

CD24 Controls Expansion and Persistence of Autoreactive T Cells in the Central Nervous System during Experimental Autoimmune Encephalomyelitis

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Abstract

In the development of experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS), autoreactive T cells must be activated and clonally expand in the lymphoid organs, and then migrate into the central nervous system (CNS) where they undergo further activation. It is unclear whether the autoreactive T cells further expand in the CNS and if so, what interactions are required for this process. We have demonstrated previously that expression by the host cells of the heat-stable antigen (CD24), which was recently identified as a genetic modifier for MS, is essential for their susceptibility to EAE. Here we show that CD24 is essential for local clonal expansion and persistence of T cells after their migration into the CNS, and that expression of CD24 on either hematopoietic cells or nonhematopoietic antigen-presenting cells in the recipient is sufficient to confer susceptibility to EAE.

Key words: costimulatory molecules • autoimmune diseases • central nervous system • multiple sclerosis • clonal expansion

Introduction

Induction of experimental autoimmune encephalomyelitis (EAE) by pathogenic T cells is a multiple step process. The autoreactive T cells are primed in the lymphoid organ by immunization with myelin components. Activated myelin antigen-specific T cells must penetrate the blood brain barrier and be recruited into the central nervous system (CNS) to exert their effector function. More recently, it has been elegantly demonstrated that the autoreactive T cells express new activation markers after their entry into the CNS, presumably by interacting with local APCs (1–3). It is unclear, however, whether the cells in the CNS undergo further clonal expansion. This issue has an important bearing on how the autoimmune disease is sustained in the CNS, as T cells in the CNS appear to be prone to programmed cell death (4–7).

CD24 is a GPI-anchored cell surface glycoprotein and is expressed in a broad range of cell types, including developing hematopoietic cells and mature B cells (8). It is also

known that CD24 is abundantly expressed in the CNS, although its expression on the cells in the CNS that have immune function has yet to be carefully defined. We have reported that mice with a targeted mutation of CD24 are resistant to the induction of both actively induced and adoptively transferred autoreactive T cells and that the development of EAE requires a functional CD24 gene in both T cells and non-T host cells (9). More recently, our genetic analysis revealed that CD24 polymorphism is a genetic modifier for risk and progression of multiple sclerosis (MS; reference 10). Because CD24 is a costimulatory molecule that functions independently of CD28 (11–15), it is possible that CD24 in the CNS mediates local costimulation of T cells. Alternatively, CD24 also modulates very late antigen (VLA)4–vascular cell adhesion molecule (VCAM)-1 interaction (16) that regulates T cell recruitment to the CNS (17). Therefore, CD24 may regulate the recruitment of autoreactive T cells to the CNS.

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Abbreviations used in this paper: BrdU, bromodeoxyuridine; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; RPA, RNase protection assay; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

To test these two hypotheses, we adoptively transferred myelin oligodendrocyte glycoprotein (MOG)-reactive T cells into WT and CD24^{-/-} mice, and analyzed the recruitment of T cells. We found that although the pathogenic T cells can be recruited at a comparable efficiency to the CNS of the WT and CD24^{-/-} mice, the T cells persist and expand only in WT CNS. In consistency with this, we found that susceptibility to adoptively transferred T cells can be conferred by cells of either hematopoietic or nonhematopoietic origin. Interestingly, CD24 deficiency significantly reduced the *in vitro* costimulatory activity of microglia and astrocytes, two major APCs in the CNS, and that the CD24 deficiency in the hosts leads to a significantly reduced local proliferation in the CNS. Finally, we found that expression of CD24 does not affect apoptosis of T cells in the CNS. These results demonstrate that CD24 controls a novel checkpoint for EAE, most likely by controlling local proliferation of autoreactive T cells in the CNS.

Materials and Methods

Mice. CD24-deficient mice were produced using embryonic stem cells from C57BL6/j mice as described previously (18). C57BL6/j mice were purchased from the National Cancer Institute. All mice were maintained in animal facilities at the Ohio State University Medical Center under specific pathogen-free conditions. Thy1.1 congenic mice were purchased from The Jackson Laboratory.

Bone Marrow Chimera Mice. Bone marrow cells were isolated by flushing femur and tibia bones with PBS. Recipient mice were lethally irradiated (1,000 rads) and constituted with 10–20 × 10⁶ bone marrow cells by intravenous injection. Engraftment took place over a 6–8-wk period.

Culture Medium and Reagents. Click's EHAA medium supplemented with 10% FCS, 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin, and 1 mM 2-ME were used for all lymph node T cell cultures. For culture of glial cells, DMEM supplemented with 10% FCS, 2 mM L-glutamine, and 100 µg/ml penicillin/streptomycin was used. Recombinant murine IFN-γ, IL-12, and recombinant human IL-2 were purchased from PeproTech. The immunogen MOG peptide 35–55 (MEVGWYRSPFSRV-VHLYRNGK) was purchased from Genemed Synthesis, Inc. The purity of the peptide was >90%.

Adoptive Transfer EAE. 8–12-wk-old C57BL/6j or B6.PL/J (Thy1.1) mice were immunized subcutaneously with 100 µg MOG peptide in CFA in a total volume of 100 µl. 10 d after immunization, draining lymph nodes were harvested and stimulated at a density of 2 × 10⁶/ml in Click's EHAA medium supplemented with 15% FCS, 20 ng/ml recombinant IL-12, and 50 µg/ml MOG peptide for 4 d. Two different protocols were used in this study. Protocol A was used for most of the studies, except for those described in Fig. 7, which used protocol B. Protocol A: 10 × 10⁶ MOG peptide-activated lymph node cells were injected *i.v.* into each recipient mouse in a total volume of 200 µl PBS. Each mouse received 160 ng of pertussis toxin (Sigma-Aldrich) in 200 µl PBS in the tail vein immediately after the cell transfer and again 48 h later. Protocol B: 20–50 × 10⁶ MOG-activated T cells were injected *i.p.* into each recipient mouse that had been γ irradiated (350 rads) 1 h earlier. The mice were observed every day and scored on a scale of 0–5 with gradations of 0.5 for intermediate scores: 0, no clinical signs; 1,

loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, moribund; and 5, death.

Immunohistochemistry. Frozen sections of brain or spinal cords were fixed with acetone and incubated with anti-CD3 (2C11) and/or biotinylated anti-Thy1.1 (OX-7; BD Biosciences). After extensive washes, the mAbs were detected by biotinylated goat anti-rat mAbs followed by horseradish peroxidase-conjugated streptavidin, whose deposition was visualized upon incubation with DAB (3, 3'-diaminobenzidine) substrate.

RNase Protection Assay (RPA). Total RNA from each individual brain was isolated with Trizol reagent (Life Technologies). The concentration of RNA in each sample was assessed by spectrophotometry. The multiprobe RPA kit (RiboQuant; BD Biosciences) was used with the assay performed according to the manufacturer's protocol. In brief, a set of ³²P-labeled RNA probes synthesized from DNA templates using T7 polymerase was hybridized with 20 µg total RNA, after which free probes and other single strand RNA were digested with RNase. The remaining RNase-protected probes were purified, and then resolved on denaturing polyacrylamide gels. The template set mCD1 for mouse cell surface antigen was used in this study, which detects mRNA of TCR-δ, TCR-α, CD3ε, CD4, CD8α, CD8β, CD19, F4/80, CD45, L32, and GAPDH. For the detection of cytokines, we used template set mCK3b, which detects mRNA of TNF-β, LTβ, TNF-α, IL-6, IFN-γ, IFN-β, TGF-β1, TGF-β2, TGF-β3, MIF, L32, and GAPDH.

Isolation of Mononuclear Cells from the CNS. Fresh brain or spinal cord tissues were removed from mice and cut into ~2-mm pieces and incubated in 10 mM HEPES/NaOH buffer containing 1mg/ml of collagenase (Sigma-Aldrich) for 1 h at 37°C. The tissues were dispersed with a syringe, filtered through a 100-mm wire mesh, and centrifuged at 2,000 rpm for 5 min at 4°C. After centrifugation, cell pellets were resuspended in 15 ml of 30% Percoll (Amersham Biosciences), and centrifuged against 70% Percoll in a 50-ml tube for 15 min. The cell monolayer at the 30–70% Percoll interface was collected and washed once for further staining.

