CD24 on the Resident Cells of the Central Nervous System Enhances Experimental Autoimmune Encephalomyelitis

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CD24 on the Resident Cells of the Central Nervous System Enhances Experimental Autoimmune Encephalomyelitis

Jin-Qing Liu,* Joseph W. Carl, Jr.*, Pramod S. Joshi,* Abhik RayChaudhury,* Xin-An Pu,† Fu-Dong Shi,‡ and Xue-Feng Bai2*

CD24 is a cell surface glycoprotein that is expressed on both immune cells and cells of the CNS. We have previously shown that CD24 is required for the induction of experimental autoimmune encephalomyelitis (EAE), an experimental model for the human disease multiple sclerosis (MS). The development of EAE requires CD24 expression on both T cells and non-T host cells in the CNS. To understand the role of CD24 on the resident cells in the CNS during EAE development, we created CD24 bone marrow chimera and transgenic mice in which CD24 expression was under the control of a glial fibrillary acidic protein promotor (AstroCD24TG mice). We showed that mice lacking CD24 expression on the CNS resident cells developed a mild form of EAE; in contrast, mice with overexpression of CD24 in the CNS developed severe EAE. Compared with nontransgenic mice, the CNS of AstroCD24TG mice had higher expression of cytokine genes such as IL-17 and demyelination-associated marker P8; the CNS of AstroCD24TG mice accumulated higher numbers of Th17 and total CD4+ T cells, whereas CD4+ T cells underwent more proliferation during EAE development. Expression of CD24 in CD24-deficient astrocytes also enhanced their costimulatory activity to myelin oligodendrocyte glycoprotein-specific, TCR-transgenic 2D2 T cells. Thus, CD24 on the resident cells in the CNS enhances EAE development via costimulation of encephalitogenic T cells. Because CD24 is increased drastically on resident cells in the CNS during EAE, our data have important implications for CD24-targeted therapy of MS. *The Journal of Immunology, 2007, 178: 6227–6235.

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perimental autoimmune encephalomyelitis (EAE)3 is an animal model for human disease multiple sclerosis (MS). In the development of EAE, myelin Ag-specific CD4 T cells must be activated, clonally expanded in the lymphoid organs, and must migrate into the CNS where they undergo reactivation (1–4). Activated T cells execute their effector functions by producing proinflammatory cytokines and triggering a cascade of events, including demyelination and axonal damage (5–7). Thus, molecular interactions involved in T cell activation, T cell homing to the CNS, and T cell reactivation and survival in the CNS can affect EAE development and progression.

CD24 is a cell surface glycoprotein with extensive carbohydrate structures attached to a small protein core arched to the membrane with a glycosylphosphatidylinositol tail (8, 9). CD24 is expressed on various cells other than on T and B lymphocytes (10, 11). It has been reported that CD24 expressed on APCs mediates CD28-independent costimulation of CD4 and CD8 T cells (11–16). Recently, CD24 on dendritic cells has been shown to control the rapid homeostatic proliferation of syngenic T cells in a lymphopenic model (17). In addition to immune cells, CD24 has also been implicated to play important roles in neuronal cells. In the developing mouse brain, CD24 is widely and transiently expressed on developing neurons (18) and glial cells (19). In the adult CNS, CD24 expression is restricted to immature neurons located in two regions showing ongoing neurogenesis: the subventricular zone of the lateral ventricle pathway and the dentate gyrus of the hippocampal formation. CD24 is also strongly expressed on ciliated ependymal cells (18). In adult mice, CD24 inhibits neurogenesis by inhibiting the proliferation of immature neurons (20). Moreover, CD24 has been shown to be an inhibitor of neurite outgrowth in an in vitro culture system (21); in that study, it is proposed that CD24 modulates neurite outgrowth via a heterophilic interaction with an unknown neuronal receptor. The expression of CD24 in the CNS under pathological conditions such as EAE is unclear. Our recent studies suggest that CD24 expression is increased greatly in EAE lesions and CNS local cells close to the EAE lesion (our unpublished observation). Our previous studies have revealed that CD24 is required for the pathogenesis of EAE (19, 22). The development of EAE requires CD24 expression on both T cells and non-T host cells. It is suggested that the CNS APCs provide a necessary CD24 signal to pathogenic T cells, which allows optimal proliferation and persistence of T cells in the CNS (19). We have shown bone marrow–derived APCs from wild-type (WT) mice are sufficient to render CD24-deficient mice susceptible to EAE; it remains to be determined what is the functional role of CD24 on the CNS local cells during EAE development.

