS.[6] Furthermore, an altered peptide ligand of MBP administered to MS patients in a phase 2 clinical trial showed potential encephalitogenic activity through the activation of Th1 cells.[7] These clinical findings suggested a pivotal role of Th1 cells during MS relapse.

Approximately a decade ago, EAE animal studies showed that Th17 cells serve a critical role in the pathobiology of CNS inflammation. In an adoptive transfer model, the pathogenic potential of Th17 cells was found to exceed that of Th1 cells. However, the therapeutic effect of IL-17 blockade was not very effective,[8] and EAE was only weakly ameliorated in IL-17 knockout mice, which suggested functional roles for other effector molecules bestowed by Th17 cells.

A potentially pathogenic role of human Th17 cells in MS has been reported. Brucklacher-Waldert et al.[9] investigated the frequency of Th1 and Th17 cells in the cerebrospinal fluid (CSF) of patients with RRMS during relapse. Both Th1 and Th17 cells were significantly higher in the CSF than in the peripheral blood. T cell clones from the CSF expressed higher levels of activation markers, such as CD69, than did Th1 clones. Furthermore, adhesion molecules mediating the involvement of T cell attachment to endothelial cells (EC), such as CD49d, CD6 and the melanoma cell adhesion molecule (MCAM/CD146), were expressed more abundantly in Th17 cells than in Th1 cells. Higher adhesion molecule expression in Th17 cells resulted in greater adhesion to EC, increased proliferative capacity and reduced susceptibility to suppression, suggesting that Th17 cells have high pathogenic potential. Prat et al. have provided several intriguing findings with regard to the pathogenicity of Th17 cells in MS. Lymphocytes obtained from the blood of relapsed MS patients showed an increased propensity to expand into IFN-γ-producing Th17 cells.[10] In brain tissues of MS patients, numerous T lymphocytes co-expressed IL-17 and IFN-γ. Furthermore, IFN-γ+ Th17 cells preferentially crossed the BBB in an *in vitro* human BBB model, suggesting a potential for pathogenicity in this cell population. Another study showed that IL-17+IL-22+ T cells can efficiently traverse a modeled BBB *in vitro*.[11] Intriguingly, this subset expressed granzyme B, a cytotoxic molecule, and killed human neurons *in vitro*. The same group suggested that MCAM/CD146 is a marker of human Th17 cells with pathogenic potential.[12]

The diversity of lesion locations has long been a mystery of MS; however, results from several reports have implied that Th cell types can influence the localization of lesions in the CNS. Results from mouse EAE model studies have suggested that Th1 cells are more likely to induce a classical-type EAE, which mainly affects the spinal cord, whereas Th17 cells tend to induce an atypical-type EAE characterized by the presence of brain or cerebellar lesions, although the details of each study were different.[13–15] Collectively, these studies suggest that both Th1 and Th17 cells mediate the relapse of MS, possibly through different immunological mechanisms and at different locations in MS patients.

Plasticity and functional diversity of Th17 cells

Recent studies have shown that Th17 cells might have higher plasticity and functional diversity than originally thought. Th17 cells and regulatory T cells (Tregs) share several common features.

Differentiation of both cell types requires transforming growth factor-β (TGF-β), which induces expression of the Th17-related transcription factor, RORγt, and the Treg-related transcription factor, Foxp3. The combined activities of TGF-β to gether with other inflammatory cytokines, such as IL-6 and IL-1β influence decisions regarding further differentiation into Th17 or Treg cells.[16] Furthermore, it has been shown that Foxp3 expression in some Treg cells is unstable, and these cells might convert to Th17 cells with pathogenic potential.[17] In humans, peripheral blood and lymphoid tissue contains significant numbers of CD4+Foxp3+ T cells that possess regulatory functions and express CCR6.[18] This subset had the capacity to produce IL-17 on activation by inflammatory cytokines, a phenomenon observed even at the single cell level.[19] To summarize, the prevailing evidence suggests the potential for phenotypic fluctuation of Th17 cells, both in mice and in humans.

Additionally, evidence suggests that Th17 cells might be able to transform into Th1 cells. For example, Hirota et al.[20] generated IL-17A reporter mice to track IL-17A expression *in vivo*. They found that during EAE development, IFN-γ and other pro-inflammatory cytokines were produced in an IL-23-dependent manner almost exclusively by IL-17-producing cells before their conversion ("ex-TH17 cells"), supporting a model of phenotypic change of Th17 cells into Th1 cells. The epigenetic mechanism underlying Th cell plasticity was investigated by generating genome-wide histone H3 lysine 4 (H3K4) and lysine 27 (H3K27) trimethylation maps for various Th cell subsets.[21] Wei et al. found that genes encoding transcription factors critical for the Th cell differentiation, such as T-bet, Foxp3 or Rorc, showed a broad spectrum of epigenetic states, suggesting high plasticity among differentiated effector T cells.

