CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis

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CD73 is a cell surface enzyme of the purine catabolic pathway that catalyzes the breakdown of AMP to adenosine. Because of the strong immunosuppressive and antiinflammatory properties of adenosine, we predicted that cd73−/− mice would develop severe experimental autoimmune encephalomyelitis (EAE), an animal model for the central nervous system (CNS) inflammatory disease, multiple sclerosis. Surprisingly, cd73−/− mice were resistant to EAE. However, CD4+ T cells from cd73−/− mice secreted more proinflammatory cytokines than wild-type (WT) mice and were able to induce EAE when transferred into naive cd73+/+ T cell-deficient recipients. Therefore, the protection from EAE observed in cd73−/− mice was not caused by a deficiency in T cell responsiveness. Immunohistochemistry showed that cd73−/− mice had fewer infiltrating lymphocytes in their CNS compared with WT mice. Importantly, susceptibility to EAE could be induced in cd73−/− mice after the transfer of WT CD73+CD4+ T cells, suggesting that CD73 must be expressed either on T cells or in the CNS for disease induction. In the search for the source of CD73 in the CNS that might facilitate lymphocyte migration, immunohistochemistry revealed a lack of CD73 expression on brain endothelial cells and high expression in the choroid plexus epithelium which regulates lymphocyte immunosurveillance between the blood and cerebrospinal fluid. Because blockade of adenosine receptor signaling with the A2a adenosine receptor-specific antagonist SCH58261 protected WT mice from EAE induction, we conclude that CD73 expression and adenosine receptor signaling are required for the efficient entry of lymphocytes into the CNS during EAE development.

Adenosine | Multiple sclerosis | Inflammation | Choroid plexus

Multiple sclerosis (MS) is a chronic debilitating inflammatory disease that affects the CNS (1). Patients with MS experience a progressive loss in neurological function caused by immune-mediated axonal demyelination in multiple areas of the brain and spinal cord. Despite years of research, the etiology of MS is still unknown. Although there is evidence for genetic (2) and environmental components (3), many studies demonstrate that the immune system plays an integral role in the progression of MS (1). For example, studies using the dominant MS animal model, experimental autoimmune encephalomyelitis (EAE), demonstrate that myelin antigen-specific CD4+ T cells can induce CNS inflammation, demyelination, and neurodegeneration, resulting in the loss of motor function (paralysis) (4).

For MS or EAE to develop, autoreactive immune cells must gain entry into the CNS (5). Typically, the anatomical characteristics of the brain, such as the specialized endothelial cell tight junctions that constitute the framework of the blood–brain barrier (BBB), limit the entry of lymphocytes into the CNS (6). Although the brain is subject to immunosurveillance under normal physiological conditions (7), disease progression in MS and EAE is commonly associated with increased lymphocyte infiltration/extravasation (5). In recent years, experimental MS therapies directed at either inhibiting lymphocyte trafficking to the CNS or suppressing inflammation have had varying degrees of success in clinical trials (8). Because adenosine has been shown to regulate leukocyte migration across endothelial barriers (9, 10) and the production of inflammatory cytokines (11), we asked whether CD73 (ecto-5′-nucleotidase), a cell surface enzyme that catalyzes the formation of extracellular adenosine, has a role in EAE disease progression.

CD73 is a 70-kDa cell surface enzyme expressed on many cell types including subsets of lymphocytes (12), endothelial cells (13), and epithelial cells (14). During inflammation and the initiation of primary immune responses, ATP is released from damaged target cells into the extracellular environment and is converted to AMP by CD39 (15). This AMP is converted to extracellular adenosine by the catalytic action of CD73. Extracellular adenosine has potent immunosuppressive effects, mediated through its four G protein-coupled receptors: A1, A2a, A2b, and A3 (16). Although extracellular ATP has been shown to function as a proinflammatory, immunostimulatory mediator, inducing immune cells to secrete cytokines such as IL-1β, TNF-α, IL-12, and IL-18 (17), adenosine inhibits the production of proinflammatory cytokines and promotes the induction of IL-10 (17). Thus, adenosine acts as a negative feedback signal to counteract ATP-mediated immune stimulation, preventing uncontrolled inflammation and lessening the collateral damage to healthy tissues. Recently, it has also been suggested that the generation of adenosine by CD73 mediates the immunosuppressive ability of regulatory T cells (Tregs) (18, 19).