To understand the role of CD24 expressed on resident CNS cells during EAE development, we generated CD24 bone marrow chimeras and transgenic mice in which CD24 was expressed in the CNS under the control of the glial fibrillary acidic protein (GFAP)
promoter (AstroCD24TG mice). Mice lacking CD24 expression in the CNS developed more mild EAE while mice with overexpressed CD24 in the CNS developed a more progressive disease than nontransgenic (NTG) littersmates. The enhanced EAE progression in AstroCD24TG mice was associated with higher expression of cytokine genes such as IL-17 and demyelination-associated marker P8. However, CD24 does not seem to be involved in remyelination or neurorepair as genes associated with these processes remain largely unchanged. Because CD24 expression is increased drastically on resident cells in the CNS during EAE, our data have important implications for CD24-targeted therapy of MS.

Materials and Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute. 2D2 TCR-transgenic mice (23) were provided by Dr. V. K. Kuchroo (Harvard Medical School, Boston, MA). Mice with CD24 overexpression in the CNS (designated as AstroCD24TG mice) were generated as we described below. CD24-deficient mice in the C57BL6 background have been described previously (19, 22). All mice were maintained in a specific pathogen-free animal facilities of Ohio State University. The animal facilities are fully accredited by American Association for Accreditation of Laboratory Care.

Production of AstroCD24TG mice

The transgenic construct used for microinjection of C57BL/6 oocytes was generated based on the transgenic construct pGfa2-C.lac (24) (provided by Dr. M. Brenner, University of Alabama, Birmingham, AL). The construct contains a 2.2-kb DNA fragment of the human GFAP promoter sequence (24 –26), a fragment of the mouse protamine-1 gene that supplies an intron, and a fragment of the mouse CD24 gene (1.8 kb) to replace the -untranslated region (UTR) and a polyadenylation signal. We used the full-length mouse CD24 cDNA (1.8 kb) to replace the LacZ gene in the original vector. After excising the vector backbone, the full-length DNA fragment used for microinjection was 4.6 kb. Oocytes were injected using conventional microinjection technology by the core facilities of Ohio State University.

Immunofluorescence and confocal laser scanning microscopy

The Abs used for immunofluorescence staining of spinal cord sections were the following: biotinylated CD24 (M1/69; BD Pharmingen); Alexa-633-streptavidin ( Molecular Probes); FITC-anti-rat mouse CD24 (M1/69; BD- Pharmingen); Alexa-488-GFAP ( Molecular Probes); Alexa-488-IgM ( Molecular Probes); and biotinylated anti-CNPase (Chemicon International). Spinal cord sections were harvested and frozen in Tissue-Tek OCT medium (Sakura Finetek), and 10-μm-thick slices were cut. Tissue sections were fixed in 1% paraformaldehyde and were blocked with 10% normal mouse serum for 1 h at 24°C. Sections were then stained with the corresponding fluorescent Abs for another 2 h at 24°C. After washing with PBS, slides were mounted with Fluoromount G mounting medium (Southern Biotechnology Associates) and were examined on a confocal laser-scanning microscope (Zeiss 510).

Induction and assessment of EAE

The immunogen, myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (MEGVYWRSPSSRVHVLNYRGK), was purchased from Genemed Synthesis. Mice of 8–12 wk of age were immunized s.c. with 200 μg of MOG peptide in CFA (containing 5 mg of Mycobacterium tuberculosis H37Ra) in a total volume of 100 μl. They received 200 μg of pertussis toxin (List Biological Laboratories) in 200 μl of PBS in the tail vein immediately after the immunization and again 48 h later. The mice were observed every day and were scored on a scale of 0–5 with gradations of 0.5 for intermediate states: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, moribund; and 5, death.

Generation of bone marrow chimeras and adoptive transfer EAE

We prepared bone marrow cells by flushing donor mice femur and tibia bone with PBS. Recipient mice were lethally irradiated (1000 rad) and reconstituted with 10 × 10⁶ bone marrow cells by i.v. injection. Engraftment took place over a 6–8 wk period. We have generated two types of bone marrow chimeras. Chimera 1 is WT>CD24 mouse. In these mice, bone marrow cells from C57BL6 mice were injected into irradiated CD24 mice. Chimera 2 is WT>WT mice. In these mice, bone marrow cells from C57BL6 mice were injected into irradiated C57BL6 mice. We used an adoptive transfer method to induce EAE in the bone marrow chimeric mice. Briefly, 8- to 12-wk-old C57BL6 mice were immunized s.c. with 100 μg of MOG peptide in CFA in a total volume of 100 μl. Ten days after immunization, draining lymph nodes were harvested and stimulated at a density of 2 × 10⁷/ml in Click’s Eagle’s Hanks’ amino acid medium supplemented with 15% FCS, 20 ng/ml IL-12, and 50 μg/ml MOG peptide for 4 days. MOG-activated lymphoid cells measuring 30 × 10⁴ were injected i.p. into each recipient mouse that had been irradiated (350 rad) 1 h earlier. The mice were observed every day and scored as we described above.