Culture of Microglia and Astrocytes from Brains of Newborn Mice. Primary glial cell cultures were prepared from brains of newborn CD24^{+/-} or CD24^{-/-} mice as described previously (19, 20). In brief, after removal of the meninges, the brains were mechanically dissociated by nylon sieves. The cells were seeded in culture medium (DMEM) containing 20% FCS in 75 cm² tissue culture flasks. On day 4 the medium was replaced with DMEM containing 10% FCS and medium was changed every 3 d thereafter. For isolation of microglial cells, confluent cultures between days 12 and 14 were vigorously shaken and the floating cells were collected and incubated at 37°C in flasks for another 2 h. The cultures were then shaken at 90 rpm and the nonadherent cells were washed out. The adherent cells were identified as microglial cells by positive staining with isolectin IB4 conjugated with Alexa Fluor 488 (Molecule Probes) and anti-CD45 antibodies (BD Biosciences). The firmly adherent cells were stained with anti-glial fibrillary acidic protein (GFAP) antibody to confirm their identity as astrocytes (21).

MOG-specific T Cell Line. Draining lymph node cells were isolated 10 d after immunization of C57BL6 mice with MOG peptide. 2 × 10⁶ cells/ml were stimulated with 50 µg/ml MOG peptide in the presence of 20 ng/ml IL-12 for 4 d. The viable cells were isolated and cultured in Click's EHAA medium in the presence of 5 ng/ml recombinant human IL-2 for another 2 wk before they were used for the proliferation assay.

Antibodies and Flow Cytometry. FITC- or PE-conjugated antibodies against CD3 (2C11), CD80 (16-10A), CD86 (GL1),

CD24 (M1/69), D^b (KH95) I-A^b (AF6-120), IFN- γ (XMG1.2), IL-4 (11B11), and rat IgG1 isotype control were all purchased from BD Biosciences. APC-Thy1.1 (HIS51) was purchased from eBioscience. Alexa Fluor 488-labeled isolectin IB4 was purchased from Molecular Probes. Purified mouse anti-GFAP cocktail was purchased from BD Biosciences. The antibody against CD24 (20C9) has been described (11). For cell surface staining, cells were incubated with antibodies in staining buffer for 30 min on ice. Cells were then washed three times and fixed. For intracellular staining of GFAP, astrocytes were first fixed with Cytofix/Cytoperm buffer (BD Biosciences) followed by washing with Perm/Wash buffer (BD Biosciences), and incubated with anti-GFAP in Perm/Wash buffer for 30 min on ice. After washing with Perm/Wash, cells were incubated with FITC-conjugated rabbit anti-mouse IgG antibody (Accurate Chemical & Scientific Corp.). For the staining of intracellular cytokines, lymphocytes were incubated with GolgiStop (BD Biosciences) in the last 5 h of culture. Viable cells were first fixed in the Cytofix/Cytoperm buffer followed by staining with PE-labeled anti-mouse cytokine antibodies and isotype control in Perm/Wash buffer. Cells were analyzed on a FACSCalibur™ cytometer (Becton Dickinson).

To analyze T cell apoptosis, mononuclear cells isolated from the CNS of WT or CD24^{-/-} mice were stained with anti-Thy1.1 antibody in conjunction with 7-AAD and FITC-labeled annexin V. The annexin V⁺ 7AAD⁺ cells are at late stages of apoptosis, whereas the annexin V⁺ 7AAD⁻ cells are at early stages of apoptosis.

Bromodeoxyuridine (BrdU) Incorporation. MOG-specific T cells were injected into mice i.v. At various times after adoptive transfer, mice were given an i.p. injection of 1 mg BrdU (BD Biosciences). 12 h later, mice were killed and perfused with PBS through the left ventricle. The brain and spinal cord were re-

moved from each mouse. Mononuclear cells were prepared from the brain and spinal cord, and were stained with anti-BrdU antibody using a BD Biosciences kit.

T Cell Proliferation Assay. To assess the antigen-presenting functions of cultured astrocytes and microglia, the WT MOG-specific T cell line was used as a responder. In brief, irradiated (3,000 rads) astrocytes or microglia were cultured in round-bottomed microtiter plates in 200 μ l DMEM medium containing 100 U/ml IFN- γ . 3 d later, the medium was removed and 2–5 \times 10⁴ T cells in Click's EHAA medium and the indicated concentrations of MOG peptide were added into each well. After 48 h, the cultures were pulsed with 1 μ Ci/well [³H]thymidine (ICN Pharmaceuticals) for another 12 h, and incorporation of [³H]thymidine was measured in a liquid scintillation β plate counter.

Results

Persistence and Expansion of MOG-reactive T Cells in the CNS of CD24^{+/+} But Not CD24^{-/-} Mice. Because CD24 is known to modulate the VLA4-VCAM-1 interaction (16), an attractive hypothesis is that CD24 may determine the pathogenicity of autoreactive T cells by controlling their recruitment to the CNS. To test this hypothesis, we injected MOG peptide-immune CD24^{+/+} T cells into WT or CD24-deficient mice and followed the clinical scores in the recipient mice. In addition, the T cells in the brain were analyzed by flow cytometry and quantitative RPA.

The T cells used were of the Th1 cell type as they produced IFN- γ , but not IL-4, in response to MOG peptide stimulation in vitro (unpublished data). As shown in Fig. 1 A, when we adoptively transferred MOG peptide-acti-

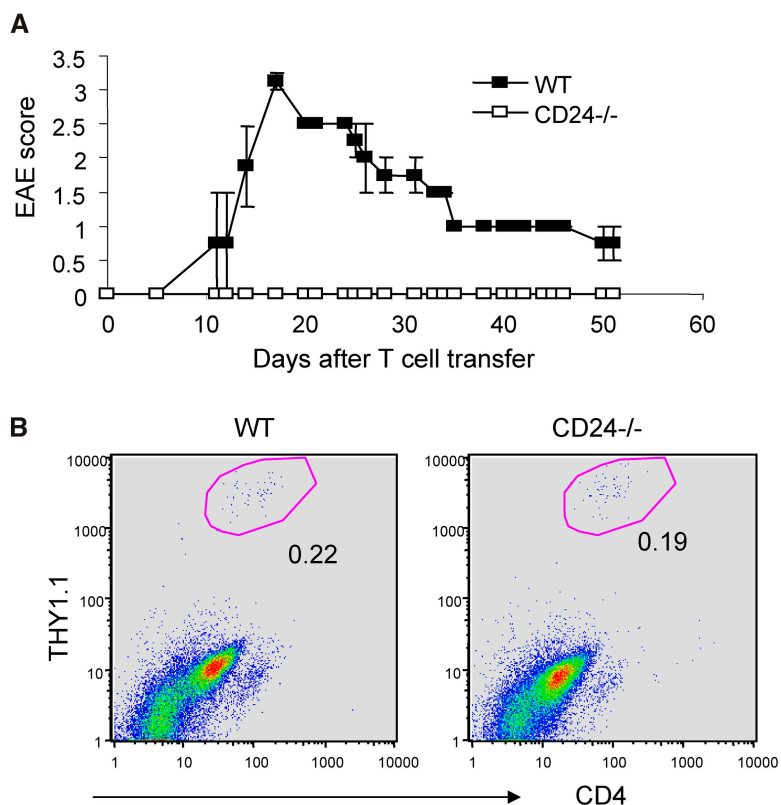


Figure 1. CD24 is required for persistence but not recruitment of autoreactive T cells in the EAE model. (A) CD24^{-/-} recipient mice are resistant to EAE induced by adoptively transferred T cells. WT and CD24^{-/-} mice (five per group) received 10 \times 10⁶ MOG peptide-activated lymph node cells i.v. The clinical signs were scored daily. The data shown are means and SD of EAE scores at different times after adoptive transfer and are representative of three independent experiments. (B) Migration of adoptively transferred T cells into the brain of WT and CD24^{-/-} mice. A MOG-immune T cell line prepared from Thy1.1 congenic mice was adoptively transferred into WT or CD24^{-/-} hosts. At 3 d after adoptive transfer, the brains were perfused with PBS and single cell suspensions were analyzed by FACS® using anti-Thy1.1 and anti-CD4 antibodies. Data shown have been repeated at least three times.

vated draining lymph node cells into CD24^{+/+} syngeneic recipient mice, all mice developed EAE at ~12–14 d after cell transfer. The clinical scores peaked at ~18 d after T cell transfer and declined gradually over the next 3 wk. In contrast, none of the CD24^{-/-} recipient mice developed EAE, which is in agreement with our previous report (9).

