CD44 Reciprocally Regulates the Differentiation of Encephalitogenic Th1/Th17 and Th2/Regulatory T Cells through Epigenetic Modulation Involving DNA Methylation of Cytokine Gene Promoters, Thereby Controlling the Development of Experimental Autoimmune Encephalomyelitis

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CD44 Reciprocally Regulates the Differentiation of Encephalitogenic Th1/Th17 and Th2/Regulatory T Cells through Epigenetic Modulation Involving DNA Methylation of Cytokine Gene Promoters, Thereby Controlling the Development of Experimental Autoimmune Encephalomyelitis

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CD44 is expressed by a variety of cells, including glial and T cells. Furthermore, in the demyelinating lesions of multiple sclerosis, CD44 expression is chronically elevated. In this study, we demonstrate that targeted deletion of CD44 attenuated myelin oligodendrocyte glycoprotein peptide-induced experimental autoimmune encephalomyelitis (EAE) through novel regulatory mechanisms affecting Th differentiation. Specifically, by developing chimeras and using adoptive transfer experiments, we noted that CD44 deficiency on CD4+ T cells, but not other cells, conferred protection against EAE induction. CD44 expression played a crucial role in Th differentiation, inasmuch as deletion of CD44 inhibited Th1/Th17 differentiation while simultaneously enhancing Th2/regulatory T cell differentiation. In contrast, expression of CD44 promoted Th1/Th17 differentiation. When osteopontin and hyaluronic acid, the two major ligands of CD44, were tested for their role in Th differentiation, osteopontin, but not hyaluronic acid, promoted Th1/Th17 differentiation. Furthermore, activation of CD44+ encephalitogenic T cells with myelin oligodendrocyte glycoprotein peptide led to demethylation at the Ifn/Il17a gene promoter region while displaying hypermethylation at the Il4/Foxp3 gene promoter. Interestingly, similar activation of CD44-deficient encephalitogenic T cells led to increased hypermethylation of Ifn/Il17a gene and marked demethylation of Il4/Foxp3 gene promoter. Together, these data suggested that signaling through CD44, in encephalitogenic T cells, plays a crucial role in the differentiation of Th cells through epigenetic regulation, specifically DNA methylation of Th1/Th17 and Th2 cytokine genes. The current study also suggests that molecular targeting of CD44 receptor to promote a switch from Th1/Th17 to Th2/regulatory T cell differentiation may provide a novel treatment modality against EAE. The Journal of Immunology, 2011, 186: 6955–6964.

Multiple sclerosis (MS) starts with increased migration of autoreactive lymphocytes across the blood-brain barrier (1–3). As an immune-privileged site, activated lymphocytes normally enter and leave the CNS in hours without causing damage. However, in MS, these cells can overcome the CNS protective microenvironment, probably through failure of suppressive function from the regulatory T cells (Treg), reside in CNS, and cause inflammation, resulting in sclerotic plaques and neurologic symptoms (4, 5). Autoreactive T cells, such as Th1 and Th17 cells and their recruited inflammatory cells, produce a variety of cytokines. Local production of cytokines in CNS varies significantly during the disease progress, and changes in discrete sets of cytokines are associated with acute response and recovery phases of the disease. In this regard, Th1–Th2–Th17 cytokines or immune responses that regulate these cytokines are especially highlighted during the disease. Their balance affects the disease progress or recovery, such as a specific Th2 accumulation in CNS or a shift from Th1-type to Th2-type immune response rendering protection against the disease. Therefore, the search for new drugs that specifically target pathogenic Th1 and Th17 cells is tremendously interesting and important. Some drugs play immunomodulatory roles in polarizing Th cells toward Th1, Th2, or Th17 effectors, such as copolymer-I and Berberine (6–10). Experimental autoimmune encephalomyelitis (EAE) is a commonly used and well-established animal model with many similarities to human MS, including episodes of relapsing and remitting paralysis, which is induced by immunization of myelin Ags such as myelin oligodendrocyte glycoprotein (MOG) or a MOG peptide of aa 35–55 (MOG35–55) in CFA.