Pathogenic and non-pathogenic Th17 cells

With respect to encephalitogenicity, accumulating evidence suggests that Th17 cells can be conceptually subdivided into pathogenic and non-pathogenic categories. Ghoreschi et al. reported that "pathogenic" Th17 cells efficiently induced EAE, and were generated in response to IL-23 signaling independently of TGF-β signaling. This class of Th17 cells co-expressed RORγt and T-bet.[22] Kuchroo et al. reported that TGF-β3, together with IL-6, induced pathogenic Th17 cells. TGF-β3 was produced by developing Th17 cells in an IL-23-dependent manner, implying a critical role of IL-23. With regard to transcription factors bestowing pathogenicity, the Th1-related T-bet was reported to be a key factor in inducing the pathogenic functions of Th17 cells,[23] although another study did not reach the same conclusion.[24] Most likely, several factors cooperate to determine the fates of various Th17 cell types. Interestingly, a study by Kuchroo et al. showed the existence of a dynamic regulatory network with as many as 39 regulators controlling Th17 cell differentiation.[25]

Two simultaneous publications showed that granulocyte-macrophage colony-stimulating factor (GM-CSF) is required for the acquisition of pathogenic capacity in Th17 cells.[26,27] Based on the numerous studies using EAE models, Kuchroo proposed a model whereby mouse Th17 cells comprise a wide spectrum with various effector phenotypes.[28] In that model, TGF-β and IL-23 play major roles in shifting the Th17 phenotype towards regulatory (non-pathogenic) and alternative (pathogenic) phenotypes, respectively, although numerous other cytokines are thought to contribute to fine-tuning of the Th17 spectrum. The non-pathogenic Th17 subtype is characterized by the production of IL-9 and IL-10, and expression of the transcription factors, c-Maf and AhR, whereas the pathogenic Th17 subtype is distinguished by IFN-γ, GM-CSF and IL-22 production, and T-bet expression.

The observation of a pathogenic versus non-pathogenic dichotomy has been reported for human Th17 cells as well. The importance of IL-1β and IL-12 in the induction of pathogenic IL-17/IFN-γ double producing phenotype has been emphasized.[29] Recently, a new human pathogenic Th17 cell subset defined by chemokine receptor expression patterns was reported.[30] A fraction of CCR6+CXCR3^{hi}-CCR4^{lo}CCR10−CD161+ cells expressing the P-glycoprotein (P-gp)/multidrug resistance type 1 protein (MDR1) showed a pro-inflammatory phenotype and showed a transcriptional signature akin to pathogenic mouse Th17 cells. Such MDR1+Th17 cells were enriched and activated in the gut of patients of Crohn's disease, and were refractory to several glucocorticoids, possibly conferring a treatment-resistant phenotype. To summarize, the spectrum of Th17 cells is broadening to reflect an expanding appreciation of various functional phenotypes with high plasticity.

Chemokine system and pathogenic Th cells

Chemokines are a superfamily of small cytokines, the name "chemokine" being derived from their

ability to induce chemotaxis in responsive cells with corresponding chemokine receptors (**chemo**tactic cyto**kines**).[31,32] Chemokines are subdivided into the CC, CXC, CX3C and C subfamilies, on the basis of the organization of two positionally conserved cysteine residues near their N-terminal ends. Approximately 50 chemokines have been identified thus far in humans. Chemokines exert chemoattractant effects through cognate chemokine receptors that are expressed on the surface of targeted leukocytes. Chemokine receptors are G protein-coupled receptors containing seven transmembrane domains. Chemokines are categorized functionally as being either constitutive (homeostatic) or inducible (inflammatory). Constitutive chemokines direct the normal trafficking of leukocytes under physiological conditions. For example, CCL19 and CCL21 bind CCR7 to control normal immune homeostasis. However, inducible chemokines are produced in response to inflammatory or immune signals, and account for the increased recruitment of leukocytes under inflammatory conditions. For example, the binding of CCL2 (MCP-1) to CCR2 is an important step in inflammatory responses. One notable characteristic of the chemokine system is that they are promiscuous in that a given chemokine might bind to multiple chemokine receptors, and conversely, a given chemokine receptor is able to respond to two or more chemokines.[33]