Because CD24 reportedly modulates the interaction between VLA4 and VCAM-1 (16), we tested whether expression of CD24 in the host is required for T cell migration into the CNS. We generated MOG-specific T cells from Thy1.1 congenic mice and adoptively transferred them into WT and CD24^{-/-} hosts. Based on published observations by others, most of the adoptively transferred T cells migrate into the CNS by the third day after adoptive transfer (1–3). Therefore, we harvested recipient brains after perfusion at 3 d after adoptive transfer and analyzed the number of T cells by flow cytometry. As shown in Fig. 1 B, despite their low percentages, a well-defined population of donor cells was found in the brains of WT recipients. The same subset was found in the CD24^{-/-} brains at a comparable frequency. Thus, CD24 expression in the host is not required for T cell migration into the brain.

We used RPA to measure the amount of T cells in the brain. At various time points after adoptive transfer of T cells, groups of two mice were perfused with PBS through the left heart ventricle immediately after death, and the brains were harvested for RNA isolation. Expression of several T cell-specific genes was determined by RPA. As directly shown in Fig. 2, CD3 and CD4 mRNA were undetectable in the CNS of normal CD24^{+/+} and CD24^{-/-} mice that received no transfer, which was expected. By day 8 after the adoptive transfer, weak but significant signals for the T cell-specific genes were detected in both groups of recipients. High and comparable amounts of CD3 and CD4 mRNA transcripts were detected on days 10–12 after T cell transfer, again in both groups. In the CD24^{+/+} brain, the amount of T cells continued to rise on day 20 and remained detectable on day 40 after the adoptive transfer. In contrast, the T cells were greatly reduced on day 20 and became undetectable by day 40 in the CD24^{-/-} brain. The majority of the T cells in the brain belonged to the CD4 subset, as the CD4 mRNA was more abundant than that of CD8. Thus, the autoreactive T cells persist and expand in CD24^{+/+} but not in the CD24^{-/-} CNS. These data demonstrate a critical role of CD24 in sustaining T cells in the CNS. The mRNA for macrophage marker F4/80 also increased with that of CD3 and CD8, consistent with an ongoing T cell-initiated inflammation in the host.

It has been established that in the adoptive transfer model, the host T cells are also recruited into the CNS after initiation of inflammation by the donor T cells (22, 23). To distinguish the adoptively transferred T cells versus T cells that were nonspecifically recruited into the CNS secondary to the antigen-specific T cells, we immunized Thy1.1 congenic mice and immune lymph node cells were cultured in the presence of MOG peptide and IL-12 for 4 d. The T cells were mostly of Th1 cell type, as the majority of them produced IFN- γ in response to PMA and iono-

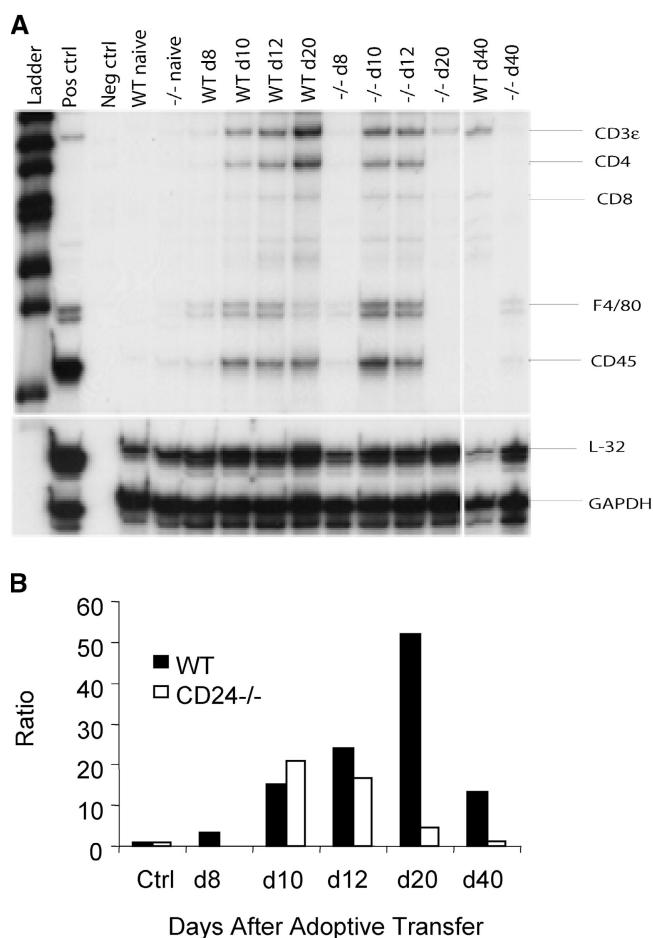


Figure 2. Quantitation of T cells in the brains of WT and CD24^{-/-} mice. (A) RPA. Mice that had received MOG-specific T cell transfers were killed at the indicated time points. Immediately after death, the mice were perfused with PBS through the left heart ventricle to wash blood out of the brain. The brain was removed and total RNA was isolated by the Trizol method. Each lane represents RNA samples mixed from two individual mice at a 1:1 ratio. A total of 20 μ g RNA was used per lane. Data are representative of four independent experiments. (B) Quantitation of the relative amount of T cells based on intensity of the CD3 bands shown in A. Intensity in the negative control samples was artificially set as 1.0.

mycin, although a small portion of T cells were capable of producing IL-4 (unpublished data). After adoptive transfer of these cells into either CD24^{+/+} or CD24^{-/-} recipient mice, we could detect Thy1.1⁺ T cells in the CNS of both CD24^{+/+} and CD24^{-/-} mice on day 10 after transfer (Fig. 3). The immunohistochemical analysis of Thy1.1⁺ T cells revealed several interesting points. First, despite comparable levels of CD3 mRNA in the brains of WT and CD24^{-/-} mice, a significant difference was observed in the number of Thy1.1⁺ T cells (Fig. 3, A–D). Second, in addition to the difference in the number of Thy1.1⁺ T cells, there was also a significant difference in their distribution. In the WT host, a substantial number of T cells were found in the brain parenchyma. In contrast, essentially all of the donor T cells were localized in the meninges and perivascular regions in the CD24^{-/-} brain and spinal cord. Third, as shown in Fig. 3 E, the donor T cells appeared in clusters in