CD44 is a widely distributed cell surface glycoprotein expressed by a variety of lymphoid and nonlymphoid cells. CD44 is encoded by 20 exons, 7 of which form the invariant extracellular region of the so-called standard form (CD44s). By alternate splicing, up to 10 variant exons (CD44 v1–v10) can be inserted within the extracellular region (11–13). The extensive alternative splicing of CD44 is believed to contribute to its sophisticated implication in the immune response and immune regulation. Studies from our laboratory and elsewhere have shown that CD44 and its isoforms

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Abbreviations used in this article: Ct, cycle threshold; EAE, experimental autoimmune encephalomyelitis; HA, hyaluronic acid or hyaluronan; KO, knockout; MNC, mononuclear cell; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; OPN, osteopontin; PTA, pertussis toxin; Treg, regulatory T cell; WT, wild-type.

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participate in lymphocyte migration, proliferation, and activation not only by establishing specific transmembrane complexes, but also by organizing signaling cascades through association with its partner proteins such as p185HER2 and c-Src kinase (12, 14–20). CD44 is recruited to the immunological synapse during dendritic cell and T cell interactions and affects the subsequent T cell activation, IL-2 and IFN-γ production, and phosphotyrosine and protein kinase C-0 enrichment at the synapse (20). Regarding Th differentiation, targeted deletion of CD44 was shown by us to induce a Th2-biased immune response to the Ags of SRBC and OVA (21). Also, Th1 and Th2 cells express CD44 and rely on CD44 for their rolling and adhesion to the endothelium (17, 18, 20–22). Hyaluronic acid or hyaluronan (HA) and osteopontin (OPN) are the main ligands for CD44 molecule (12, 23).

There is strong evidence to indicate that CD44 and its ligands may play a critical role in the regulation of MS or EAE. CD44 is expressed at low levels in astrocytes and microglia, but the expression level is elevated in demyelinated lesions. Oligodendrocytes only express detectable levels of CD44 in vitro, but they are induced to express CD44 in vivo during MS development (24–26). It was reported that CD44 is chronically elevated in demyelinating lesions, alters the HA-based extracellular matrix and subsequent signaling, and causes failure of remyelination (27). Also, patients with MS have been shown to have elevated levels of OPN in their serum; mice deficient in OPN exhibit milder form of EAE (28, 29). Together, such studies indicate the crucial role played by CD44 and its ligands in the regulation of neuro-inflammation during MS. However, how such interactions involving CD44 influence the differentiation of encephalitogenic T cells into Th1/Th2/Th17/Treg subsets and its consequences on the clinical disease has not been previously investigated. In this study, we provide mechanistic evidence for the role of CD44 in encephalitogenic T cell differentiation and ensuing pathogenesis.

Materials and Methods

Mice and reagents

Wild-type (WT) C57BL/6 (or CD44−/−) mice were purchased from the National Cancer Institute. CD44 knockout (CD44 KO or CD44−/−) mice were generated at Angen Institute and provided to us by Dr. Tak. Mak. These mice are on C57BL/6 background and have been extensively characterized in our previous studies (15, 16). CD4+ T cell–deficient (CD4−/−) mice were purchased from The Jackson Laboratory. Mice were housed in the University of South Carolina Animal Facility. Animal procedures were performed according to National Institutes of Health guidelines under protocols approved by the Institute Animal Care and Use Committee of the university. MOG35–55 peptide was purchased from NeoMPS. IFA and desiccated Mycobacterium tuberculosis were purchased from Sigma-Aldrich. PMA and ionomycin were purchased from Jackson ImmunoResearch Laboratories. PMA and ionomycin were purchased from R&D Systems. Isotype control for the OPN IgG was synthesized, as previously described (30). OPN-specific goat IgG was purchased from NeoMPS. IFA and desiccated Mycobacterium tuberculosis were purchased from Sigma-Aldrich. For isolation of infiltrating mononuclear cells (MNCs) from pooled spinal cord and brain, mice were perfused with 30 ml heparin PBS. Single-cell suspensions were prepared and subjected to Percoll gradient (70%/30%) centrifugation. Isolated MNCs were incubated with anti-CD3, anti-CD4, or anti-CD8 Abs for 30 min at 4˚C before blocking of nonspecific staining. The staining was analyzed using a flow cytometer (Beckman Coulter; CXP FC500).