Histology

Mice were sacrificed by inhaling CO₂. Spinal cords were removed by in- sufflation and fixed in 10% formalin/PBS. Paraffin sections were prepared and stained by the histology core facilities of Department of Pathology (Ohio State University) for H&E, Luxol fast blue (myelin staining), and Bielchowsky silver stainings (axon staining). To calculate the extent of spinal cord pathology in mice, an interactive digital analysis system and camera lucida attached to a photomicroscope (Zeiss) were used. The percentages of spinal cord inflammation, demyelination, and axonal damage per mouse were calculated by first determining the total white matter area for each spinal cord section, manually tracing a region of interest (ROI) of spinal cord inflammation, demyelination, and axonal damage were determined by manually tracing each of the sections. Pathological changes of each spinal cord was scored as follows: 0, no changes; 1, focal area involvement; 2, <5% of total myelin area involvement; 3, 5–10% of total myelin area involvement; 4, 10–20% of total myelin area involvement; and 5, >20% of total myelin area involvement.

Isolation of total RNA from spinal cords and real-time RT-PCR

Total RNA was isolated from each spinal cord by using the TRIzol method (Invitrogen Life Technologies). The first-strand cDNA of each sample was synthesized using a reverse transcription kit (Invitrogen Life Technologies). Quantitative real-time PCR was performed using an ABI Prism 7900HT sequence system (Applied Biosystems) with the QuantiTect SYBR Green PCR kit (Qiagen) in accordance with the manufacturer’s instructions. The primers used were: IL4.F, 5’-TGG AGC CAT ATC CA-3’, and IL4.R, 5’-TTC TTC GTT GCT GTT AGG AC-3’; IL10.F, 5’-AGC TGT CTC CCT TGT TT-3’; IL10.R, 5’-CAG CTG CTC TTT TGT AA-3’; IL12b.F, 5’-TGG CTG CTG TCT CCA CTC AT-3’; IL12b.R, 5’-GAGG AGT CCA GTC CAC CTC TA-3’; IL17F, 5’-CCT CCA GAA TGT GAA GGT CA-3’; and IL17R, 5’-CTA TCA GGG TCT TTA TGG CG-3’. IL17A.F, 5’-TGG CTG CTG TCT CCA CTC AT-3’; and IL17A.R, 5’-GAGG AGT CCA GTC CAC CTC TA-3’. TNF.a.F, 5’-AGG AGT AGT CAA ATG GC-3’; TNF.a.R, 5’-CCT CAC TTG GTT GTC TGG TC-3’; and NGF.L, 5’-GAC GAT GGT GGA A-3’. Nerve growth factor (NGF) was amplified and served as a reference gene. PCR was performed using an optimal condition. One microliter of first-strand cDNA product was amplified with platinum Taq polymerase (Invitrogen Life Technologies) and gene-specific primer pairs. Each sample was assayed in triplicate and experiments were repeated twice. The relative amounts of mRNAs were calculated by plotting the threshold cycle (Ct) (cycle number), and average relative expression was determined by the comparative method (2^-ΔΔCt).

Preparation of mononuclear cells from the CNS, intracellular cytokine staining, and flow cytometry

At days 17–20 after EAE induction, mice were sacrificed and perfused with PBS through the left heart ventricle to eliminate contaminating blood cells in the CNS. The CNS mononuclear cells were then prepared, as we have described previously (19). Cells were stained for the appropriate cell surface markers as indicated in the text. For intracellular cytokine staining, the CNS-permeating cells from mice were stimulated in vitro with PMA (50 ng/ml) and ionomycin (1 μM) for 5 h. Golgiplug (BD Pharmingen) were added (1/1500) during the last 2 h of incubation. The cells were stained for the cell surface marker CD4, followed by a standard intracellular cytokine staining for IFN-γ and IL-17. The following Abs were used: anti-CD4...
FITC (GK1.4; BD Pharmingen), anti-IFN-γ allophycocyanin (XMG1.2; BD Pharmingen), anti-IL-17 PE (TC11-18H10; BD Pharmingen), and rat IgG1 isotype control PE or allophycocyanin (BD Pharmingen). Cells were analyzed on a FACS Calibur flow cytometer, and respective isotype controls were obtained from BD Pharmingen. Cells were analyzed on a FACS Calibur flow cytometer.

**BrDU incorporation assay**

At days 19 after EAE induction (immunization), mice were given an i.p. injection of 1 mg of BrdU (BD Pharmingen). Brains and spinal cords of recipient mice were harvested 12 h later. The CNS-infiltrating mononuclear cells were isolated and stained for cell surface markers such as CD4, followed by intracellularly stained with anti-BrdU Ab (BD Pharmingen). Flow cytometry was used to analyze BrdU incorporation into the CNS-infiltrating cells.