Human memory T cells can be categorized into two functionally distinct populations related to the course of their differentiation from naive T cells. CCR7 is a key regulatory chemokine receptor that controls homing to secondary lymphoid organs. Effector memory T cells (TEM) possess a capacity for migration toward inflamed tissues, show immediate effector functions and are CCR7 negative. In contrast, central memory T cells (TCM) are CCR7+ cells that express lymph node homing receptors, lack immediate effector function, but efficiently stimulate dendritic cells.[34] Recent studies have identified a third subset, termed tissue-resident memory (TRM) cells,[35,36] which resides in peripheral tissues, such as the skin or intestine, for years without circulating in peripheral blood and can elicit rapid responses *in situ*. It has not yet been made clear whether this population resides in the CNS under physiological or pathological conditions. As aforementioned, during the differentiation process of naive T cells to Th1 or Th2 cells, T cells lose CCR7 expression, while acquiring constitutive expression of other inflammatory chemokine receptors. CCR5 and CXCR3 are preferentially expressed on Th1 cells, whereas CCR3, CCR4 and CRTh2 expression is characteristic of Th2 cells.[37–39] In this manner, migration and effector functions are closely linked to provide a mechanism of recruiting appropriate immune cells to appropriate inflammation sites.

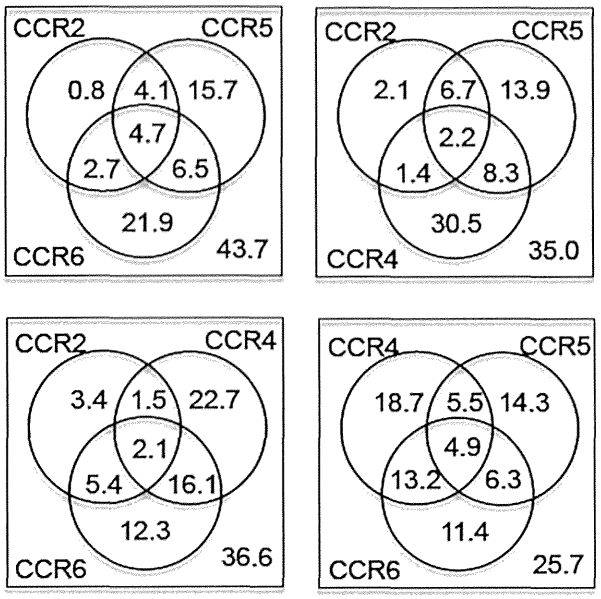
Around 2005, Th17 cells were recognized as a new subset distinct from Th1 or Th2 cells in mice. In 2007, Acosta Rodriguez et al.[40] reported that human Th17 cells were found in CCR6+, but not in CCR6- populations. By subdividing CCR6+ cells into CCR4+ and CCR4− cells, they identified CCR4+CCR6+ cells that produced IL-17 with little IFN-γ or IL-4. Our group used a similar strategy by classifying human memory Th cell populations according to their expression of CCR2 and CCR5. We found that the CCR2+ populations contained both IL-17- and IFN-γ-producing cells. CCR2+ T cells consisted of CCR5+ and CCR5− subsets. After sorting both populations by flow cytometry, we found that CCR2+CCR5− cells produced mainly IL-17, whereas CCR2+CCR5+ cells mainly produced IFN-γ, suggesting that Th17 cells are included in CCR2+CCR5− populations.[41]

Successive studies established that CCR6 was most closely associated with Th17 cells; both mouse and human Th17 cells universally express CCR6. In fact, the majority of CCR2+CCR5− T cells co-expressed CCR6 (Fig. 1a). It is noteworthy that CCR6 is also expressed on other T cell subsets, such as Th1 or Treg cells.[42,43]

Chemokines and chemokine receptors in multiple sclerosis pathology

Given the strong relationship between chemokine receptor expression and Th cell subset, we studied chemokine receptor expression patterns in T cells of MS patients to identify a Th cell subset relevant to MS pathogenesis. The relative abundance of lymphocytes in the CSF is much lower than that found in peripheral blood (0–3 lymphocytes/μL in CSF from normal subjects and in relapsing MS patients), and most lymphocytes in the CSF are memory CD4+ T cells.[44] Therefore, 5 mL of CSF might only contain several thousand memory CD4+ T cells, making it narrowly possible to estimate the frequency of chemokine receptor +/− cells in the CSF by flow cytometry. Previous studies confirmed that CCR5+ Th1 cells are enriched in the CSF compared with the periphery.[45] CCR5+ Th1 cells, although small in number, might be critical for immune surveillance of the CNS. Natalizumab, a drug that blocks T cells entry into the CNS, increases the risk of progressive

(a)



(b)