Histopathology

Brain and spinal cords were removed from mice after heparin–PBS perfusion and fixed in 10% paraformaldehyde overnight. Paraffin-embedded 10-μm sections were stained with H&E or Luxol Fast Blue (American MasterTech Scientific) and examined under light microscope. Sections were scored for the degree of inflammation, as described elsewhere (32).

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated from spinal cord, and cDNA was synthesized from 1 μg RNA. Real-time PCR for amplifying Opn was performed by using SYBR Green on StepOne Plus cycler (Applied Biosystems). Relative fold expression values were calculated based on the expression of GAPDH gene. Opn forward primer, 5′-TGAGCATTCCAAGAGAGCCAGGA-3′; Opn reverse primer, 5′-ACTAGCTGTGCTTGGTCTGTA-3′; GAPDH forward primer, 5′-TCAACAGCAACTCCTACTTCCA-3′; GAPDH reverse primer, 5′-ACCTCGTGTGTCGCGTATTTCA-3′.

T cell reconstitution and cytokine measurement

To analyze MOG-specific Th1, Th2, or Th17 cells, splenocytes or CNS-infiltrating MNCs were stimulated with 30 μg/ml MOG35–55 for 24 h, followed by stimulation with 50 ng/ml PMA and 1 μg/ml ionomycin in the presence of 2 μm monensin for 5 h. Part of the cultures was supplemented with anti-OPN Ab or isotype control goat IgG (3 μg/ml each) or Pep-1 peptide or scrambled control peptide (100 μg/ml each). For intracellular staining, cells were stained for surface CD4, then fixed, permeabilized, and stained for intracellular cytokines with anti–IL-17, anti–IL-4, or anti–IFN-γ (Cytofix/Cytoplperm intracellular staining kit; BD Pharmingen). IFN-γ production was also measured by ELISPOT assay (ELISpot kit; R&D Systems). Cell supernatants were collected at 24 h of MOG35–55 stimulation. Cytokine production in the supernatants was measured by multiplexed microsphere cytokine immunossay (Bio-Plex cytokine assay kit; Bio-Rad). Sera were collected between days 20 and 22 postimmunization. IL-4 and IFN-γ production in sera were measured by sandwich ELISA.

Th cell differentiation

Naïve CD4+ T cells were isolated from spleen of naive mice, as previously described (21). Cells were stimulated for 4 d with plate-bound anti-CD3 and soluble anti-CD28 (3 μg/ml each) plus irradiated WT splenocytes under Th1-, Th2-, or Th17-polarizing condition. Th1 condition: IL-12 (10 ng/ml) and anti–IFN-γ (10 μg/ml); Th2 condition: IL-4 (2 ng/ml), anti–IL-12 (10 μg/ml), and anti–IFN-γ (10 μg/ml); and Th17 condition: TGFβ1 (10 ng/ml), IL-6 (20 ng/ml), IL-23 at day 3 (20 ng/ml), anti–IL-10 (10 μg/ml), anti–IFN-γ (10 μg/ml). On day 4, cells were stimulated with PMA and ionomycin for 4–5 h in the presence of monensin and processed for intracellular cytokine staining of IL-4, IL-17, and IFN-γ, as described above. TGFβ1, IL-6, and IL-23 were purchased from R&D Systems. Other cytokines and Abs were purchased from ebioScience.