**Purification of CD4+ T cells from 2D2 TCR-transgenic mice**

CD4+ T cells were purified by negative selection. Briefly, spleen and lymph node cells from 2D2 TCR-transgenic mice were incubated with a mixture of mAbs (anti-CD8 mAb TIB210, anti-FcR mAb 2.4G2, and anti-CD11c mAb N418). After removing the unbound Abs, the cells were incubated with anti-Ig-coated magnetic beads (Dynal Biotech). A magnet removed the Ab-coated cells. The remaining cells were CD4+ T cells, typically with a purity of >90%. The purified CD4+ T cells were used for the proliferation assays.

**Preparation of astrocytes from newborn mice and proliferation assay**

Primary glial cell cultures were prepared from brains of newborn AstroCD24TG/CD24 or CD24−/− mice as described previously (19, 27, 28). After removal of the meninges, the brains were dissociated mechanically by nylon sieves. The cells were seeded in DMEM containing 20% FCS in 75-cm2 tissue culture flasks. On day 4, the medium was replaced with DMEM containing 10% FCS and subsequently changed every 3 days thereafter. The firmly adherent cells were stained with anti-CD45 Ab to confirm their identity as astrocytes (24). To assess the Ag-presenting functions of cultured astrocytes, the purified MOG-specific 2D T cells were used as a responder. In brief, irradiated (3000 rad) astrocytes were cultured in round-bottom microtiter plates in 200 μl of DMEM containing 100 U/ml IFN-γ. Three days later, the medium was removed, and 5 × 104 T cells in Click’s Eagle’s Hanks’ amino acid medium and the indicated concentrations of MOG peptide were added into each well. After 48 h, the cultures were pulsed with 1 μCi/well [3H]thymidine (ICN Pharmaceuticals) for another 12 h, and incorporation of [3H]thymidine was measured in a liquid scintillation beta plate counter.

**Results**

**CD24 on the local cells in the CNS contributes to EAE development**

To understand the role of CD24 on the CNS resident cells in EAE development and progression, we created two groups of bone marrow chimeras. For group 1, we used bone marrow cells from WT C57BL/6 mice to replace bone marrow of WT C57BL/6 mice (WT>WT). In this group of mice, both bone marrow-derived cells and the CNS local cells expressed CD24. For group 2, we used WT bone marrow cells to replace the bone marrow of CD24−/− mice (WT>−/−). In these mice, bone marrow-derived cells expressed CD24, whereas cells in the CNS parenchyma were CD24 deficient. Our previous studies have shown that CD24−/− mice are resistant to T cell adoptive transfer-induced EAE whereas WT>−/− chimeras become sensitive (19). As shown in Fig. 1, after adoptive transfer of encephalitogenic T cells, both groups of mice developed EAE at around day 10 after T cell transfer. WT>WT chimeras developed more severe disease than WT>−/− chimeras. The difference was reflected by the increased mean accumulating score and mean maximal score in WT>WT chimeras compared with WT>−/− chimeras (p < 0.05, Table I). The increased severity suggests that CD24 on the CNS parenchymal cells contributes to EAE development.

**Overexpression of CD24 in the CNS enhances EAE progression**

To understand how CD24 on the CNS cells contributes to EAE development, we have produced CD24-transgenic mice in which CD24 transgene expression is under the control of the GFAP promoter (AstroCD24TG mice). The transgenic construct used for microinjection of C57BL/6 oocytes was generated based on a transgenic construct pGfa2-cLac (24) (provided by Dr. M. Brenner). The construct contains a 2.2-kb DNA fragment of human GFAP promoter sequence (24–26), a fragment of the mouse protamine-1 gene that supplies an intron to stabilize 3′-UTR and a polyadenylation signal. As shown in Fig. 2, we used the full-length mouse CD24 cDNA (1.8 kb) to replace the LacZ gene in the original vector. After excising the vector backbone, the full-length DNA fragment used for microinjection was 4.6 kb (Fig. 2, a and b). Fertilized eggs were injected using conventional microinjection technology by the core facilities of The Ohio State University. As shown in Fig. 2c, we obtained a strain of mice with abundant expression of CD24 in the CNS, whereas the CNS of NTG mice did not have an abundant expression of CD24. We also observed that CD24 expression colocalized with GFAP+ cells (astrocytes; Fig. 2c) but not with other CNS cells such as neurons (NeuN+), microglial cells (IB4+), and oligodendrocytes (CNPase+) (data not shown).