	HS (%)	MS (%)
2+5+4+6-	0.69±0.46	1.05±0.59
2+5+4+6+	0.88±0.87	1.09±0.78
2+5+4-6+	2.91±2.47	3.6±1.72
2+5+4-6-	3.05±1.91	3.08±1.05
2+5-4+6-	0.27±0.24	0.4±0.37
2+5-4+6+	0.57±0.49	0.95±0.79
2+5-4-6+	1.35±1.35	1.78±1.21
2+5-4-6-	0.46±0.25	0.36±0.19
2-5+4+6-	3.49±1.52	4.43±1.82
2-5+4+6+	3.63±1.95	3.84±1.39
2-5+4-6+	2.19±0.92	2.67±1.22
2-5+4-6-	9.38±4.61	11.26±6.79
2-5-4+6-	18.72±5.07	18.32±5.01
2-5-4+6+	11.91±3.91	12.21±3.34
2-5-4-6+	9.04±3.10	9.62±1.53
2-5-4-6-	31.49±8.55	25.33±4.83

Figure 1 Multiple chemokine receptor expression profiles and its overlap in memory CD4+ T cells. (a) For flow cytometric analysis, peripheral blood mononuclear cells depleted of CD14+ T cells were stained with differentially labeled anti-CD4, -CD45RA, -CCR2, -CCR5, -CCR4 and -CCR6 monoclonal antibodies simultaneously. Venn diagrams show the frequency (%) of the cells expressing CCR2, CCR4, CCR5 and CCR6 in memory CD4+ T cells in peripheral blood from 11 multiple sclerosis (MS) patients in remission. The combination of three chemokine receptors out of four is shown. (b) Memory CD4+ T cells were divided into 16 subsets based on the expression of CCR2, CCR5, CCR4 and CCR6. Data from 11 healthy subjects (HS) and 11 MS patients in remission (modified from Sato et al.[5], with permission).

multifocal leukoencephalopathy (PML) caused by John Cunningham virus, which could reflect the importance of steady state immune surveillance of the CNS by CD4+ T lymphocytes.[46]

Expression levels of chemokines in the CSF and chemokine receptors on the CSF lymphocytes and brain infiltrating T cells of MS patients have been analyzed.[45,47–49] Th1-related chemokines, such as CXCL9 (Mig), CXCL10 (IP-10) or CCL5 (RANTES), were reportedly increased in the CSF during acute relapses. CXCL9 and CXCL10 bind CXCR3, and CCL5 binds CCR5, both of which are expressed on Th1 cells. CXCR3 and CCR5 were upregulated in both CSF lymphocytes and brain infiltrating cells, suggesting that Th1 cells might contribute to the pathogenesis of MS relapse.

Regarding the role of the Th17-related chemokine receptor, CCR6, Reboldi et al.[50]found that during the early phase of EAE, CCR6+ cells penetrated into the CNS through the choroid plexus, a villous structure that extends into the lumen of the ventricles and produces CSF. Examining T cells in the CSF and in the peripheral blood of clinically isolated syndrome (CIS) patients, they observed that a substantial proportion of T cells in the CSF expressed CCR6 on their surface. CIS is the first episode of neurological symptoms caused by inflammation and demyelination of the CNS, and is potentially the first episode of MS. Interestingly, the CCL20 protein was constitutively expressed at high levels in the choroid plexus in brains of both healthy subjects and MS patients. These results suggested that CCR6-expressing Th17 cells enter into the CNS parenchyma through the choroid plexus, as guided by the CCL20–CCR6 interaction.

The chemokine receptor, CCR2, is expressed on multiple cell types, including monocytes and T cells. Although CCR2 expression is comparatively weaker in T cells than that in monocytes, results from several reports suggest that CCR2 serves an important role in T cells relating to MS pathology. CCL2, the most potent ligand of CCR2, and CCL5 were shown to be critical for adhesion of encephalitogenic T cells to brain EC in a mouse EAE model.[51] Intriguingly, CCL2 was decreased in the CSF of MS patients during relapses.[45,52,53] Mahad et al.[53] found that CCR2+ T cells efficiently migrate across the BBB, using an *in vitro* BBB model. CCL2 bound to CCR2 was internalized, which effectively decreased the CCL2 concentration in the medium. This mechanism

potentially occurs during MS relapse, such that CCR2+ T cell transmigration through the BBB might reduce the extracellular concentration of CCL2. The sparse immunoreactivity of CCR2 seen on infiltrating T cells could reflect downregulated CCR2 expression after CCL2 binding and internalization.[54] More recently, it was shown that CCR2 plays a pivotal role in transendothelial migration of effector CD4+ T cells through inflamed EC.[55] The unique roles of CCL2 and CCR2 in CNS pathology have been reviewed previously.[56,57]