DNA methylation analysis

To analyze methylation status of ifny, il4, il17a, or foxp3 loci, quantitative Methylight assays were developed (33). Splenocytes from immunized or unimmunized mice were stimulated with 30 μg/ml MOG35–55 for 24 h. CD4+ T cells were purified with magnetic beads (StemCell) from the culture. For analysis of foxp3, noncultured splenic CD4+ T cells isolated from immunized or unimmunized mice were used. Genomic DNA was extracted and subjected to bisulfite-mediated C > T conversion. Primers were designed to amplify the bisulfite-converted promoter sequences and outside of CpG sites to ensure unbiased amplification of methylated and unmethylated alleles. TaqMan real-time PCR probe pair detects simultaneously the methylated state (C|-C|) and the unmethylated state (C|G|C). Assays were run on StepOne Plus cycler (ABI). Table 1 lists primer and probe sequences.
Chimeric development

The chimeras were developed, as described previously (21). Briefly, CD4−/− mice (CD44−/−) were lethally irradiated (950 rad from a 137Cs source) and reconstituted with a total of 10^3 bone marrow in a mixture consisting of 1 part CD44−/− bone marrow plus 3 parts CD4+ (CD44+/+) bone marrow, which resulted in all CD4+ T cells being CD44−/−, whereas all other cells were CD44+, as shown by us and others previously (21, 34). For the control, the mixture consisted of 1 part CD44−/− bone marrow plus 3 parts CD4+−/− (CD44−/−) bone marrow, which resulted in all cells that were CD44−/−. The level of chimerism was measured, as described previously (21). Mice were allowed to reconstitute for at least 6 wk before the immunization with MOG35–55.

Statistical analysis

The differences between experimental and control groups were analyzed using the Student t test, except for comparing EAE clinical scores using the Mann–Whitney U test, with p < 0.05 being considered statistically significant.

Results

Amelioration of EAE by CD44 deletion correlates with decreased Th1, but enhanced Th2 responses

We first investigated effects of CD44 targeted deletion in the development of EAE using CD44−/− mice. CD44 deletion resulted in a significant reduction in disease severity, and it delayed the clinical onset (Fig. 1A) (Table II). In additional experiments, we noted that in EAE-induced CD44−/− mice, there was a marked reduction in the number of CNS-infiltrating MNCs; specifically, the percentage of CNS-infiltrating CD4+ T cells was significantly decreased, whereas that for CD8+ T cells was not altered (Fig. 1B, and data not shown). The histopathology showed a markedly decreased inflammation and demyelination in CNS of EAE-induced CD44−/− mice when compared with CD44+/+ mice (Fig. 1C, 1D). We even observed three relapses in MOG-immunized CD44+/+ mice, but without a single one occurring in CD44−/− mice. With the stimulation ex vivo, CD4+ T cells from CD44−/− mice showed a significantly different polarization from Th1 to Th2 to the eliciting MOG35–55 peptide when compared with the CD44+/+ T cells. Thus, CD4+ T cells that were deficient in CD44 produced decreased level of Th1 cytokines, including IFN-γ, but increased amounts of Th2 cytokines, including IL-4, IL-5, and IL-13 (Fig. 2). These data suggested a switch in Th differentiation from Th1 to Th2 caused by CD44 deletion. Importantly, this Th1 to Th2 switch was also demonstrable in CNS-infiltrating CD44−/− CD4+ T cells (Fig. 3).

CD44 deletion in encephalitogenic T cells plays a critical role in EAE

To investigate whether the signaling for such a switch arose in the encephalitogenic T cells, CD44−/− or CD44+/+ encephalitogenic T cells were induced and transferred into naïve recipient CD44+/+ mice. The data indicated that transfer of CD44+/+ encephalitogenic T cells caused robust disease progression in recipient mice, whereas CD44−/− encephalitogenic T cells caused markedly milder signs of EAE (Fig. 4A). Again, the CNS-infiltrating CD44−/− encephalitogenic T cells showed a preference to Th2 polarization in response to the eliciting MOG35–55 stimulation, whereas Th1 polarization was inhibited (Fig. 4B). Furthermore, to corroborate these studies, we created chimeras in which only the CD4+ T cells were CD44−/− and noted that such mice were completely resistant to EAE when compared with mice that had CD44+/+CD4+ T cells (Fig. 4C). Collectively, these data suggested that CD44 deletion in CD4+ T cells directly promotes a switch from Th1–Th2 differentiation of encephalitogenic Th cells and ameliorates clinical disease. To further evaluate the functional consequence of CD44 deletion under varying culture conditions that promoted Th cell differentiation, naïve CD4+ T cells were stimulated with anti-CD3 and anti-CD28 Abs under Th1-, Th2-, or Th17-polarizing condition. As shown in Fig. 5, CD44 deficiency inhibited Th1 and Th17 polarization, whereas Th2 polarization was enhanced. These data provided further evidence that CD44 deletion promotes Th2 differentiation while inhibiting the proinflammatory Th1 and Th17 differentiation.