To understand the role of constitutively expressed CD24 in the CNS in EAE development, we induced EAE in AstroCD24TG mice and their NTG littersmates. Within 2 wk after immunization with MOG peptide and pertussis toxin, disease was initiated in both groups of mice. By days 17–25, the disease score reached peak. However, while paralysis gradually recovered to lower levels in the NTG mice after day 20, paralysis in AstroCD24TG mice maintained at high levels and persisted throughout the observation period (Fig. 3a). The mean accumulating scores and mean maximal scores were significantly different between these two groups (p <

**Table I. EAE induction in bone marrow chimeras**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Onset (day)</th>
<th>Mean Accumulating Score</th>
<th>Mean Maximal Score</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT&gt;WT</td>
<td>11.2 ± 1.3</td>
<td>51.4 ± 24.2</td>
<td>2.0 ± 0.5</td>
<td>6/8</td>
</tr>
<tr>
<td>WT&gt;−/−</td>
<td>11.7 ± 1.5</td>
<td>28.9 ± 21</td>
<td>1.44 ± 0.4</td>
<td>8/9</td>
</tr>
<tr>
<td>p Value</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>
Probes were used to label astrocytes. Spinal cord tissue sections (10 μm) were used to label CD24, and Alexa-488-GFAP (Molecular probes) were used to label CD24 (M1/6230 CD24 IN THE CNS ENHANCES EAE). Thus, AstroCD24TG mice suffered enhanced EAE progression. We examined histology of mice in both groups. At day 50 after EAE induction, inflammatory lesions could be detected in all spinal cord sections from AstroCD24TG mice, and in some cases, very diffused inflammatory infiltration, which crossed the gray matter of spinal cords, was detected (Fig. 3b, left panel). But in NTG mice, inflammatory lesions that were usually located in proximity to meninges (Fig. 3b, left panel) were less detectable or almost completely undetectable in the spinal cords. Large areas of demyelination could be detected in the spinal cords of AstroCD24TG mice (Fig. 3b, middle panel), whereas only focal or little demyelination was detected in the spinal cords of NTG mice (Fig. 3b, middle panel). Axonal staining of the spinal cord sections suggested that broad areas of axonal damage were present in AstroCD24TG mice (Fig. 3b, right panel) but not in NTG mice. NTG mice usually show axonal damage limited to the submeninges area (Fig. 3b, right panel). Quantitation of inflammation, demyelination, and axonal degeneration areas revealed significant differences between TG and NTG mice (Fig. 3c). Thus, AstroCD24TG mice suffered enhanced EAE progression with broad demyelination and axonal damage.

Enhanced EAE progression in AstroCD24TG mice is associated with increased expression of proinflammatory cytokines in the CNS

Enhanced EAE signs and myelin/axonal damage in AstroCD24TG mice may be a result of enhanced damaging factors during EAE development. Therefore, we compared expression of cytokine genes in the spinal cords of AstroCD24TG mice with NTG mice at day 20 (peak of EAE) and day 40 (EAE recovery) by using real-time PCR. On day 20, dramatic up-regulation of cytokine genes,
were calculated by plotting the Ct (cycle number), the average relative expression was determined by the comparative method (2^(-ΔΔCt)) compared with NTG mice. No significant differences were detected among other cytokine genes between TG and NTG mice. On day 40 after EAE induction, the majority of cytokine genes measured was dramatically lower compared with day 20 in both groups of mice; however, the expression of IL-12, IL-17, IFN-γ, and TNF-α was still significantly higher in the spinal cords of TG mice in comparison with NTG mice. Up-regulation of IL-10 and IL-4 genes were also observed in both groups of mice on day 40, but no significant differences were observed between TG and NTG mice.

The most recent research in EAE pathogenesis has revealed that IL-17-producing T cells, but not IFN-γ-producing T cells, are linked with pathogenicity in the CNS (29, 30). Moreover, the development of Th17 cells is negatively regulated by Th1 cells (30, 31). Therefore, we directly quantitated Th1 vs Th17 cells in the CNS of AstroCD24TG mice and controls. As shown in Fig. 5, more Th17 cells were detected in the CNS of TG mice than in controls on day 20. However, the overall difference in Th1 cells was not different between the two groups (Fig. 5b). Thus, the intracellular cytokine-staining data correlated with real-time PCR data and suggested that overexpression of CD24 in the CNS increased Th17 but not Th1 responses. Interestingly, we also detected similar numbers of IFN-γ/IL-17 double-positive cells in the spinal cords of both groups of mice. IFN-γ/IL-17 double-positive cells has been detected by other groups in the spinal cords of EAEC mice (32, 33); the significance of these cells remains unclear.

### Overexpression of CD24 enhances local T cell proliferation in the CNS

To understand whether overexpression of CD24 in the CNS enhances EAE via enhancing T cell proliferation, we first quantitated total CD4+ T cell numbers in the CNS of TG mice vs control mice. As shown in Fig. 6a, we detected significantly increased numbers of IFN-γ/IL-17 double-positive cells in the spinal cords of both groups of mice. IFN-γ/IL-17 double-positive cells has been detected by other groups in the spinal cords of EAEC mice (32, 33); the significance of these cells remains unclear.