Because of the immunomodulatory and immunosuppressive properties of adenosine, we evaluated the role of CD73 in EAE. Based on a report of exacerbated EAE in A1 adenosine receptor (AR)-deficient mice (20), we expected that cd73−/− mice that are unable to catalyze the production of extracellular adenosine would experience severe EAE. Surprisingly, cd73−/− mice were highly resistant to the induction of EAE. However, CD4+ T cells from cd73−/− mice do possess the capacity to generate an immune response against CNS antigens and cause severe EAE when adoptively transferred into cd73+/+ T cell-deficient mice. CD73+CD4+ T cells from WT mice also caused disease when transferred into cd73−/− recipients, suggesting that CD73 expression, either on lymphocytes or in the CNS, is required for lymphocyte entry into the CNS.


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the brain during EAE. Because cd73<sup>−/−</sup> mice were protected from EAE induction by the broad-spectrum AR antagonist caffeine and the A<sub>2a</sub>-AR-specific antagonist SCH58261, our data suggest that the extracellular adenosine generated by CD73, and not CD73 itself, regulates the entry of lymphocytes into the CNS during EAE. Our results demonstrate a role for CD73 and adenosine in regulating the development of EAE.

Results

**cd73<sup>−/−</sup> Mice Are Resistant to EAE Induction.** To determine whether CD73 plays a role in controlling inflammation during EAE progression, cd73<sup>−/−</sup> and WT (cd73<sup>+/+</sup>) mice were subjected to the myelin oligodendrocyte glycoprotein (MOG) EAE-inducing regimen (21, 22). Daily monitoring for EAE development revealed that cd73<sup>−/−</sup> mice consistently displayed dramatically reduced disease severity compared with their WT counterparts (Fig. 1). By 3 weeks after disease induction, cd73<sup>−/−</sup> mice had an average EAE score of only 0.5 (weak tail) compared with 2.0 (limp tail and partial hind limb paralysis) for WT mice (Fig. 1).

**CD4<sup>+</sup> T Cells from cd73<sup>−/−</sup> Mice Respond to MOG Immunization.** We asked whether the resistance of cd73<sup>−/−</sup> mice to EAE induction could be explained by either an enhanced ability of cd73<sup>−/−</sup> lymphocytes to suppress an immune response or an inability of these lymphocytes to respond to MOG stimulation. Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells, or Tregs, can regulate actively induced EAE (23). Because Tregs have recently been shown to express CD73 and some reports suggest that the enzymatic activity of CD73 is needed for Treg function (18, 19), we asked whether the number and suppressive activity of Tregs were normal in cd73<sup>−/−</sup> mice. As shown in Fig. 2A, there were no significant differences in the frequencies of CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs in WT and cd73<sup>−/−</sup> mice, either before or after EAE induction. Similarly, the percentage of CD4<sup>+</sup> T cells that expressed CD73 changed only modestly after EAE induction in WT mice (Fig. 2B). Additionally, no significant difference was observed in the suppressive capacity of WT and cd73<sup>−/−</sup> Tregs to inhibit MOG antigen-specific CD4<sup>+</sup> effector T cell proliferation (data not shown). To determine whether cd73<sup>−/−</sup> T cells can respond when stimulated with MOG peptide, the capacity of these cells to proliferate and produce cytokines was assessed. CD4<sup>+</sup> T cells from MOG-immunized cd73<sup>−/−</sup> and WT mice displayed similar degrees of in vitro proliferation in response to varying concentrations of MOG peptide (data not shown). However, CD4<sup>+</sup> T cells from MOG-immunized cd73<sup>−/−</sup> mice secreted higher levels of IL-17 and IL-1β after in vitro MOG stimulation, compared with those of WT CD73<sup>+</sup>CD4<sup>+</sup> or CD73<sup>−</sup>CD4<sup>+</sup> T cells (Fig. 2C). Elevated levels of IL-17 are associated with MS (24) and EAE development (25), whereas high levels of the proinflammatory IL-1β cytokine are a risk factor for MS (26) and an enhancer of IL-17 production (27). No difference in IL-2, IL-4, IL-5, IL-10, IL-13, INF-γ, and TNF-α secretion was observed between WT and cd73<sup>−/−</sup> T cells after MOG stimulation (Fig. 2C and data not shown). Overall, the results from these assays suggest that cd73<sup>−/−</sup> T cells can respond well to MOG immunization.