CD44–OPN interactions regulate epigenetic changes leading to promotion of Th2

Th1 and Th2 polarization is also associated with epigenetic changes in chromatin structure and DNA methylation at the ifnγ and il4 loci (35–38). To investigate whether CD44 signals are implicated in epigenetic imprinting of the ifnγ and il4 loci, DNA methylation at the promoter of the ifnγ and il4 loci in encephalitogenic CD4+ T cells was assessed (Fig. 6). In CD44−/−CD4+ T cells isolated from naïve mice, following activation with MOG35–55 for 24 h, the CpG dinucleotides within both promoters were found to be hypermethylated, exhibiting 77–87% methylation. However, additional comparisons revealed that there was no difference in methylation of ifnγ promoter between naïve CD44−/−CD4+ T cells and CD44+/+CD4+ T cells, whereas less methylation of il4 promoter was noted in naïve CD44−/−CD4+ T cells when compared with CD44+/+CD4+ T cells (77 versus 87%, p < 0.01; Fig. 6). In contrast, in encephalitogenic CD44−/−CD4+ T cells, methylation of ifnγ promoter was significantly more than that found in encephalitogenic CD44−/−CD4+ T cells (62 versus 45%, p < 0.001). Moreover, encephalitogenic CD44−/−CD4+ T cells exhibited dramatic decrease in DNA methylation of il4 promoter when compared with similar cells from CD44+/+CD4+ T cells (45 versus 87%). These data together demonstrated that

| Primer | For | reverse | forward | reverse'
|---|---|---|---|---|
| Methyalted DNA | AGGAGGTATACCCGCGGCTTGTGG | AGGATCCGATTTTTGAACGAGGTT | AGGAGGTATACCCGCGGCTTGTGG | AGGATCCGATTTTTGAACGAGGTT
| Unmethylated DNA | AGGTAATTGTTGTGTTGTTGAAGTGN | AGGATTTTAGTTTCAATCCCTTATTTT | AGGTAATTGTTGTGTTGTTGAAGTGN | AGGATTTTAGTTTCAATCCCTTATTTT

Table I. Primer and TaqMan probe sequences used for quantitative MethyLight assay of DNA methylation
activation of CD44 affects epigenetic imprinting by DNA hypomethylation of the Ifng and hypermethylation of il4 promoters, thereby promoting Th1 differentiation, whereas in the absence of CD44 activation, this process is reversed, thereby promoting Th2 differentiation.

To identify which signaling pathways were involved, we targeted HA and OPN, two important ligands of CD44. Pep-1 is a HA-binding peptide known to block CD44–HA interactions (30, 39). Thus, we used Pep-1 or neutralizing anti-OPN Ab in cultures of T cells activated with MOG 35–55. As shown in Fig. 7A, neutralization of OPN markedly inhibited IFN-γ production of CD44 +/+ CD4+ T cells (60% inhibition, from 18.3 to 7.2%). The addition of Abs failed to exhibit a similar effect on IFN-γ production in CD4 + T cells from CD44−/− mice, thereby suggesting that these Abs were inhibiting CD44–OPN interactions. The Abs against OPN failed to exhibit a significant effect on IL-4 production. Blockade with Pep-1 did not significantly affect IFN-γ or IL-4 production in any of the CD4 + T cell cultures. It should be noted that the concentration of Pep-1 tested in this study was validated previously to functionally block CD44–HA interaction (30). These data demonstrated that CD44–OPN interactions may potentiate Th1 polarization. In support of this finding, when we analyzed the spinal cords for Opn mRNA levels, we found that EAE-induced CD44 +/+ mice had elevated levels of Opn mRNA when compared with naive CD44 +/+ mice, thereby suggesting that there is OPN induction during EAE pathogenesis. Furthermore, EAE-induced CD44−/− mice had significantly lower levels of Opn mRNA when compared with EAE-induced WT mice (Fig. 7B). These data also suggested that OPN induction during CNS inflammation