#### Table II. EAE induction in AstroCD24TG mice

<table>
<thead>
<tr>
<th></th>
<th>Mean Onset (day)</th>
<th>Mean Accumulating Score</th>
<th>Mean Maximal Score</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG</td>
<td>11.4 ± 1.8</td>
<td>25.4 ± 11.7</td>
<td>3.68 ± 1.1</td>
<td>7/8</td>
</tr>
<tr>
<td>TG</td>
<td>11.7 ± 1.5</td>
<td>40.1 ± 14.3</td>
<td>3.8 ± 0.7</td>
<td>9/9</td>
</tr>
<tr>
<td>p Value</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

#### FIGURE 4. Assessment of cytokine gene expression in the spinal cords of AstroCD24TG mice and controls by real-time PCR. We first induced EAE in AstroCD24TG mice and their NTG littermates by active immunization. On days 20 (peak of EAE) and 40 (recovery of EAE), we sacrificed mice, and total RNA was prepared from diseased mice and naive C57BL/6 mice. Real-time PCR was used to quantify cytokine gene expression. The HPRT gene was amplified and served as endogenous control. Each sample was assayed in triplicate, and experiments were repeated twice. The relative amounts of mRNA were calculated by plotting the Ct (cycle number), the average relative expression was determined by the comparative method (2^(-ΔΔCt)), and cytokine gene expression in naive spinal cord was set as 1. Three mice per group were used and data were expressed as mean ± SD.

#### FIGURE 5. Th1 and Th17 cells in the spinal cords of AstroCD24TG mice and controls under EAE conditions. EAE was induced in AstroCD24TG and control mice by active immunization. On day 20 after immunization, we sacrificed mice and perfused mice with PBS (pH 7.4), and then we isolated the CNS-infiltrating cells from the brain and spinal cord of each mouse. Cells were incubated with PMA and ionomycin for 5 h. During the last 2 h of culture, GolgiStop (BD Pharmingen) was added into the cell culture. Cells were first stained for CD4 followed by intracellular cytokine staining. We performed intracellular staining for cytokines by using a kit from BD Pharmingen. The following Abs were used for the staining: anti-CD4 FITC (GK1.4; BD Pharmingen), anti-IFN-γ allophycocyanin (XMG1.2; BD Pharmingen), anti-IL-17 PE (TC11-18H10; BD Pharmingen), and rat IgG1 isotype control PE or allophycocyanin (BD Pharmingen). Cells were analyzed on a FACSCalibur flow cytometer. a, Example of intracellular cytokine staining. b, Quantitation of Th1, Th17, and Th1/Th17 cells among total CD4 cells. Student’s t test was used for the statistical analysis. Four mice per group were quantitated. Data are representative of at least three experiments with similar results.
of CD4+ T cells in the CNS of TG mice in comparison with NTG mice; moreover, CD4+ T cells in the CNS of TG mice underwent more significant proliferation, as revealed by a short-term BrdU incorporation assay (Fig. 6, b and c). To determine whether the CNS-resident APC are more efficient in stimulating encephalitogenic T cells, we cultured astrocytes from CD24-deficient mice with the CD24 transgene (TG+/CD24−/−) and without CD24 transgene (TG+/CD24+/−). The cultured astrocytes from TG+/CD24−/− and TG+/CD24+/− mice expressed similar levels of MHC class II (MHC II) and CD80, but the CD40 expression was only marginal in both groups (Fig. 7a) upon activation with IFN-γ. We used cultured astrocytes as APCs to stimulate purified 2D2 TCR-transgenic T cells (specific for MOG 35–55 (23)). Astrocytes with CD24 transgene were more efficient in stimulating myelin Ag-specific T cells than astrocytes that were deficient for CD24 (Fig. 7b). Moreover, CD24-specific Ab 20C9 (19, 34) specifically blocked the stimulatory effects of TG+/CD24−/− astrocytes (Fig. 7c).

FIGURE 7. Transgenic expression of CD24 in CD24-deficient astrocytes promotes their costimulatory activity to MOG-specific T cells. We have bred AstroCD24TG mice with CD24−/− mice and generated mice with CD24 expression only on astrocytes (AstroCD24TG/CD24−/−). Astrocytes were generated from newborn (day 2) mice with TG+/CD24−/− or TG+/CD24+/− genotypes. a, Cell surface staining for CD24, I-Aβ, CD40, and CD80. The cultured astrocytes were stimulated with recombinant murine IFN-γ (100 U/ml; PeproTech) for 72 h, and cells were then stained for cell surface markers and were analyzed on a FACS Calibur flow cytometer. b, Astrocytes from TG+/CD24−/− and TG+/CD24+/− mice were tested for their ability to stimulate MOG-specific 2D2 T cells to proliferate in the presence of MOG peptide ([3H]thymidine incorporation assay). Data shown were after 5 days coculture and represents four experiments with similar results. c, Anti-CD24 Ab 20C9 blocked the stimulatory effects of TG+/CD24−/− astrocytes. 20C9 Ab or control IgG (10 μg/ml) was used in the culture.