Multiple chemokine receptor expression in multiple sclerosis

As aforementioned, human Th17 cells are enriched in CCR4+CCR6+ or CCR2+CCR5− populations. Therefore, by examining the expression of CCR2, CCR4, CCR5 and CCR6 on T cells, it could be possible to evaluate the functional significance of various Th17 cell subtypes in MS. With the development of high-throughput multicolor flow cytometry, it has become feasible to study the expression of multiple chemokine receptors on cells simultaneously. We obtained both CSF and peripheral blood samples from MS patients in relapse during their admission for treatment, and compared the proportion of multiple chemokine receptor-positive cells (such as CCR2+CCR4−CCR5+CCR6− cells), in memory CD4+ T cells, CSF and peripheral blood (Fig. 1).[5] In agreement with previous reports,[48,49] CCR5+ T cells were enriched in CSF samples from both MS patients and control patients (non-inflammatory neurological disease patients). However, CCR2+CCR5−, CCR4+CCR6+ or CCR6+ subsets, presumably comprising the Th17 cell population, were not enriched. Unexpectedly, the CCR2+CCR5+ population was increased in the CSF of the relapsing MS patients, but not in control subjects. Because the enrichment was observed only in MS patients, we hypothesized that they serve a pathogenic role during the relapse of MS. Interestingly, the increase of CCR2+CCR5+ T cells in the CSF was not detected in patients with a disease history of >10 years. This result could be explained by the model proposed by Weiner[58], which suggests that in later stages of MS, the contribution of adaptive immunity declines, while innate immune and neurodegenerative components are more influential. As previously described, Reboldi et al.[50] observed an increase of Th17 cells in the CSF of CIS patients. One possible reason for these contradictory results is that different cohorts of patients were tested, namely, CIS patients and established RRMS patients who had experienced several relapses. Further analyses showed that the CCR2+CCR5+ T cells were mostly CCR7− effector memory phenotypes, and showed a Th1/Th17 phenotype with a large amount of IFN-γ and IL-17 production. The reactivity to MBP, a putative autoantigen in MS, was also investigated. CCR2+CCR5+ T cells from peripheral blood of MS patients in relapse selectively responded to MBP by producing a large amount of IFN-γ, suggesting an important role of IFN-γ -producing cells in the cohort (Fig. 2a). Further analyses showed that CCR2+CCR5+ T cells could be subdivided by CCR6 expression. As expected, CCR2+CCR5+CCR6− T cells produced much IFN-γ and little IL-17. Furthermore, expression patterns of transcription factors in CCR2+CCR5+CCR6− T cells were characteristic of Th1 cells, namely, high T-bet and low RORC. Taken together, we regard CCR2+CCR5+CCR6− cells as Th1 cells. In our cohort, CCR2+CCR5+CCR6− populations, but not CCR2+CCR5+CCR6+ populations, were increased in the CSF of patients with relapsing MS, showing a determinant role for Th1 cells rather than Th17 cells (Fig. 2b).

Blood–brain barrier and the migration of pathogenic T cells

Homeostasis is critical for the proper function of neurons. The CNS is thus isolated from systemic circulation by the BBB.[59] The BBB is a functional unit consisting of three cell types, namely, specialized EC, astrocytes and pericytes. The BBB is composed of two basement membrane (BM) layers, namely, a BM of blood EC and a BM of astrocyte end-feet (referred to as the glia limitans). EC and astrocytes secrete extracellular matrix (ECM) proteins to generate and maintain BM. ECM receptors, such as integrins and dystroglycans, are also expressed in the brain microvasculature, and mediate the connections between cellular and matrix components during normal physiology and during the development of various pathologies. The BBB is not a static barrier, but rather is a functionally active, dynamic interface between systemic circulation and the CNS. The composition of the ECM can be altered by inflammation, which affects inflammatory processes in the CNS.[60,61]

The transmigration of T cells into the CNS is a multistep process characterized by a series of sequential and tightly controlled steps. These steps proceed in the following order: rolling, activation, arrest, crawling and transmigration. The final step is further sub-