Table II. Clinical symptoms of EAE

<table>
<thead>
<tr>
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<th>WT</th>
<th>CD44KO</th>
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<tbody>
<tr>
<td>Mean day of onset</td>
<td>11.4 ± 0.98</td>
<td>14.1 ± 1.07*</td>
</tr>
<tr>
<td>Mean maximum score</td>
<td>3.3 ± 0.34</td>
<td>0.8 ± 0.14*</td>
</tr>
<tr>
<td>Mean peak day</td>
<td>15.6 ± 0.53</td>
<td>21 ± 0.82*</td>
</tr>
</tbody>
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*p < 0.01 comparison between WT and CD44 KO groups.
may also be regulated by CD44. Furthermore, blocking CD44–OPN interaction affected the methylation at the *ifn* 

The status of Th17 and FOXP3+ Treg

Next, we also assessed the status of Th17 and FOXP3+CD4+ Treg. Deletion of CD44 markedly suppressed Th17 polarization of naive CD44+/−CD4+ T cells in vitro (Fig. 5). In EAE-induced CD44−/− mice, there was a dramatic decrease in IL-17 production in T cells restimulated with MOG35–55 both in the periphery and in the CNS (Fig. 8A). This response correlated with a significantly decreased frequency of IL-17–producing CD4+ T cells in the periphery (Fig. 8B, 8C), as well as in the CNS (Fig. 8D). CD44–OPN rather than CD44–HA signaling pathway promoted Th17 differentiation inasmuch as blocking the former with anti-OPN Abs rather than the latter with Pep-1 significantly inhibited IL-17 production from CD44+/− CD4+ T cells (57% inhibition, from 9.4 to 4.1%) (Fig. 9A). When we investigated the methylation status of *il17* promoter, MOG-activated T cells from CD44−/− EAE-induced mice displayed hypermethylation when compared with the WT T cells. Also, blocking CD44–OPN interaction in WT, but not CD44+/− T cells increased the methylation at the *il17* promoter (Fig. 9B). Conversely, CD44−/− mice exhibited a significant increase in the percentages of FoxP3+CD4+ Treg in the periphery at different stages of the disease (Fig. 10). Methylation status of *foxp3* promoter revealed that on day 13, T cells from CD44−/− EAE-induced mice had significantly lower methylation than similar cells from WT mice (Fig. 10). All together, these data demonstrated that the unique promotion of Th differentiation between Th1, Th2, Th17, and/or Treg was induced by deletion of CD44 and regulated through epigenetic modulation.

**Discussion**

CD44 has previously been shown to regulate lymphocyte migration, proliferation, and activation; however, its role in Th differentiation is not well understood. Our laboratory previously reported that CD44 regulates Th1–Th2 differentiation when activated with particulate or soluble Ags such as SRBC or OVA (21). In this study, we found that CD44 can regulate the differentiation and activity of Th1, Th2, Th17, and Treg in an autoimmune disease. Overall, the current study indicated that CD44 sufficiency promotes Th1/Th17 differentiation, whereas CD44 deficiency favors Th2/Treg differentiation. Furthermore, such a switch in T cell differentiation appears to be regulated by epigenetic mechanisms.