FIGURE 6. Overexpression of CD24 in the CNS enhances CD4+ T cell proliferation in the CNS. a, Quantitation of total CD4+ T cells in the CNS of mice with EAE. EAE was induced in AstroCD24TG and control mice by active immunization. On day 20 after immunization, we sacrificed mice and perfused mice with PBS (pH 7.4). We then isolated the CNS-infiltrating cells from the brain and spinal cord of each mouse. Cells were stained for CD4 marker, and total CD4+ T cells were quantitated by flow cytometry. Data were expressed as mean ± SD, with five mice per group being used. *, p < 0.05. Student’s t test was used. b, Analysis of BrdU incorporation in the CNS infiltrating cells. On day 19, we injected 1 mg of BrdU (BD Pharmingen) i.p. into each mouse. Twelve hours later, we sacrificed mice and isolated CNS-infiltrating cells from each mouse. We performed BrdU staining by using a kit from BD Pharmingen. c, Quantitation of BrdU incorporation in CD4+ T cells. Data were expressed as mean ± SD. n = 5/group. *, p < 0.05. Student’s t test was used.

Neurogenesis and remyelination levels are similar in CD24 transgenic vs NTG mice during the course of EAE

We wanted to determine whether the persistent demyelination in AstroCD24TG mice was attributed to persistent active demyelination. The stress-associated gene P8 is a newly identified marker that is associated with active demyelination (35). We used real-time PCR to quantitate P8 gene expression in the spinal cords of AstroCD24TG mice and NTG mice on day 20 (peak of disease) and day 40 (EAE recovery) after immunization. Increased P8 gene expression was detected in both groups of mice compared with normal spinal cords. However, the overall expression dramatically reduced in the recovery phase (day 40) compared with the acute phase (day 20) (Fig. 8). P8 gene expression was significantly higher in the spinal cords of AstroCD24TG mice compared with that of NTG mice at both time points. These data suggested that the demyelinating activity is more active in AstroCD24TG mice than in NTG mice.
Because persistent demyelination in AstroCD24TG mice with EAE was observed, it is possible that overexpressed CD24 in the CNS inhibits remyelination. CD24 has been shown to inhibit proliferation of immature neuron in vivo (20) and neurite extension in vitro (21); it is conceivable to hypothesize that overexpressed CD24 may also inhibit neural repair in inflammatory-demyelinating disease. Therefore, we tested whether defective remyelination and neurogenesis were operative in AstroCD24TG mice during EAE. We observed significantly down-regulated expression of the IFN-γ gene in the spinal cords of TG mice unpaired with naive C57BL/6 mice. Real-time PCR was used to quantify P8, NG2, and DCX gene expression. The HPRT gene was amplified, and it served as an endogenous control. Each sample was assayed in triplicate, and experiments were repeated twice. Three mice per group per time point were used, and data were expressed as mean ± SD. **p < 0.01; *p < 0.05, Student's t test was used.

Discussion

In this study, we have evaluated the role of CD24 on the CNS cells in EAE development. Our bone marrow chimera experiments showed that, if CD24 was missing from the CNS cells, EAE scores were lower. In contrast, if CD24 was overexpressed in the CNS cells, EAE development was enhanced. Taken together, these data suggest an enhancing role of CD24 on CNS cells in EAE development.

Previous studies have suggested that CD24 provides a CD28-independent costimulatory signal to T cells (11–16). Recently, we have shown that CD24 expression in the CNS is necessary for myelin Ag-specific T cells to persist in the CNS (19). Because mice with overexpressed CD24 in the CNS had enhanced EAE progression, we have tested the hypothesis that abundant CD24 in the CNS costimulates myelin Ag-specific T cells and promotes their effector function. Several lines of evidence support this hypothesis. First, the enhanced EAE progression was associated with higher expression of cytokine genes such as IL-17 and demyelination-associated marker P8 in the spinal cords of AstroCD24TG mice compared with NTG mice. Second, the CNS of AstroCD24TG mice accumulated higher numbers of total CD4+ T cells and Th17 cells compared with NTG mice. Moreover, BrdU incorporation assay revealed that CD4+ T cells from the CNS of TG mice underwent more proliferation compared with that of NTG mice. Third, we showed that transgenic expression of CD24 in CD24-deficient astrocytes significantly enhanced their costimulatory activity to MOG-specific, TCR-transgenic 2D2 T cells.