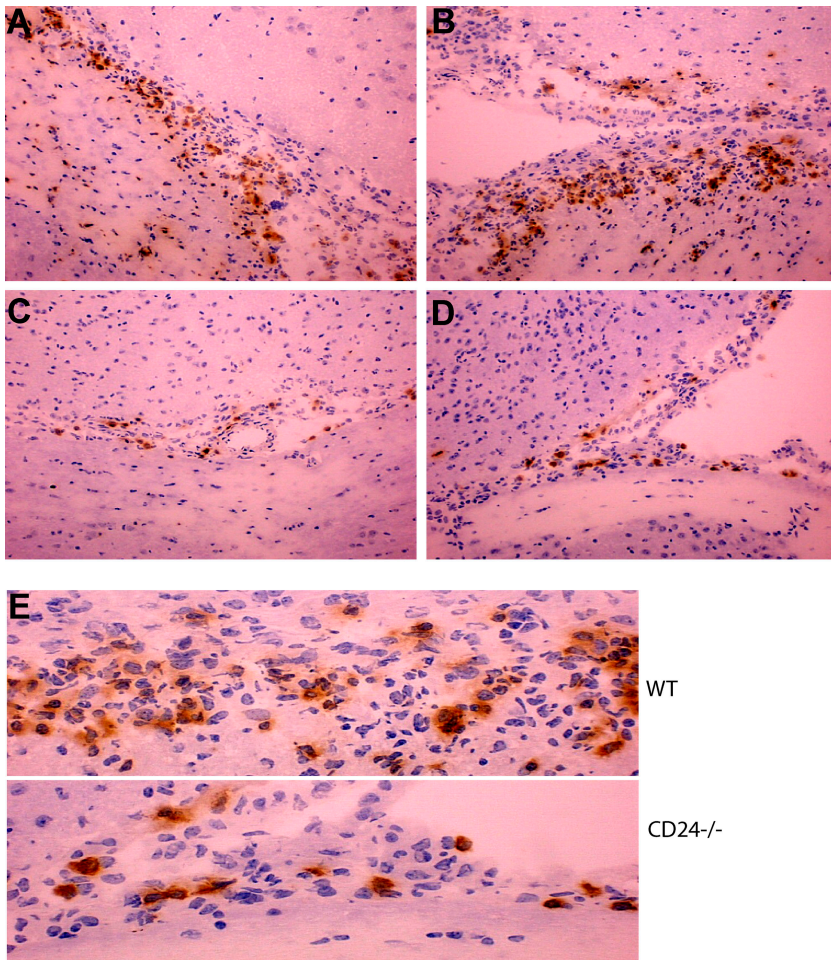


Figure 3. CNS infiltration of the MOG-activated T cells from Thy1.1 congenic mice. MOG peptide-activated Thy1.1⁺ T cells were injected into either CD24^{+/+} or CD24^{-/-} recipient mice, and 160 ng of pertussis toxin was injected i.v. at the time of T cell transfer at 48 h. On day 10 after T cell transfer, the mice were killed and frozen brain tissue sections were prepared and stained with anti-Thy1.1 antibody. A and B represent brain sections of two individual WT mice that received T cell transfer, and C and D represent those two CD24^{-/-} mice that received MOG peptide-activated Thy1.1⁺ T cells. Magnification: A–D, 50; E, 200. Data shown are representative of three independent experiments involving a total of five mice per group.

the WT CNS. In the CD24^{-/-} hosts, the donor cells rarely formed clusters.

Reduced Local Proliferation in the CNS of the CD24^{-/-} Mice. The failure of CD3⁺ T cells to persist in the CNS of CD24^{-/-} mice could be due to reduced proliferation of CD24^{+/+} T cells, or due to accelerated apoptosis of T cells. To address this issue, we adoptively transferred MOG-specific Thy1.1 congenic T cells into CD24^{+/+} and CD24^{-/-} mice, and determined the proliferation of the donor T cells by a pulsing of BrdU on days 3–20 after T cell transfer. 12 h after BrdU injection, the recipients were killed and perfused through the left ventricle to avoid blood contamination in the CNS. Mononuclear cells were isolated from the brain, spinal cord, and spleen, and stained for BrdU incorporation.

As shown in Fig. 4 A, on day 3 after T cell transfer, comparable numbers of Thy1.1⁺ T cells were detected in the spleens of both CD24^{+/+} and CD24^{-/-} mice (0.45 vs. 0.57% of total lymphocytes). These cells were undergoing vigorous proliferation, as 19.1 versus 23% of Thy1.1⁺ T cells were incorporating BrdU within 12 h. At this point, 0.39 and 0.33% of Thy1.1⁺ T cells were detected among the CNS mononuclear cells of CD24^{+/+} and CD24^{-/-} mice, respectively. Interestingly, in WT mice, 43.2% of Thy1.1⁺ T cells were incorporating BrdU in the CNS of WT mice. This is more than twofold higher than those

Thy1.1⁺ T cells in the spleen, presumably reflecting the effect of local antigen stimulation in the CNS. However, only 27.1% of Thy1.1⁺ T cells in the CD24^{-/-} mouse brains were BrdU⁺. Because the rate of BrdU incorporation in the CNS is comparable to that of the spleen, it is less clear to what extent their proliferation was in response to stimulation in the periphery or in the CNS, as T cells are known to be able to continue their proliferation if a threshold proliferation has been observed (24).

By day 12 after T cell transfer, fewer donor T cells were detected in the CD24^{-/-} spleen than in the CD24^{+/+} spleen (0.8% in CD24^{+/+} mice vs. 0.018% in CD24^{-/-} mice). About 2% of Thy1.1⁺ T cells in the CD24^{+/+} spleen were BrdU⁺. However, none of the Thy1⁺ T cells in the CD24^{-/-} spleen were incorporating BrdU in the spleen. We observed a significant increase in the number of Thy1.1⁺ T cells in both CD24^{+/+} and CD24^{-/-} CNS on day 12 compared with day 3. More than 4% of the donor T cells in the CD24^{+/+} mouse brain were actively incorporating BrdU, whereas only 1.5% of donor T cells underwent division in the CD24^{-/-} mouse brain. Similar differences were observed in the T cells isolated from the spinal cord (unpublished data). The significance of CD24-mediated T cell clonal expansion is further substantiated by a >10-fold difference in the number of T cells accumulated

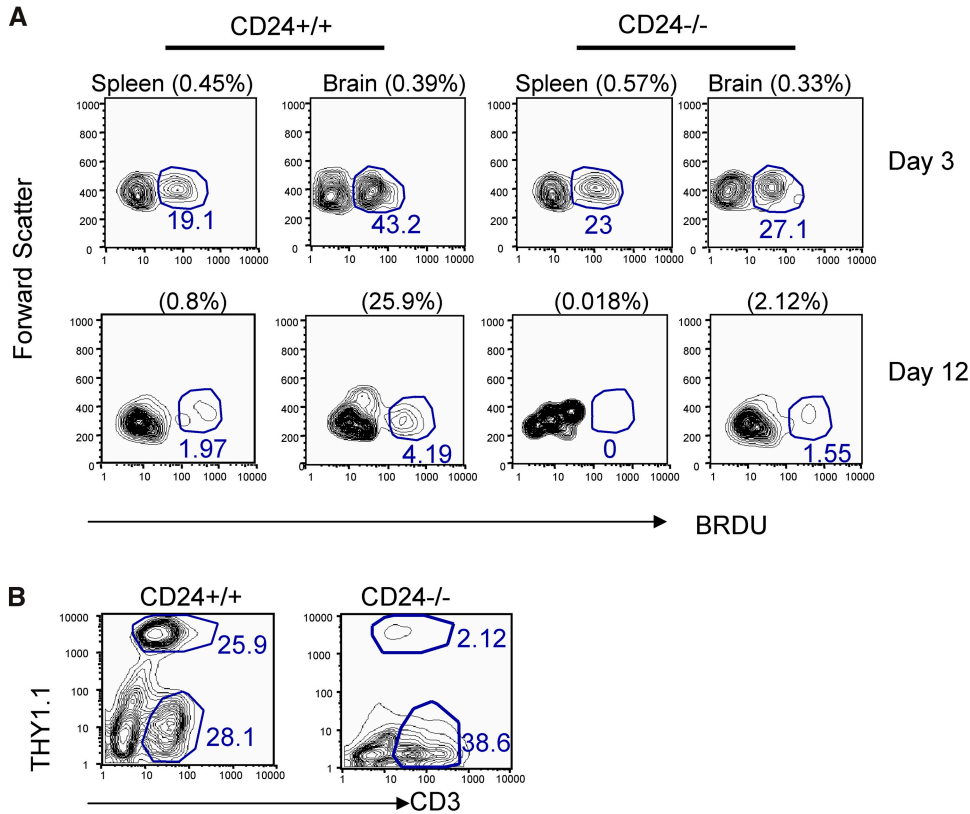


Figure 4. Requirement for CD24 for optimal T cell proliferation in the CNS. WT and CD24^{-/-} mice adoptively transferred with congenic T cells received an i.p. injection of BrdU (1 mg/mouse) 12 h before they were killed. Immediately after death, the mice were perfused with PBS through the left heart ventricle. The brain, spinal cord, and spleen were removed, and mononuclear cells were prepared and stained for Thy1.1 marker and BrdU incorporation. (A) BrdU incorporation in antigen-specific donor T cells. Data shown are profiles of gated Thy1.1⁺ T cells. The percentage of donor T cells among mononuclear cells in the spleen or brain is presented in the parentheses on top of each panel, and the proportion of BrdU⁺ T cells are indicated underneath the gates. (B) T cell accumulation in the CNS at day 12 after T cell transfer. Note that both donor-derived and host-derived T cells accumulated in the CNS. The substantially reduced incorporation of BrdU and the accumulation of donor cells in the brain of CD24^{-/-} mice have been reproduced in three independent experiments.

in CD24^{+/+} (26%) versus CD24^{-/-} (2%) mouse brains on day 12.