The role of CD44 was first demonstrated by diminished Th1 and enhanced Th2 response in EAE mice in which CD44 was genetically deleted and further corroborated by adoptive transfer experiments showing loss of encephalitogenicity of in situ deletion of CD44 in CD4+ T cells. It was evident that CD44 promoted Th1...
those transcription factors for epigenetic memory of differentiation, DNA methylation can occur later, independent of dependent expression of OPN is essential for efficient skewing of Th1/Th2 cells at the development stage. A number of studies have demonstrated that OPN critically contributes to development of Th1-associated markers and activities, such as production of pro-inflammatory cytokines like IL-12, IL-17, and IFN-γ, as well as expression of pro-inflammatory chemokines. The additional ligands include OPN, collagen, fibronectin, fibrinogen, laminin, chondroitin sulfate, mucosal vascular addressin, the MHC class II invariant chain, L-selectin, E-selectin, and galectin-8 (12, 13). A number of studies have demonstrated that OPN critically contributes to development of Th1-mediated immunity and disease. It was established that T-bet-dependent expression of OPN is essential for efficient skewing of CD4+ T and CD8+ T cells toward Th1 and Tc1 pathway, respectively. In MS patients, increased levels of OPN protein were found in the serum and plasma as well as cerebrospinal fluid. In EAE-induced mice, the sclerotic plaques contain high levels of Opn transcripts, and OPN-deficient mice showed decreased development of the disease correlating with decreased Th1 response (28, 29, 40, 41). Despite such studies on the important role of OPN in EAE, previous studies have not identified the target receptor of OPN in regulating EAE. In the current study, we noted that use of anti-OPN Ab in cultures caused dramatic reduction in IFN-γ production by CD44+/CD4+ T cells, but not CD44+/CD4+ T cells. This effect also modulates the epigenetic modification at the il4 gene promoter. These data indicated CD44–OPN signaling participates in Th1 differentiation of encephalitogenic T cells, and furthermore, deletion of CD44 may deprive Th1-polarizing signaling and promote Th2 differentiation. We also noted that the levels of OPN mRNA increased significantly during EAE in the CNS of CD44+/+ mice, whereas EAE induction in CD44−/− mice failed to increase the levels of OPN. These data suggested that OPN may play a negative role in the pathogenesis of EAE and that CD44 expression may also regulate the production of OPN, which is considered to be a cytokine, in the CNS.

Inasmuch as other Th effectors are implicated in MS/EAE, we were also interested in evaluating the status of Th17 and Treg, two critical players in pathogenesis of EAE (42). It was reported that Treg can prevent EAE, and this effect takes place before the disease onset (43, 44). We did observe the highest percentage of Th17 and Treg on the preonset stage (day 13). Actually, CD44 deficiency induced an expansion of total FOXP3+ population at all three stages of EAE, including preonset, peak, and prerelapse (days 13, 20, and 30). We also noted a significant increase in peripheral Treg on the preonset stage (day 13). Currently, CD44 deficiency induced an expansion of total FOXP3+ population at all three stages of EAE, including preonset, peak, and prerelapse (days 13, 20, and 30). We also noted a significant increase in peripheral Treg on the preonset stage (day 13). It was reported that Treg can prevent EAE, and this effect takes place before the disease onset (43, 44). We did observe the highest percentage of Th17 and Treg on the preonset stage (day 13).

![Figure 3](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
pathway may also promote encephalitogenic Th17 differentiation, and that deficiency of CD44, in contrast, may enhance Treg differentiation. Notably, these data were corroborated with epigenetic imprinting of the il17 and foxp3 loci following CD44 signaling. Together, our findings also provide mechanistic clues on how Abs against CD44 can inhibit neuroinflammation during EAE (48–50).

Pathogenesis of MS also depends upon the balance of Th-shaping cytokines such as IL-12 and IL-10, as well as distinct activities of Th subsets. IL-12 is essential for the generation of autoreactive EAE-inducing Th1 cells, whereas IL-10 antagonizes the disease-promoting effects of IL-12 and has been associated with remission from EAE. Modulation of IL-10/IL-12 cytokine circuit by IFN-β inhibits the development of epitope spreading and disease progression in EAE (51–53). In OPN−/− EAE mice, IL-10 also assists toward Th2 skewing (28, 29). Our findings showed

concomitant induction of IL-10 and downregulation of IL-12 production in CD44−/− EAE mice, which reflects decreased Th1 and skewing toward Th2 immune response, as well as fortified

FIGURE 5. CD44−/− CD4+ T cells preferentially polarize to Th2 cells in vitro. Naive CD4+ T cells from WT or CD44 KO mice were stimulated in vitro with anti-CD3 and anti-CD28 Abs accompanied with Th1-, Th2-, or Th17-polarizing condition. On day 4 of the culture, cells were stimulated with PMA and ionomycin for 4–5 h, and IFN-γ, IL-4, and IL-17 production was detected by intracellular staining. Data were expressed as mean ± SEM from three independent experiments.