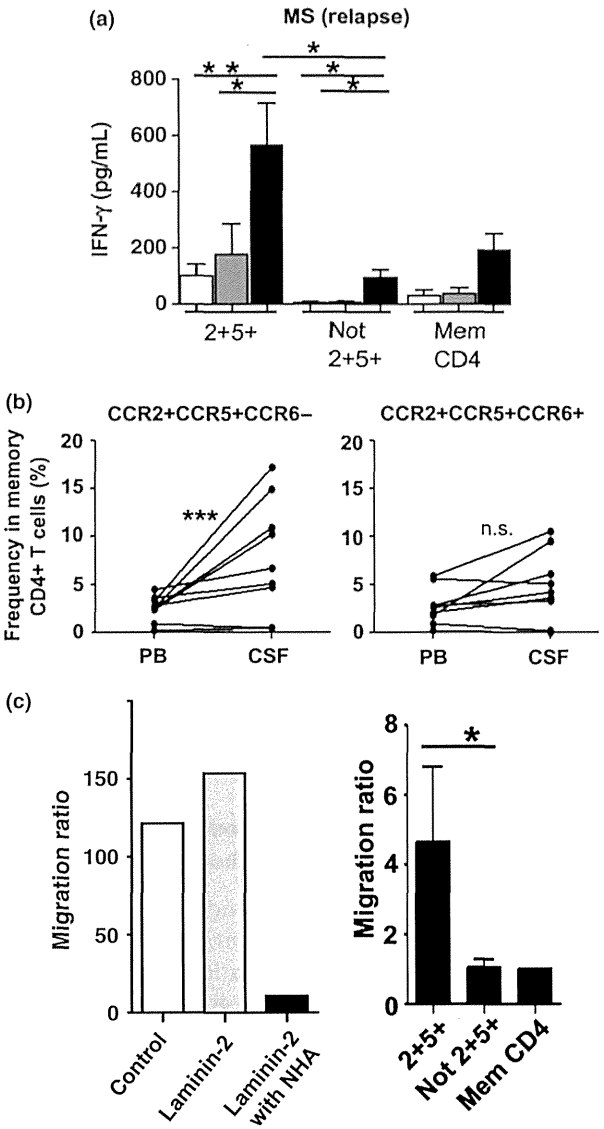


Figure 2 Features of CCR2+CCR5+CCR6− Th1 cells: potentially pathogenic T cells in multiple sclerosis (MS). (a) Purified memory CD4+ T cell subsets were cultured with irradiated antigen presenting cells in the presence of myelin basic protein (100 μg/mL) for 5 days. Concentrations of as interferon-g (IFN-γ) and interleukin (IL)-17 in the supernatants were measured by enzyme-linked immunosorbent assay. Data represented as mean ± SD of six MS patients in relapse. (b) CCR2+CCR5+CCR6− T cells are enriched in the cerebrospinal fluid (CSF) of MS patients in relapse. Peripheral blood mononuclear cells depleted of CD14+ cells were stained with differentially labeled anti-CD4, -CD45RA, -CCR2, -CCR5, -CCR4 and -CCR6 monoclonal antibodies simultaneously. Comparison of the frequencies of CCR2+CCR5+CCR6− and CCR2+CCR5+CCR6+CD4+ T cells in the CSF and peripheral blood (PB) samples from the same MS patients in relapse (n = 8). Lines connect data of paired CSF and PB samples from the same patients. (c) T cell migration across an *in vitro* glia limitans model. (Left) The upper sides of Transwell membrane inserts were coated with laminin-2, and normal human astrocytes (NHA) were seeded on the lower sides of the membrane inserts. T cells isolated from peripheral blood mononuclear cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 18 h, and seeded onto the upper chambers. A total of 8 h later, absolute numbers of migrated cells were counted by flow cytometer. Data shown are the percent inhibition of the migration, calculated as follows: ([migrated cell number through uncoated membrane]−[migrated cell number through membrane coated with laminin alone or laminin and NHA]) × 100/(migrated cell number through uncoated membrane). Data represent mean values ± SD of four independent experiments. (Right) Peripheral blood mononuclear cells from healthy subjects were sorted into memory CD4+CCR2+CCR5+ T cells (2+5+), memory CD4+ T cells depleted of CCR2+CCR5+ T cells (Not 2+5+) and unfractionated memory CD4+ T cells (Mem CD4) by flow cytometry. The cells were stimulated with plate-bound anti-CD3/CD28 monoclonal antibodies for 60 h, and seeded onto the upper chambers whose membrane were coated with laminin-2 and NHA. A total of 8 h later, absolute numbers of migrated cells were counted by flow cytometry. To normalize individual variance, data are expressed as the migration ratio of the number of migrated cells to the number of migrated unfractionated memory CD4+ T cells. Data are represented as mean values ± SD of four independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (modified from Sato et al.[5], with permission).

divided into transmigration from blood vessels into the perivascular space, and transmigration from perivascular space into the CNS parenchyma. The precise mechanism of transmigration through glia limitans has not yet been elucidated; however, the development of multi-cell culture systems possibly recapitulating the glia limitans is beginning to uncover the molecular mechanism regulating this step.[62–64] Our group developed an *in vitro* BBB model to recapitulate the function of the glia limitans. The model consists of a human astrocyte cell line and laminin-1 or laminin-2, which are components of glia limitans. The barrier function of this model was evaluated by blocking the transmigration of activated memory CD4+ T cells. However, CCR2+CCR5+ T cells efficiently transmigrate through the model, implying the high transmigration capacity (Fig. 2c). Because of the paucity of cells, we could not examine the transmigration of CCR2+CCR5+CCR6− cells as compared with that of CCR2+CCR5+CCR6+ cells. Therefore, it has not yet been determined if CCR2+CCR5+CCR6− Th1 cells have high transmigration capacity. The next question would be why CCR2+CCR5+ cells show such a high capacity to transmigrate across the glia limitans model.