A comparison between the total number of T cells in the CNS detected by RPA (Fig. 2) and those detected by Thy1.1-specific antibody in both immunohistochemistry (Fig. 3) and flow cytometry (Fig. 4 A) suggests that the overwhelming majority of T cells detected by RPA in the CD24^{-/-} mouse brain are of host origin. This is confirmed by flow cytometry, which revealed that more than ~95% of T cells in the CD24^{-/-} mouse brain are of host origin, whereas about an equal number of host and donor cells were found in the CD24^{+/+} brain (Fig. 4 B).

Local Activation and Apoptosis of T Cells in the CNS. In addition to proliferation, a major factor that determines the number of T cells is apoptosis. To test whether the lack of T cell persistence in the CNS of CD24^{-/-} mice is due to accelerated cell death, we stained CNS-infiltrating cells for apoptosis markers 7-AAD and annexin V, as shown in Fig. 5. On day 10 after T cell transfer, ~30% of Thy1.1⁺ brain-infiltrating T cells were undergoing apoptosis in both CD24^{+/+} and CD24^{-/-} mice. More than 50% of Thy1.1⁺ T cells were undergoing apoptosis in the spinal cords in both CD24^{+/+} and CD24^{-/-} mice. However, no difference was observed between WT and CD24^{-/-} hosts. Thus, the failure of T cell persistence in the CNS of CD24^{-/-} mice is not due to accelerated T cell apoptosis.

Because expression of cytokines in the CNS is widely used to measure T cell activation in situ (25), we used the RPA assay to measure inflammatory cytokine mRNA ex-

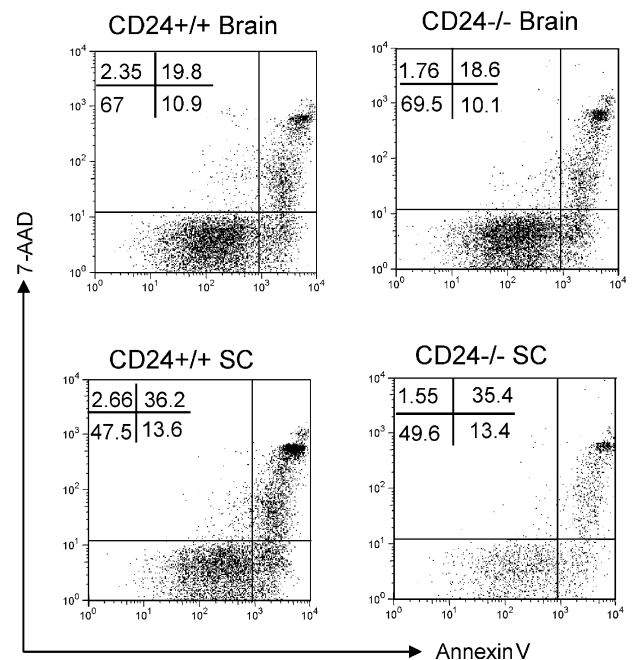


Figure 5. Antigen-specific T cells undergo apoptosis at similar rates in WT and CD24^{-/-} CNS. MOG peptide-activated Thy1.1⁺ T cells were injected into either CD24^{+/+} or CD24^{-/-} recipient mice, and 160 ng of pertussis toxin was injected i.v. at the time of T cell transfer and at 48 h. On day 10 after T cell transfer, mice were killed and CNS-infiltrating cells were prepared as described in Fig. 3. The CNS mononuclear cells were triple labeled for Thy1.1, 7-AAD, and annexin V markers, as described in Materials and Methods. Data presented here are gated on Thy1.1⁺ T cells. Two independent experiments were performed with similar results.

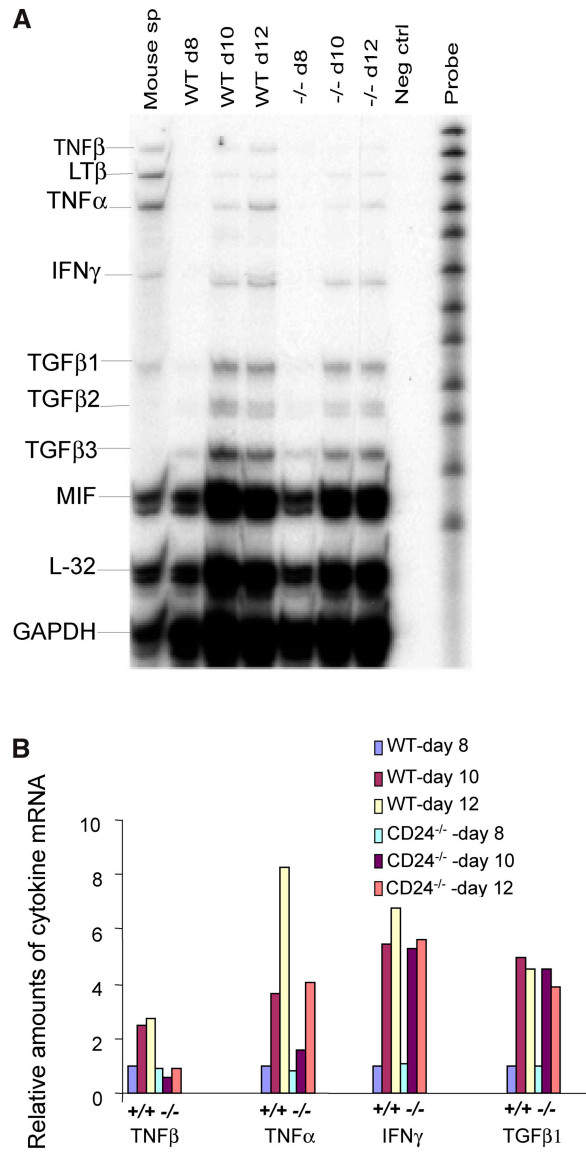


Figure 6. Cytokine gene expression in brains of WT and CD24^{-/-} mice on days 8, 10, and 12 after adoptive transfer of T cells. (A) An autoradiograph of a representative RPA. (B) Quantitation of the relative abundance of cytokine mRNA. The intensity of bands were normalized with L32 to avoid variations of total RNA used. The mRNA level on day 8 in the WT mouse brains is defined as 1.0. The data presented are representative of three experiments. The samples used in this figure are the same as those used in Fig. 2.

pression in the CNS. As shown in Fig. 6, although little IFN- γ , TNF- α , TNF- β , and TGF- β were detected in both CD24^{+/+} and CD24^{-/-} CNS on day 8 after T cell transfer, significant up-regulation of all of these cytokine genes was detected in WT mouse brains on days 10 and 12. With a notable exception of TNF- β , significant induction of IFN- γ , TNF- α , and TGF- β was also observed in the CD24^{-/-} brains, although the overall levels were somewhat lower than in the WT mice. The reduction in cytokine mRNA that can be attributable to CD24 deficiency was not as drastic as that in the number of donor T cells, perhaps because some of the host T cells also synthesize cytokine mRNA.