FIGURE 6. DNA methylation analysis. Splenocytes at day 14 post-immunization were restimulated with MOG35-55 peptide for 24 h. CD4+ T cells were further purified from the culture. Genomic DNA was isolated from the CD4+ T cells and subjected to bisulfite conversion. DNA methylation at ifnγ and il4 loci was analyzed by Methylight quantitative real-time PCR. The schematic depiction of the protocol for the PCR and average percentage of DNA methylation has been shown. Data were expressed as mean ± SEM from three independent experiments. *p < 0.001 comparison between naive and EAE group, **p < 0.001 between WT EAE and CD44 KO EAE group, #p < 0.05 comparison between naive WT and EAE WT group for il4.
functionality of Treg, which together may account for reversal of the disease.

In summary, our study demonstrates that CD44 plays a critical immunoregulatory role in EAE. Specifically, CD44 promotes Th1/Th17 differentiation, whereas deficiency of CD44 inhibits Th1/Th17 differentiation and simultaneously enhances Th2/Treg differentiation. Expression of CD44 on encephalitogenic T cells leads to potential interactions with OPN, and consequent epigenetic

FIGURE 7. Interaction between CD44 and OPN is required for Th1 differentiation. A, Splenocytes at day 14 postimmunization were restimulated with MOG35–55 peptide for 24 h in the presence of anti-OPN Ab, Pep-1 peptide, or their respective controls. Th1/Th2 cells were analyzed by intracellular staining of IFN-γ and IL-4. Representative dot plots from three independent experiments were shown. Gates were on CD4 subset. B, Expression of Opn mRNA in the spinal cords on day 14 postimmunization was detected by real-time PCR. Average of relative fold changes was expressed as mean ± SEM from three independent experiments. #p > 0.05 comparison between naive WT and naive CD44 KO group, *p < 0.001 comparison between WT EAE and CD44 KO EAE group. C, CD4+ T cells were purified from the culture and subjected to the DNA methylation analysis, as described. Comparison for IFN-γ: **p < 0.001 between naive WT and EAE WT group, #p > 0.05 comparison between naive CD44 KO and EAE CD44 KO group. Comparison for IL-4: *p < 0.01 for anti-OPN or p < 0.001 for isotype comparison between WT and CD44 KO group.

FIGURE 8. Deletion of CD44 decreases generation of myelin-reactive Th17 cells. Splenocytes and CNS infiltration MNCs at day 22 postimmunization were restimulated with MOG35–55 peptide for 24 h. Production of IL-17 in the supernatants was detected by the multiplexed cytokine immunoassay. Th1/Th17 cells were analyzed by intracellular staining of IFN-γ and IL-17. A, IL-17 measurement in the culture supernatants; B, percentage of Th17 cells in spleen; C, representative dot plots of Th17 cells in spleen; D, percentage of Th17 cells in CNS and representative dot plots. All cytokine measurements and percentages are expressed as mean ± SEM from three independent experiments.
regulation, including hypomethylation of \textit{ifn}γ and \textit{il17a} genes and enhanced differentiation of Th1 and Th17 cells. In contrast, CD44 deficiency leads to hypermethylation of \textit{ifn}γ and \textit{il17a} and hypomethylation of \textit{il4} gene, leading to Th2 cell differentiation. Our study elucidated the role of CD44 and provides mechanisms of the action in EAE that will benefit the designing of therapeutic strategy by targeting CD44 in EAE or human MS. Thus, molecular targeting of CD44 receptor to promote a switch from Th1/Th17 to Th2/Treg differentiation may provide a novel treatment modality against EAE.
Disclosures

The authors have no financial conflicts of interest.

References