We next asked whether T cells from cd73<sup>−/−</sup> mice possess the ability to cause EAE. To test this possibility, CD4<sup>+</sup> T cells from the spleen and lymph nodes of MOG-immunized cd73<sup>−/−</sup> mice were evaluated for their ability to induce EAE after transfer into tcr<sup>−/−</sup> (cd73<sup>+/+</sup>) recipient mice; tcr<sup>−/−</sup> mice lack endogenous T cells and cannot develop EAE on their own (ref. 28 and data not shown). cd73<sup>−/−</sup>tcr<sup>−/−</sup> recipient mice that received CD4<sup>+</sup> T cells from cd73<sup>−/−</sup> donors developed markedly more severe disease compared with those that received WT CD4<sup>+</sup> T cells (Fig. 2D). CD4<sup>+</sup> T cells from cd73<sup>−/−</sup> donors displayed equal degrees of expansion after transfer into cd73<sup>−/−</sup>tcr<sup>−/−</sup> recipient mice (data not shown). Thus, CD4<sup>+</sup> T cells from cd73<sup>−/−</sup> mice are not only capable of inducing EAE, but cause more severe EAE than those derived from WT mice when transferred into cd73<sup>+/+</sup>tcr<sup>−/−</sup> mice. These results are consistent with in vitro assays in which cd73<sup>−/−</sup> CD4<sup>+</sup> T cells secreted elevated levels of IL-17 and IL-1β (which are known to exacerbate EAE) in response to MOG stimulation (Fig. 2C), and they suggest that cd73<sup>−/−</sup> mice are resistant to MOG-induced EAE because of a lack of CD73 expression in nonhematopoietic cells (most likely lack of expression in the CNS).

**cd73<sup>−/−</sup> Mice Exhibit Little/No Lymphocyte Infiltration into the CNS After EAE Induction.** EAE is primarily a CD4<sup>+</sup> T cell-mediated disease (29); and during EAE progression, lymphocytes must
first gain access into the CNS to mount their inflammatory response against CNS antigens, resulting in axonal demyelination and paralysis (4). To determine whether CNS lymphocyte infiltration is observed after EAE induction in cd73−/− mice, brain and spinal cord sections were examined for the presence of CD4+ T cells and CD45+ cells by immunohistochemistry. cd73−/− mice displayed a dramatically lower frequency of CD4+ (Fig. 3 D–G) and CD45+ [Supporting information (SI) Fig. S1] lymphocytes in the brain and spinal cord compared with WT mice (Fig. 3 A–C and G) at day 13 after MOG immunization. Additionally, in lymphocyte-tracking experiments where MOG-specific T cells from 2d2 TCR transgenic mice (30) were transferred into either WT or cd73−/− mice with concomitant EAE induction, the percentage of 2d2 cells in the CNS increased severalfold with time in WT recipient mice, but not at all in cd73−/− recipients (Fig. S2). Overall, these results suggest that the observed protection against EAE induction in cd73−/− mice is associated with considerably reduced CNS lymphocyte infiltration. Nevertheless, CD4+ T cells from MOG-immunized cd73−/− mice possessed the ability to gain access to the CNS when transferred into cd73+/+ tcra−/− mice that were concomitantly induced to develop EAE (Fig. 3 K and L). In fact, earlier and more extensive CNS CD4+ lymphocyte infiltration was observed in cd73+/+ tcra−/− mice that received cd73−/− CD4+ T cells (Fig. 3 K and L) than in those that received WT CD4+ T cells (Fig. 3 H–J). Therefore, these results demonstrate that donor T cells from cd73−/− mice have the ability to infiltrate the CNS of cd73+/+ recipient mice.