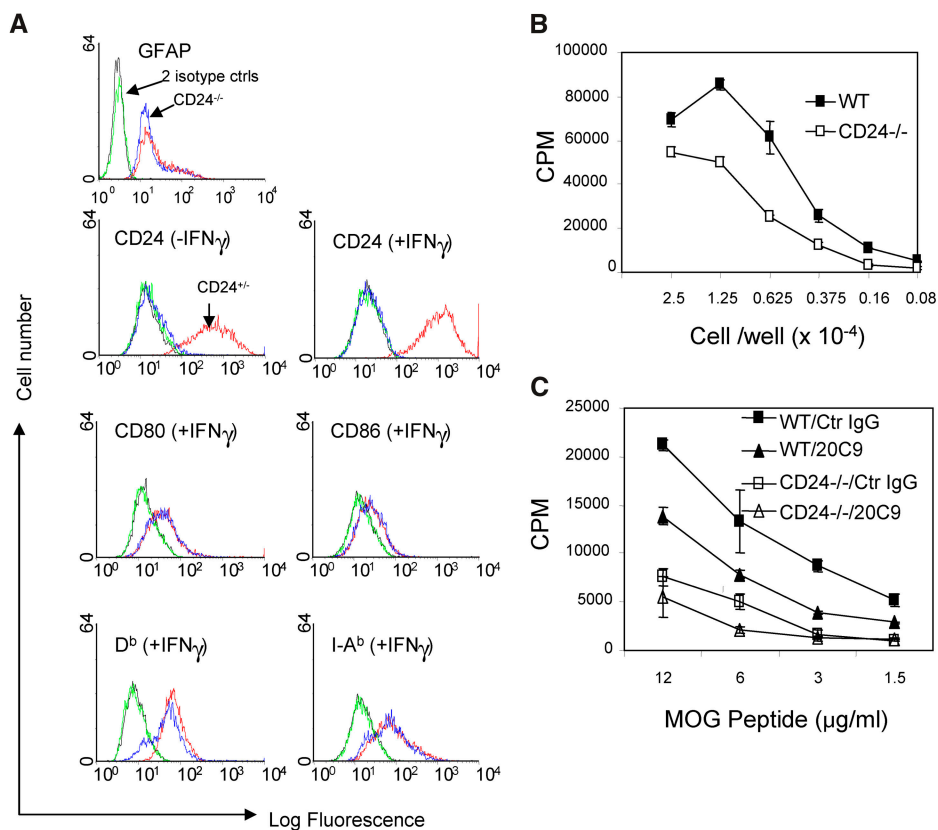
CD24-deficient CNS APCs Have Reduced Capacity to Stimulate Proliferation of Autoreactive T Cells. Because T cells proliferated less in the CNS of CD24^{-/-} recipient mice, we reasoned that CD24 may contribute to EAE development by regulating the functions of the CNS APCs. To test this hypothesis, we compared astrocytes and microglia from brains of newborn CD24^{+/+} or CD24^{-/-} mice for their ability to stimulate WT T cells. As shown in Fig. 7 A, essentially all of the cells in the astrocyte culture expressed GFAP. CD24^{+/+} astrocytes constitutively expressed CD24, and after stimulation with IFN- γ , the level of CD24 increased about threefold. The levels of CD80 and CD86 were low even after IFN- γ stimulation. As previously reported by others (19, 26), IFN- γ induced significant expression of MHC class I and II in both groups.

Although both groups of astrocytes stimulated proliferation of these T cells, WT astrocytes were approximately fourfold more efficient in inducing the proliferation than the CD24^{-/-} astrocytes on a cell to cell basis (Fig. 7 B). When the MOG peptide was titrated, the WT astrocytes were again about fourfold more potent. The enhanced proliferation was blocked by the addition of an anti-CD24 antibody, 20C9 (Fig. 7 C), which is known to block the costimulatory activity of the CD24 molecule (13).

We also isolated microglia cells from newborn CD24^{+/+} or CD24^{-/-} mice. As shown in Fig. 8 A, comparable levels of the CD24^{+/+} and CD24^{-/-} microglia were strongly positive for isolectin IB4 staining and CD45 (not depicted; references 27 and 28). CD24^{+/+} microglia constitutively expressed high levels of CD24 and after stimulation with IFN- γ , they expressed even higher levels of CD24. IFN- γ induced significant expression of MHC class I and II and low levels of CD80 and CD86 on microglia. To compare the costimulatory activity of the CD24^{+/+} and CD24^{-/-} microglia cells, we stimulated MOG-specific T cell lines with these cells in the presence of different concentrations of MOG peptide. Based on the titration of antigen, WT microglia were approximately fivefold more efficient in inducing T cell proliferation (Fig. 8 B). These results suggest that CD24 expressed on microglia can promote proliferation of MOG-reactive T cells.

Expression of CD24 on Radiosensitive and Radioresistant Host Cells Confers Susceptibility to Pathogenic T Cells in the CNS. The resistance of the CD24^{-/-} mice to EAE induction by WT T cells suggests that at least some host cells are required to express CD24. Because local APCs can be of either hematopoietic or nonhematopoietic origin, we created radiation bone marrow chimeras to determine whether expression of CD24 on hematopoietic cells is sufficient to convey EAE susceptibility. As shown in Fig. 9 A, a single round of bone marrow transplantation resulted in an essentially complete replacement of CD24-expressing cells in the spleen, which demonstrates the efficacy of irradiation. Reciprocal chimeras were used to determine the nature of the host cells required for EAE susceptibility, whereas syngeneic chimeras were used as controls.

As shown in Fig. 9 B, all CD24^{+/+} > CD24^{+/+} bone marrow chimeras developed EAE after CD24^{+/+} T cell



indicated wells at a concentration of 2.5×10^4 /well in the presence of given concentrations of MOG peptide. Anti-CD24 mAb 20C9 and a control hamster IgG were used at $1 \mu\text{g/ml}$. Data are means and SD of cpm and are representative of three experiments.

Figure 7. CD24^{-/-} CNS astrocytes had a reduced capacity to stimulate MOG-specific T cell proliferation. (A) Characterization of astrocytes prepared from newborn CD24^{+/+} and CD24^{-/-} littermates. GFAP was stained after permeabilization and the intact cells were used for analysis of other markers. Red lines and black lines represent profiles of fluorescence intensity of CD24^{+/+} astrocytes when stained with either specific antibodies (red) or controls (black), and blue lines (specific staining) and green lines (control staining) depict the fluorescence intensity of CD24^{-/-} astrocytes, as indicated by arrows. CD24 staining of IFN- γ -treated and -untreated astrocytes is shown, whereas only IFN- γ -stimulated astrocytes were analyzed for CD80, CD86, D^b, and I-A^b expression. (B) The MOG-reactive T cell line (5×10^4 /well) was stimulated by serial titrated astrocytes in the presence of $50 \mu\text{g/ml}$ MOG peptide. Data are representative of two independent experiments with similar results. (C) Blocking of T cell proliferation by CD24-specific antibody. CD24^{+/+} or CD24^{-/-} astrocytes were irradiated (3,000 rads) and seeded into U-bottomed 96-well plates in DMEM culture medium containing 100 U/ml IFN- γ . 3 d later, medium was discarded and a T cell line specific for MOG peptide was added into the

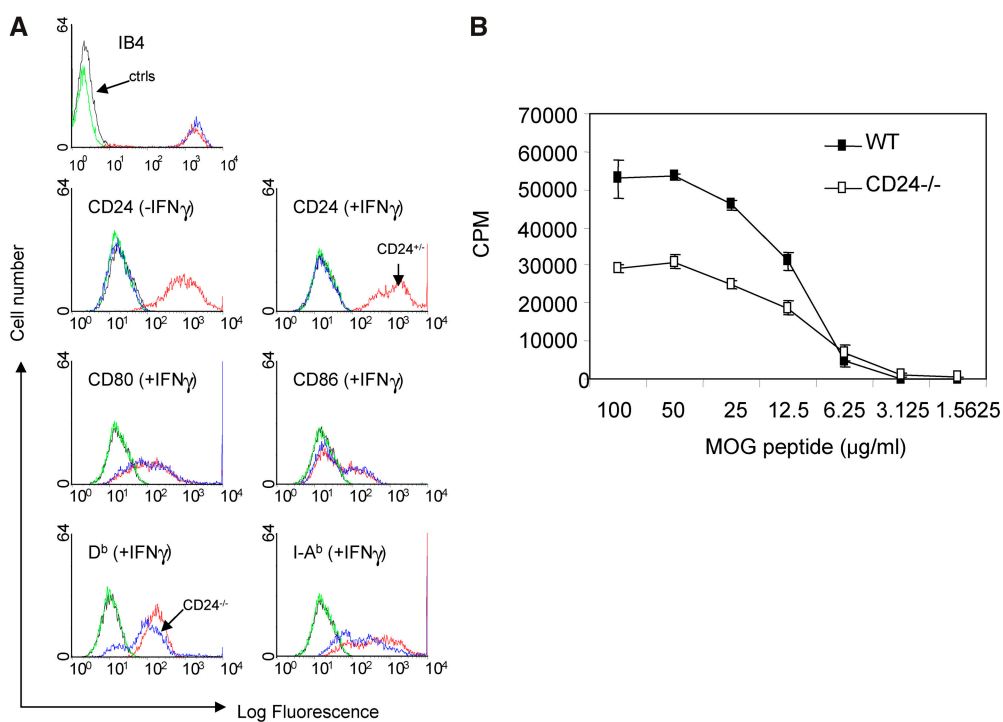


Figure 8. CD24^{-/-} CNS microglia had a reduced capacity to stimulate MOG-specific T cell proliferation. (A) Characterization of microglia prepared from newborn CD24^{+/+} and CD24^{-/-} mice. Microglia preparation was either left untreated or stimulated with 100 U/ml IFN- γ for 48 h. Cell surface IB4, CD80, CD86, D^b, and I-A^b were analyzed by flow cytometry. Red lines and black lines represent profiles of fluorescence intensity of CD24^{+/+} astrocytes when stained with either specific antibodies (red) or controls (black), and blue lines (specific staining) and green lines (control staining) depict the fluorescence intensity of CD24^{-/-} astrocytes, as indicated by arrows. (B) CD24^{-/-} brain microglia had a reduced capacity to stimulate MOG-specific T cell proliferation. Irradiated microglia were stimulated with 100 U/ml IFN- γ for 3 d and were used to present MOG peptide to a MOG-specific T cell line (5×10^4 /well). Data are means and SD of cpm and are representative of five individual experiments.