Th17 cells, but not Th1 cells, have been shown recently to be correlated with disease pathogenicity for T cells (29, 43). In this study, we showed that, in the spinal cords of mice with more severe disease (AstroCD24TG mice), IL-17 gene expression and number of Th17 cells were significantly higher than in control mice that had lower EAE scores. At this stage, it is not clear whether CD24 costimulation directly causes CD4+ T cells to differentiate into Th17 cells in the CNS. However, it is clear that, in the peripheral lymphoid organs, the generation of Th17 cells does not require CD24 (our unpublished data). Further studies are required to clarify this issue. Our data also suggested that impaired Th2 cytokine production (IL-4 and IL-10) was not responsible for the differential recovery of EAE in TG and NTG mice.

We observed significantly down-regulated expression of the IFN-γ gene in the spinal cords of TG mice than in control mice at the peak of disease. However, absolute numbers of total Th1 cells in the CNS were similar at the peak of disease in the spinal cords of AstroCD24TG mice compared with NTG mice. We consider several possibilities that may cause this discrepancy. First, we quantitated infiltrating cells from the whole CNS (brain plus spinal cord) of each mouse, while we analyzed cytokine gene expression only in spinal cords because cells attack different areas of the CNS via different mechanisms (44), which can potentially cause the difference. Second, it remains possible that on the per cell basis, Th1 cells from NTG mice had higher IFN-γ gene expression compared with Th1 cells from TG mice. It remains unclear why Th1 responses were down-regulated in the spinal cords of AstroCD24TG mice. It is possible that Th17 cells in the spinal cords directly inhibit Th1 cell differentiation as Th1 cells do to Th17 cells (31). Alternatively, the microenvironment in the spinal cords of AstroCD24TG mice may favor differentiation of Th17 cells but inhibit Th1 differentiation. Finally, both lineages of cells may compete with each other for differentiation from the same limited pool of precursors.
Because AstroCD24TG mice show persistent demyelination during EAE, it is possible that overexpressed CD24 in the CNS inhibited remyelination. However, using real-time PCR to quantitate NG2, which is a specific marker for OPCs (36–39), in spinal cords of TG and NTG mice, it revealed no significant difference between these two groups. Therefore, overexpressed CD24 did not inhibit remyelination in AstroCD24TG mice during EAE recovery. Recent studies have suggested that increased neurogenesis may also contribute to EAE recovery (45–48). CD24 has been shown to inhibit immature neuronal cells to proliferate (20). Moreover, CD24 expressed on other cells may interact with its unknown receptor on immature neurons and inhibits neurite extension (21). Therefore, it is conceivable to hypothesize that CD24 may inhibit neural repair in inflammatory-demyelinating disease. However, our direct evaluation of DCX, which is a gene that has been shown to be correlated with neurogenesis, suggested that the neurogenesis level during EAE was very limited; moreover, no difference was observed between AstroCD24TG mice and NTG mice. Therefore, the enhanced EAE progression in AstroCD24TG mice was not caused by inhibition of neurorepair by CD24. Taken together, our data suggest that CD24 on CNS-resident cells enhance EAE development by costimulating encephalilitogenic T cells and making T cells more aggressive.

So far, the identity of the CNS resident cells that provide CD24 costimulation to T cells remains unclear. In the developing mouse brain, CD24 is widely and transiently expressed on developing neurons (18) and glial cells (19). In the adult CNS, CD24 expression is restricted to immature neurons located in two regions showing ongoing neurogenesis: the subventricular zone of the lateral ventricle pathway and the dentate gyrus of the hippocampal formation (18). CD24 is also strongly expressed on ciliated ependymal cells (18). Under EAE conditions, CD24 is strongly up-regulated in the CNS resident cells, including microglial cells, astrocytes, and neuronal cells (our unpublished data). However, because neurons do not express MHC II and costimulation requires an antigenic signal and a costimulatory signal come from the same cell (49), costimulatory contributions from MHC II− neurons should be negligible. In contrast, astrocytes and parenchymal microglial cells, which are MHC II inducible, have been shown to be potentially important APCs during EAE development (27, 50, 51) and, therefore, are likely to provide CD24 costimulatory signal to T cells. Our overexpression study suggests that astrocytes provide CD24 costimulation to pathogenic T cells.

Taken together, we have shown that CD24 on the CNS cells such as astrocytes promotes autoimmune inflammation in the CNS. The enhancement of EAE is likely mediated by costimulation of pathogenic T cells in the CNS. Because this process occurs in the effector phase of EAE, where autoreactive T cells have already migrated into the CNS, specific blocking of this process should provide a therapeutic option for EAE or MS.

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Disclosures

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References