Fig. 3. cd73−/− mice display little or no CNS lymphocyte infiltration after EAE induction; donor cd73−/− T cells infiltrate the CNS of cd73−/− tcra−/− recipient mice after EAE induction. (A–F) Frozen tissue sections from WT mice (A–C) and cd73−/− mice (D–F) day 13 after EAE induction were labeled with a CD4 antibody. (G) The mean number of CD4+-infiltrating lymphocytes in the brain and spinal cord was quantified per field in frozen tissue sections from WT and cd73−/− mice day 13 after EAE induction. Eight anatomically similar fields per brain and four fields per spinal cord per mouse were analyzed at ×10 magnification (n=5 mice per group). Error bars represent the standard error of the mean. (H–L) Frozen tissue sections of hippocampus (H, I, and K) and cerebellum (L and J) labeled with a CD4 antibody from EAE-induced tcra−/− mice that received CD4+ cells from WT (H–L) or cd73−/− (K and L) mice at day 12 (K), 18 (H and L), or 22 (I and J) after EAE induction. Immunoreactivity was detected with HRP anti-rat Ig plus AEC (red) against a hematoxylin-stained nuclear background (blue). Brackets indicate CD73 staining. Arrows indicate sites of lymphocyte infiltration. (Scale bars: 500 μm.)

CD73 Must Be Expressed Either on Lymphocytes or in the CNS for Efficient EAE Development. We next asked whether CD73 expression on CD4+ T cells can compensate for a lack of CD73 expression in the CNS and allow the development of EAE. Therefore, we adoptively transferred CD4+ T cells from MOG-immunized WT mice into cd73−/− recipients, concomitantly induced EAE, and compared disease activity with that of similarly treated WT recipients (Fig. 4A). Although WT recipients developed disease after EAE induction as expected, cd73−/− recipients also developed prominent EAE with an average disease score of 1.5 by 3 weeks after disease induction. This result was much higher than the 0.5 average score that cd73−/− mice normally showed at this same time point (Fig. 1). To define further the association of CD4+ T cell CD73 expression with EAE susceptibility, sorted CD73−/+ CD4+ and CD73−/-CD4+ T cells from immunized WT mice, or total CD4+ (CD73−) T cells from immunized cd73−/− mice, were transferred into cd73−/− recipients with concomitant EAE induction (Fig. 4B). cd73−/− mice that received CD73−/+ CD4+ T cells from WT mice developed EAE with an average score of 1.5 by 3 weeks after induction. Conversely, cd73−/− mice that received WT-derived CD73−/-CD4+ T cells did not develop significant EAE. Additionally, CD4+ cells from cd73−/− donor mice, which have the ability to cause severe EAE in CD73-expressing tcra−/− mice...
(Fig. 2D), were also incapable of potentiating EAE in recipient cd73−/− mice (Fig. 4B). Therefore, although CD73 expression on T cells can partially compensate for a lack of CD73 expression in nonhematopoietic cells, EAE is most efficiently induced when CD73 is expressed in both compartments.

The identity of the CD73-expressing nonhematopoietic cells that promote the development of EAE is not known. We considered vascular endothelial cells at the BBB as likely candidates because many types of endothelial cells express CD73 (13). However, immunohistochemistry revealed that mouse brain endothelial cells are CD73+ (data not shown). During these experiments, we observed that CD73 is, however, highly expressed in the brain on the choroid plexus (Fig. 4C), which is an entry point into the CNS for lymphocytes during EAE progression (4). Fig. 4D shows infiltrating lymphocytes in association with the choroid plexus of WT mice 12 days after EAE induction. Minimal CD73 staining was also observed on submeningeal regions of the spinal cord (data not shown). Taken together, our results suggest that CD73 expression, whether on T cells or in the CNS (perhaps on the choroid plexus), is necessary for efficient EAE development.