Molecules important for the transmigration of T cells into brain parenchyma

One of the influential factors in the transmigration of T cells into brain parenchymal is the matrix metalloproteinases (MMP).[65,66] MMP are a family of proteolytic enzymes capable of remodeling and degrading ECM, and have important roles in development and physiology. Previous studies have shown that several MMP, including MMP-2, MMP7, MMP8, MMP-9 and MMP14, are upregulated in serum, CSF and brain tissues from MS patients. The role of MMP-9 (gelatinase B) in particular has been emphasized.[67–70] MMP-2 and MMP-9 cleave β-dystroglycans, which are transmembrane receptors that anchor the astrocytic end-feet to the parenchymal BM through interactions with laminins-1 and laminin-2, perlecan, and agrin to allow cells to enter the CNS parenchyma. Tissue inhibitor of metalloproteinase 1 (TIMP-1) is a natural inhibitor of MMP. High MMP-9 and low anti-proteolytic TIMP-1 levels are reported in the CSF of MS patients. Higher MMP9/TIMP-1 ratios are predictive of development of new gadolinium-enhancing lesions, indicative of new inflammatory activity in the brain.[71–73] IFN-β treatment decreases MMP-9 expression in MS patients.[74] Intriguingly, it was reported that Th1 cells migrated through the artificial BM more efficiently than Th2 cells, and this correlated with higher levels of MMP-2 and MMP-9 in Th1 cells.[75] Interestingly, we found that activated CCR2+CCR5+ T cells in the peripheral blood of MS patients expressed high MMP-9 mRNA levels and showed significant enzymatic (collagenase) activity.

Steinman et al. reported that there were large amounts of OPN transcripts in MS lesions,[76] highlighting its role in MS pathogenesis. OPN is a member of the family of small integrin binding proteins termed SIBLING proteins that execute multiple biological functions, especially in inflammation.[77] The involvement of OPN in EAE and MS was first shown by Steinman[2] In an EAE mouse model, injection of OPN induced relapses, and OPN knockout mice were protected against CNS inflammation. OPN transcripts are also upregulated in human MS lesions,[76] and increased OPN levels in plasma in RRMS patients have been reported.[78–81] OPN levels

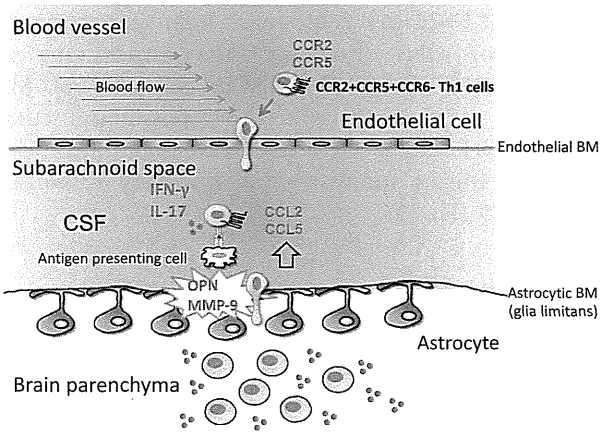


Figure 3 A model in which CCR2+CCR5+ cells (possibly CCR2+CCR5+CCR6− Th1 cells) migrate from periphery to brain parenchyma, through subarachnoid space, triggering multiple sclerosis relapse. CCR5+ T cells are enriched in the cerebrospinal fluid (CSF) and play a role for immune surveillance of the central nervous system (CNS) in a physiological situation. By repeated relapses, CCR5+ T cells transform into CCR2+CCR5+ T cells, which is more sensitive to T cell receptor stimulation with high inflammatory cytokine producing capacity and is anti-apoptotic. This population was enriched in the CSF of relapsing–remitting multiple sclerosis patients in relapse. With activation by putative myelin autoantigen, such as the myelin basic protein, CCR2+CCR5+ T cells express high MMP-9 and osteopontin (OPN) transcripts. MMP-9 presumably degrades parenchymal basement membrane of the blood–brain barrier, and OPN augments CNS inflammation. Accordingly, CCR2+CCR5+ T cells could serve as "advanced guards," which trigger CNS inflammation and multiple sclerosis relapse. BM, basement membrane; IFN-γ, interferon-γ; IL, interleukin.