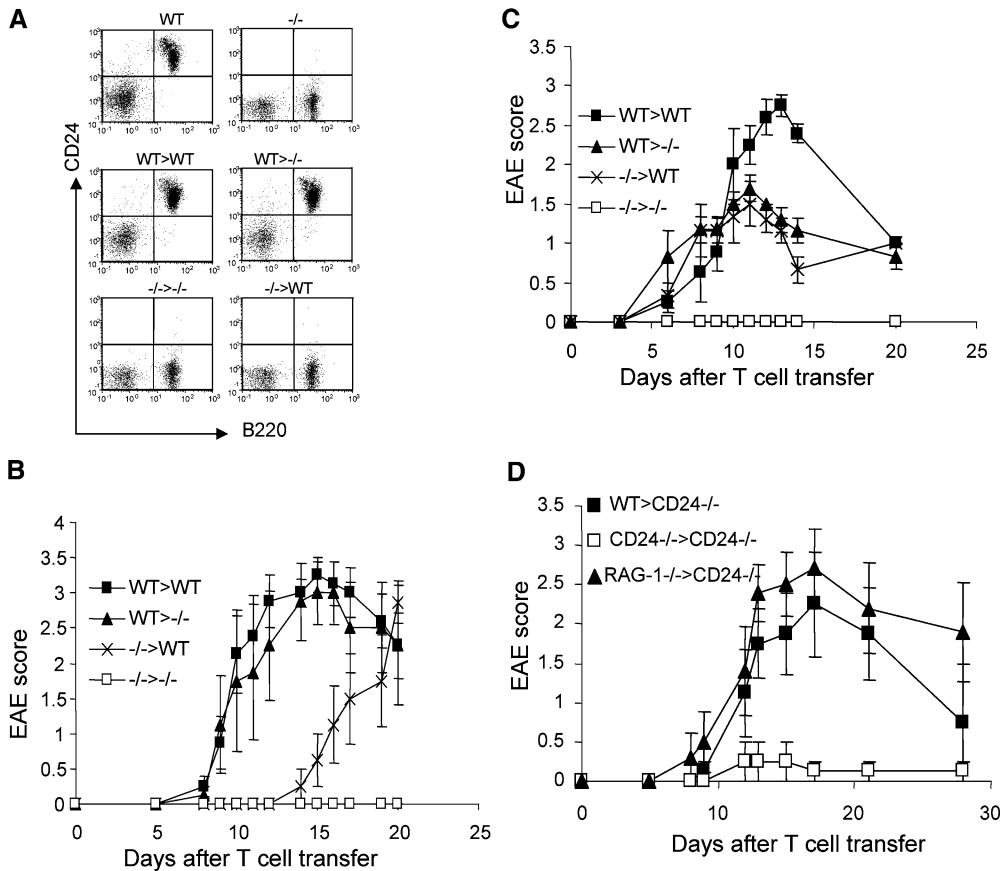


Figure 9. Expression of CD24 on either hematopoietic or non-hematopoietic cells is sufficient to confer susceptibility to adoptively transferred MOG-specific T cells. (A) FACS[®] profiles of bone marrow chimeras at 8 wk after reconstitution. Splenocytes were stained with FITC-labeled anti-B220 and PE-conjugated anti-CD24 antibodies. (B) Induction of EAE in bone marrow chimeras. 40×10^6 MOG peptide-activated lymph node cells were injected into mice that were irradiated (350 rads) 1 h before receiving pathogenic T cells. Data are means and SEM of EAE score in groups of four mice each and are representative of three independent experiments with similar results. (C) EAE susceptibility in double radiation chimeras. Mice numbers in each group: WT > WT, $n = 4$; -/- > -/-, $n = 4$; WT > -/-, $n = 3$; -/- > WT, $n = 3$. Data are representative of two independent experiments. (D) CD24^{+/+}RAG-1^{-/-}>CD24^{-/-} mice are fully sensitive to EAE induction. For induction of EAE, 50×10^6 /mouse of T cells were used. Mice numbers in each group: WT > -/-, $n = 4$; -/- > -/-, $n = 4$; RAG-1^{-/-}> -/-, $n = 5$. Data are pooled from two experiments.

transfer. In contrast, none of the CD24^{-/-}>CD24^{-/-} recipient chimeras were susceptible to pathogenic T cells. All CD24^{+/+}>CD24^{-/-} chimeras developed EAE with similar kinetics to that of the CD24^{+/+}>CD24^{+/+} mice. These results demonstrate that CD24^{+/+} bone marrow-derived cells corrected the local antigen presentation defects in the CD24^{-/-} mice. Interestingly, all CD24^{-/-}>CD24^{+/+} chimeras also developed EAE. Although the onset was delayed by ~4 d in this experiment, the peak EAE scores were equal to those observed in the CD24^{+/+}>CD24^{+/+} and CD24^{+/+}>CD24^{-/-} chimera mice. Such difference was, however, not always observed in subsequent experiments (unpublished data).

To rule out the possibility that the susceptibility of the CD24^{-/-}>CD24^{+/+} mice to EAE was due to residual bone marrow-derived CD24^{+/+} cells, we irradiated CD24^{-/-}>CD24^{+/+} chimera mice and reconstituted them with CD24^{-/-} bone marrow again. We then tested whether these mice were sensitive to EAE induction by WT MOG-specific T cell transfer. As shown in Fig. 9 C, while CD24^{-/-}>CD24^{-/-} mice were resistant to EAE induction, CD24^{-/-}>CD24^{+/+} mice developed EAE with similar kinetics to those of CD24^{+/+}>CD24^{-/-} mice. However, both groups had a lower EAE score than the CD24^{+/+}>CD24^{+/+} mice did. It is therefore likely

that although WT bone marrow can convey EAE susceptibility to CD24^{-/-} mice, replacement with CD24^{-/-} bone marrow in WT mice does not abolish the EAE susceptibility of the CD24^{+/+} mice. Thus, either hematopoietic or nonhematopoietic CD24-expressing cells can confer susceptibility to pathogenic T cells in the CNS. However, it is likely that the two cell types can act synergistically, as revealed by the partial efficacy of a single cell type in recipients after two rounds of bone marrow replacement.

B cells constitutively express high levels of CD24. Moreover, a significant number of B cells were recruited into the CNS during EAE development and regulated disease severity and recovery (29, 30). Because activated B cells delivered CD24-mediated costimulation (11), we investigated whether B cells are the bone marrow-derived CD24^{+/+} APCs that convey EAE susceptibility. As shown in Fig. 9 D, CD24^{+/+}RAG-1^{-/-}>CD24^{-/-} mice were fully susceptible to EAE induction. Thus, CD24 expression on B cells is not required for conveying EAE susceptibility to CD24^{-/-} mice.

Discussion

We have previously demonstrated that CD24-deficient hosts resist the pathogenic T cells adoptively transferred from WT mice. Because resistance to the adoptively trans-

ferred T cells was observed when the hosts were immune suppressed by irradiation before adoptive transfer (9), it is likely that the resistance is not due to host immunity to adoptively transferred T cells. To avoid complications associated with homeostatic proliferation in an irradiated host, we reproduced the above observation in nonirradiated recipients and followed the recruitment and local activation of autoreactive T cells in the CNS during EAE. The data presented here make three points.