**AR Antagonists Protect Mice Against EAE Induction.** Because CD73 catalyzes the breakdown of AMP to adenosine and ARs are expressed in the CNS (20, 31), we next determined whether AR signaling is important during EAE progression. WT and cd73−/− mice were treated with the broad-spectrum AR antagonist caffeine (32) at 0.6 g/liter in their drinking water, which corresponds to an approximate dose of 4.0 mg per mouse of caffeine per day (33), 1 day before and throughout the duration of the EAE experiment (Fig. 5A). WT mice that received caffeine were dramatically protected against EAE development (Fig. 5A). As expected, cd73−/− mice that received caffeine did not develop EAE (Fig. 5A). Because CD73 is highly expressed on the choroid plexus (Fig. 4C), we next determined whether ARs are also expressed on the choroid plexus. Using the Z310 murine choroid plexus cell line (34), only mRNAs for the A1 and A2A AR subtypes were detected by quantitative PCR (qPCR) (Fig. 5B). Because A2AR−/− mice have been shown to develop severe EAE after disease induction (20), we asked whether treatment of WT mice with SCH58261 (35), an AR antagonist specific for the A2A subtype, could protect against EAE development. WT mice were given 1 mg/kg SCH58261 in DMSO or DMSO alone both i.p. and s.c. (for a total of 2 mg/kg) 1 day before EAE induction and daily for 30 days throughout the course of the experiment (Fig. 5C). WT mice that received SCH58261 were dramatically protected against EAE development compared with WT mice that received DMSO alone (Fig. 5C). Additionally, WT mice given SCH58261 displayed a significantly lower frequency of CD4+ lymphocytes in the brain and spinal cord compared with DMSO-treated WT mice at day 15 after EAE induction (Fig. 5D). Because studies have shown that adhesion molecules (such as ICAM-1, VCAM-1, and MadCAM-1) on the choroid plexus play a role in the pathogenesis of EAE (36), we determined whether SCH58261 treatment affected adhesion molecule expression on the choroid plexus after EAE induction. Comparison of the choroid plexus from DMSO- and SCH58261-treated WT mice shows that A2A AR blockade prevented the up-regulation of ICAM-1 that normally occurs during EAE progression (Fig. S3). Based on these results, we conclude that the inability of cd73−/− mice to catalyze the generation of extracellular adenosine explains their failure to develop EAE efficiently after MOG immunization and that CD73 expression and A2A AR signaling at the choroid plexus are requirements for EAE progression.

**Discussion**

The goal of this work was to evaluate the role of CD73 in EAE, an animal model for MS. Because CD73 catalyzes the formation of extracellular adenosine that is usually immunosuppressive (17) and A1AR−/− mice exhibit severe EAE (20), we predicted that cd73−/− mice would also develop severe EAE. However, cd73−/− mice were highly resistant to EAE induction, a surprising finding considering the plethora of studies demonstrating that cd73−/− mice are more prone to inflammation. For example, cd73−/− mice are more susceptible to bleomycin-induced lung injury (37) and are more prone to vascular inflammation and neointima formation (38). Consistent with these reports, we showed that cd73−/− T cells produced higher levels of the EAE-associated proinflammatory cytokines IL-1β and IL-17 after MOG stimulation. Furthermore, the adoptive transfer of cd73−/− T cells to tcrα−/− mice, which lack T cells but express CD73 throughout their periphery, resulted in severe CNS inflammation after MOG immunization, consistent with a role for adenosine as an antiinflammatory mediator. It is interesting to note that IFN-β treatment, one of the most effective therapies for MS, has been shown to up-regulate CD73 expression on endothelial cells both in vitro and in vivo (39). Thus, although the mechanism by which IFN-β benefits MS patients is incompletely understood, increased production of adenosine accompanied by its known antiinflammatory and neuroprotective effects could be a factor.

Consistent with their resistance to EAE induction, cd73−/− mice had a lower frequency of cells infiltrating the CNS during EAE compared with WT mice. This finding was also unexpected because CD73-generated adenosine has been shown to restrict
the migration of neutrophils across the vascular endothelium during hypoxia and of lymphocytes across high endothelial venules of draining lymph nodes (5, 10). Our data, in contrast, suggest that CD73 and the extracellular adenosine generated by CD73 are needed for the efficient passage of pathogenic T cells into the CNS. Therefore, the role that CD73 and adenosine play in CNS lymphocyte infiltration during EAE is more profound than their role in modulation of neuroinflammation.