were elevated during MS relapse compared with MS patients in remission. Furthermore, OPN levels were elevated 1 month before the appearance of new gadolinium-enhancing lesions. The inflammatory effect of OPN is explained by binding to its receptor α4β1 integrin on T cells to stimulate expression of pro-inflammatory mediators, including Th1 and Th17 cytokines.[82,83] OPN signaling promotes the survival of autoreactive T cells by inhibiting apoptosis.[82] As natalizumab is an inhibitor of α4 integrin, this treatment blocks OPN signaling, which might be an important mechanism for preventing relapses.[84] OPN is produced by macrophages, microglia and astrocytes; however, we detected a significantly higher expression of OPN transcripts among activated CCR2+CCR5+ T cells in the peripheral blood. Thus, these cells, enriched in the CSF during MS relapse, might use the OPN pathway to invade into the CNS.

Collectively, these studies have shown that CCR2+CCR5+ T cells are equipped with MMP-9 and OPN, which might support the invasion of activated T cells into the CNS parenchyma. It has not yet been determined if CCR2+CCR5+CCR6−, rather than CCR2+CCR5+CCR6+ cells, have high expression of MMP-9 or OPN. However, the enrichment of CCR2+CCR5+CCR6− Th1cells in the CSF of MS relapse patients raises the possibility that CCR2+CCR5+CCR6− Th1 cells serve as an advanced guard to trigger successive inflammatory responses in the CNS (Fig. 3).

T cells with multiple chemokine receptors

As aforementioned, chemokine–chemokine receptor interactions are complex and redundant. Chemokines can form homodimers, heterodimers and oligomers. Chemokine receptors might also heterodimerize, adding another layer of complexity to this system.[85,86] CCR2 and CCR5 are phylogenetically akin to each other,[33] and CCR2 can heterodimerize with CCR5 and CXCR4. A synergistic effect of multiple chemokine–chemokine receptor signaling pathways has been proposed.[87] The threshold of activation of the cells expressing CCR2/CCR5 heterodimers was 10- to 100-fold lower than the threshold for cells expressing either chemokine alone.[88] Furthermore, it has been shown that signaling pathways activated after heterodimer receptor engagement with cognate chemokines is different from that observed after single receptor binding. In another study, Zhang et al.[89] investigated the function of CCR2+CCR5+ T cells, and found a unique character of this population. They showed a high capacity to respond to antigens, yielding high inflammatory cytokine production, showed robust proliferation and were resistant to apoptosis. A model was proposed, wherein CCR5+CCR2− cells change into CCR2+CCR5+ T cells after repeated stimulation. This model could explain why CCR2+CCR5+ T cells are increased in the CSF of MS patients during relapse. According to this model, each time relapse of MS occurs, autoreactive CCR5+ Th1 cells would be stimulated again, and the more that CCR5+ Th1 cells are stimulated, the more that CCR2+CCR5+ T cells are expanded.

Although the chemokine system has been studied extensively, and a significant knowledge base for this system has emerged, strategies for blocking chemokine receptors to treat autoimmune diseases including MS have unfortunately proven ineffective thus far. To target a single chemokine receptor might be too simplistic of an approach to alter the functions of the numerous chemokine pathways *in vivo*. Developing drugs to block multiple chemokine receptors could be a solution to overcome this problem. Development of a dual antagonist of CCR2/CCR5 or CCR2/CCR5/CXCR4 heterooligomers is currently in progress.[90–92]

Conclusion

Significant progress has been made in identifying pathogenic T cells in MS and in understanding their close connection to T cell trafficking to the CNS. Analyzing chemokine receptor expression in T cells is critical for the understanding of MS relapse, given that relapse is triggered by the invasion of pathogenic T cells into the CNS. With advances in flow cytometry, multiple chemokine receptor assays using fewer cells is becoming possible. By comparing the patterns of chemokine expressions on T cells in the CSF and peripheral blood cells, a unique T cell population that is potentially involved in MS pathology was identified. The CCR2+CCR5+CCR6− T cell population expressed the BBB-invading MMP-9 and OPN proteins, features that are distinct from those observed in CCR2−CCR5+ or other T cell populations. In this manner, single chemokine positive cells could be heterogeneous, comprising different functional subsets. In general terms, the analysis of T lymphocytes from patients to characterize the expression of multiple chemokine receptors might show novel T cell subsets that can serve as biomarkers of a disease of interest and identify therapeutic targets for the disease.

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