CD24 Expression in the Host Is Required for T Cell Persistence in, But Not Recruitment to, the CNS. Our previous studies have demonstrated that expression of CD24 on host cells is critically important for the development of EAE (9). Because CD24 was not required for the priming of autoreactive T cells (9), and because CD24 was reported to modulate VLA4 interaction with VCAM-1 (16), which is known to be required for T cell recruitment (17), we tested whether CD24 is critical for the recruitment of T cells to the CNS by immunohistochemistry, RPA, and flow cytometry.

At 3 d after adoptive transfer, a similar number of Thy1.1⁺ donor cells were detected in the CNS of both CD24^{+/+} and CD24^{-/-} recipients. On day 12, the number of donor T cells in the CNS was substantially less in CD24^{-/-} mice in comparison to WT mice. However, the number of CD3⁺ T cells recruited to the CNS was comparable in the two groups, as revealed by quantitative RPA, largely due to recruitment of host T cells. These results demonstrate that expression of CD24 in the CNS is not required for T cell migration into the CNS, regardless of their origin.

Despite the lack of a significant difference in T cell recruitment, we observed major differences in the persistence of autoreactive T cells in the CNS. By day 12, the number of donor T cells was reduced by 10-fold in the CD24^{-/-} brain in comparison to WT recipients. The reduction of donor autoreactive T cells was followed by a disappearance of bystander host T cells, such that no T cells were detected in the CD24^{-/-} mouse brain at a time when the WT brain had seen a significant increase in total T cells. Thus, CD24 expression is required for the persistence of both antigen-specific donor cells and host T cells with unknown specificity.

A potential caveat associated with the adoptive transfer experiment is that the CD24 may serve as a rejection antigen when the WT T cells are injected into the CD24^{-/-} host. We performed two types of experiments to rule out this possibility. First, we obtained serum from CD24-deficient hosts at 3 wk after they received WT T cells, and then tested for the presence of anti-CD24 antibodies. No such antibodies could be detected in the serum (unpublished data). Second, at 3 wk after adoptive transfer, we reinjected carboxyfluorescein succinimidyl ester-labeled, MOG-reactive WT T cells into the WT and CD24^{-/-} recipient that had been "primed" by the previous round of adoptive transfer and determined the amounts of donor cells in the two types of recipients. The analysis indicated that CD24^{-/-} recipients did not preferentially eliminate the donor T cells (unpublished data). Both approaches suggest

that it is highly unlikely that the failure of WT T cells to persist in the CD24^{-/-} CNS is due to host immunity specific for CD24.

The Rate of Local Expansion of Autoreactive T Cells in the CNS Is Controlled by CD24. Theoretically, the differential persistence of T cells in the WT and CD24^{-/-} hosts can be attributed to two different factors. The first is the local expansion of T cells and the second is their programmed cell death. It is well established that the T cells in the CNS are prone to programmed cell death (4–7). However, it is unknown whether T cells clonally expand in the CNS and if so, whether CD24 plays a role in this process.

Using a short-term BrdU labeling in vivo, we were able to clearly demonstrate substantial T cell division in the CNS of WT mice. Because the antigen-specific T cells migrated into the CNS en masse shortly after adoptive transfer (1), and more importantly, because the rate of T cell division was substantially higher in the CNS than in the spleen, it is likely that the majority of BrdU incorporation in the CD24^{+/+} CNS took place locally. In contrast, even though the donor T cells divided at a slightly higher rate in the spleen of CD24^{-/-} recipients than the WT counterpart on day 3, the rate of division in the CD24^{-/-} CNS was less than that in the WT CNS. The reversion of the relative BrdU incorporation rates between the spleen and the CNS of the two strains of mice reveals two interesting points. First, the BrdU incorporation in the CNS reflects local T cell proliferation, and second, CD24 plays a critical role in the process. Consequently, the number of donor T cells was ~10-fold higher in the WT CNS on day 12 when the total numbers of T cells in CNS were comparable between WT and CD24^{-/-} mice.

Our study extends the elegant work by Flugel et al. (1), who reported de novo expression of activation markers on autoreactive T cells after migration into the CNS. It is of interest to note that these authors also showed, in an adoptive transfer model of EAE, that T cell migration into the CNS is complete within 3 d of adoptive transfer. Given the rapid T cell death seen in WT CNS (4–7), it is highly likely that a more rapid T cell clonal expansion is required for a sustained immune response. Our study indicated that by controlling the efficacy of this process, CD24 expression on host cells constitutes a major checkpoint of EAE pathogenesis.

Two Lineages of CD24-expressing APCs in CNS. Recognition of CNS autoantigen is considered a prerequisite for the initiation of T cell-mediated CNS inflammation (2, 3). Recent evidence suggests that local antigen processing and presentation in the CNS is essential for EAE development because mice deficient for Ii and H-2M failed to develop EAE (31). Because our work established the importance of local T cell clonal expansion, it also raised the possibility that T cell costimulation might be required for the process.

Our comparison of T cell proliferation in CD24^{+/+} and CD24^{-/-} recipients demonstrates that CD24 is required for optimal local T cell clonal expansion in the CNS. In support of this hypothesis, we also demonstrated that activation of T cells by astrocytes and microglia in vitro depends on

CD24, as CD24^{+/-} APCs are substantially more efficient in inducing T cell proliferation, and that proliferation of CD24^{+/+} astrocytes are blocked by an anti-CD24 antibody.

Our bone marrow chimera experiments revealed that expression of CD24 on one of the two lineages of APCs is sufficient for EAE development. One of the two is bone marrow derived. Expression of CD24 on B cells was unnecessary, as bone marrow from RAG-1-deficient mice was equally competent as WT bone marrow. Although no genetic model is available to define other cell lineages as the potential local APC, our in vitro analysis demonstrated that the ability of microglia to present antigen to T cells is diminished if the CD24 gene is defective. Because perivascular microglia, although not parenchyma microglia, are mainly bone marrow derived (32, 33), it is possible that this lineage of the microglia is the radiosensitive bone marrow-derived APC.

Interestingly, we also found that CD24 expression on radioresistant cells in the host was sufficient to convey EAE susceptibility. This is not due to incomplete depletion of radiosensitive cells, as the same conclusion was reached after two rounds of irradiation. The identity of the radioresistant cells is unknown. However, at least two candidates can be proposed from our in vitro data. The first is the parenchyma microglia that have been shown to be radioresistant (34, 35), and the other is astrocytes. Both cell types constitutively express CD24, which can be further elevated by inflammatory cytokines such as IFN- γ . Moreover, both cell types require the CD24 gene for optimal induction of T cell proliferation in vitro.

In summary, we have shown that CD24 is a novel checkpoint for local clonal expansion of T cells in the CNS. Because there is no difference in apoptosis of donor T cells, the defective proliferation in the CD24^{-/-} CNS is likely the primary cause for the drastic reduction of donor T cells at the time when clinical signs of EAE were observed. The lack of effect on T cell apoptosis indicated that the function of CD24 differs from B7-1 and B7-2 (36). However, it should be pointed out that this study has not definitively proven that defective proliferation alone prevented the induction of EAE in the adoptive transfer model. CD24 may mediate other functions essential for EAE development. For instance, the transferred autoreactive T cells reside primarily in the meninges in CD24^{-/-} mice, whereas many of them migrate into the brain parenchyma in WT mice. The significance of this observation is unknown at this point, nor is it clear if this is a direct consequence of defective T cell proliferation. Nevertheless, given the high death rate of T cells in the CNS, it is highly likely that a gene that controls the rate of proliferation of autoreactive T cells in the CNS may play an essential role for pathogenesis. This checkpoint might be particularly relevant for immunotherapy of MS for two reasons. First, this checkpoint controls the function of T cells after they are primed and have migrated to the CNS. This resembles a clinical setting, as patients who seek care already have autoreactive T cells in the lymphoid organ and/or in the CNS. Second, the significance of CD24 in MS is demon-

strated by our recent observation that CD24 polymorphism in the human is a genetic modifier for the risk and progression of MS (10).

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