It is important to know where CD73 must be expressed for T cell migration into the CNS. CD73 is found on subsets of T cells (12) and on some epithelial (14) and endothelial cells (13). Our data clearly demonstrate that although cd73−/−T cells respond well to MOG immunization, they cannot enter the CNS unless CD73 is expressed in nonhematopoietic tissues (i.e., cd73−/−/tcra−/− mice that develop EAE after adoptive transfer of CD4+ T cells from cd73−/−/ mice). A lack of CD73 on nonhematopoietic cells can also be compensated for, in part, by CD73 expression on T cells (i.e., cd73−/− mice become susceptible to EAE when CD73+ but not CD73−, CD4+ T cells are adoptively transferred). Although we considered BBB endothelial cells as a relevant source of CD73 in the CNS because CD73 is expressed on human brain endothelial cells (39), immunohistochemistry revealed that mouse brain endothelial cells are CD73+. However, CD73 was found to be highly expressed on choroid plexus epithelial cells, which form the barrier between the blood and the cerebrospinal fluid (CSF) and have a role in regulating lymphocyte immunosurveillance in the CNS (40). The choroid plexus has also been suggested to be the entry point for T cells during the initiation of EAE progression (4). Although we acknowledge the well documented role of lymphocyte–brain endothelial cell interactions via VLA-4/VCAM-1 binding in both EAE and MS (41), perhaps lymphocyte trafficking across the endothelial BBB is more important for disease maintenance and progression than for disease initiation, at least in EAE.

The next issue is how CD73 facilitates the migration of T cells into the CNS. Earlier work showed that lymphocyte CD73 can promote the binding of human lymphocytes to endothelial cells in an LFA-1-dependent fashion (42). We do not believe that this is the function of CD73 in EAE, however, because CD73-deficient T cells can enter the CNS and cause severe disease in cd73−/−/tcra−/− mice (Fig. 2D). Alternatively, CD73 can function as an enzyme to produce extracellular adenosine, a ligand for cell surface ARs. It is this latter function that we believe is relevant for the current work given that AR blockade with caffeine or SCH58261 can protect mice from EAE. Although the broad-spectrum AR antagonist caffeine also inhibits certain phosphodiesterases (43), its modulation of EAE progression is most likely through its effect on AR signaling (20). This notion is supported by the fact that SCH58261 also prevents EAE progression by specifically inhibiting A2a AR signaling. Because CD73 and the A1 and A2a AR subtypes are expressed on the choroid plexus, extracellular adenosine produced by CD73 at the choroid plexus can signal in an autocrine fashion. Because the A1 and A2a ARs are functionally antagonistic to each other and have different affinities for adenosine (44), the extracellular concentration of adenosine determines how a cell expressing the A1 and A2a receptors will respond, thus creating a mechanism switch whereby low concentrations of adenosine activate the A1 subtype and higher concentrations stimulate the A2a subtype (45). In the CNS, there is evidence to suggest that this A1–A2a interaction is important in mediating neuroinflammation, where A1 signaling is protective and A2a signaling promotes inflammation. For example, mice that lack the A1 AR develop severe EAE after disease induction (20), whereas mice that are given an A2a antagonist are completely protected against EAE (Fig. 5C). Additionally, mice that lack the A2a receptor are protected from brain injury induced by transient focal ischemia (46). Therefore, CD73-generated adenosine signaling at the choroid plexus appears to play a very important role in modulating inflammation in the CNS. This adenosine signaling most likely regulates the expression of adhesion molecules at the choroid plexus. Studies have shown that the up-regulation of the adhesion molecules ICAM-1, VCAM-1, and MadCAM-1 at the choroid plexus is associated with EAE progression (36). Because mice treated with the A2a AR antagonist SCH58261 do not experience increased choroid plexus ICAM-1 expression (Fig. S3), as normally occurs after EAE induction (36), our results suggest that A2a AR signaling increases ICAM-1 during EAE progression.

In summary, our data show that CD73 plays a critical role in the progression of EAE. Mice that lack CD73 are protected from the degenerative symptoms and CNS inflammation that are associated with EAE induction. Our work demonstrates a requirement for CD73 expression and AR signaling for the efficient entry of lymphocytes into the CNS during EAE. The data presented here may mark the first steps of a journey that will lead to new therapies for MS and other neuroinflammatory diseases.

Materials and Methods

Mice. cd73−/− mice have been described in ref. 9 and have been backcrossed to C57BL/6 for 14 generations. cd73−/− mice have no overt susceptibility to infection and appear normal based on weight gain, organ and body measurements, and monitored organ and their T and B cell responses in vivo and in vitro assays (9). C57BL/6 and tcra−/− mice on the C57BL/6 background were purchased from The Jackson Laboratories. Mice were bred and housed under specific pathogen-free conditions at Cornell University or the University of Turku. For AR blockade experiments, mice were given drinking water supplemented with 0.6 μg/liter caffeine (Sigma) or 2 mg/kg SCH58261 (1 mg/kg s.c. and 1 mg/kg i.p.) in DMSO (45% by volume) or 45% DMSO alone starting 1 day before EAE induction and continuing throughout the experiment. All procedures performed on mice were approved by the relevant animal review committee.

EAE Induction and Scoring. EAE was induced as described in ref. 22. Briefly, a 1:1 emulsion of MOG35-55 peptide (3 mg/ml in PBS) (Invitrogen) and complete Freund’s adjuvant (CFA; Sigma) was injected s.c. (50 μl) into each flank. Pertussis toxin (PTX, 20 ng) (Biological Laboratories) was given intravenously (200 μl in PBS) at the time of immunization and again 2 days later. Mice were scored daily for EAE based on disease symptom severity: 0 = no disease, 0.5–1 = weak/limp tail, 2 = limp tail and partial hind limb paralysis, 3 = total hind limb paralysis, 4 = both hind limb and fore limb paralysis, 5 = death. Mice with a score of 4 were euthanized.

T Cell Preparations and Adoptive Transfer. Mice were primed with MOG35-55 peptide in CFA without PTX. Adoptive transfer. After 1 week, lymphocytes were harvested from spleen and lymph nodes and incubated with ACK buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA, pH 7.3) to lyse red blood cells. Cells were incubated with antibodies to CD8 (TIB-105), IA-B,a,b (212A.14), FcR (2b.4.G2), B220 (TIB-164), NK1.1 (HB191), and then BioMag goat anti-mouse IgG, IgM, and goat anti-rat IgG (Qiagen). After negative magnetic enrichment, CD4+ cells were used either directly or further sorted into specific subpopulations. For sorting, cells were stained with antibodies to CD4 (RM4-5), CD73 (TY/23), or FoxP3 (FJK-16s). Intracellular cytokine staining was carried out according to the manufacturer’s instructions (eBioscience). Stained cells were acquired on a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

Flow Cytometry. Cell suspensions were stained with fluorochrome-conjugated antibodies for CD4 (RM4-5), CD73 (TY/23), or FoxP3 (FJK-16s). Intracellular cytokine staining was carried out according to the manufacturer’s instructions (eBioscience). Stained cells were acquired on a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

T Cell Cytokine Assay. Sorted T cells from MOG-immunized mice were cultured in the presence of irradiated C57BL6 splenocytes with 0 or 10 μg/ml MOG peptide. Supernatants were collected at 18 h and analyzed by using the Bio-plex cytokine (Bio-Rad) assay for IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, IL-17, and TNF-α.

Immunohistochemistry. Anesthetized mice were perfused with PBS, brains, spleens, and spinal cords were isolated and snap frozen in Tissue Tek-OCT medium. Five-micrometer sections (brains in a sagittal orientation) were cut and stained with antibodies for CD4 (RM4-5), CD73 (TY/23), or FoxP3 (FJK-16s). Intracellular cytokine staining was carried out according to the manufacturer’s instructions (eBioscience). Stained cells were acquired on a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).
were affixed to SuperFrost/Plus slides (Fisher), fixed in acetone, and stored at −80°C. For immunostaining, slides were thawed and treated with 0.03% H2O2 in PBS to block endogenous peroxidase, blocked with casein (Vector Laboratories) in normal goat serum (Zymed), and then incubated with anti-CD45 (YY62.3), anti-CD4 (RM-5), or anti-ICAM-1 (3E2). Slides were incubated with biotinylated goat anti-rat Ig (Jackson Immunoresearch) and streptavidin–HRP (Zymed) and developed with an AEC (Red) substrate kit (Zymed) and a hematoxylin counterstain. Coverslips were mounted with Fluormount-G and photographed under light (Zeiss).

Real-Time qPCR. By using Trizol (Invitrogen), RNA was isolated from the Z310 chorioid plexus cell line (34). cDNA was synthesized by using a Reverse-IT kit (ABGene). Primers (available upon request) specific for ARs were used to determine gene expression levels and standardized to the GAPDH housekeeping gene levels by using a SYBR Green kit (ABGene) run on an ABI 7500 real-time PCR system. Melt curve analyses were performed to measure the specificity for each qPCR product.

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