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Silencing IFN-γ Binding/Signaling in Astrocytes versus Microglia Leads to Opposite Effects on Central Nervous System Autoimmunity

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IFN-γ, the hallmark cytokine of Th1 cells, plays an important role in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. Thus far, the role of IFN-γ in EAE has been largely studied through its effects on immune cells, whereas much less is known about its effects on CNS cells, especially in vivo. In this study, we dissected the in vivo effects and mechanisms of IFN-γ binding/signaling in astrocytes and microglia, and found that IFN-γ signaling in these cell types has opposite effects in EAE pathogenesis. Silencing IFN-γ binding/signaling in astrocytes alleviated EAE, whereas in microglia, and likely in some infiltrating macrophages, it increased disease severity. Silencing IFN-γ binding/signaling in microglia resulted in diminished expression of chemokines and fewer inflammatory cells infiltrating into the CNS, whereas blocking IFN-γ binding/signaling in microglia, probably infiltrating macrophages as well, increased disease severity through augmented activation and proliferation of microglia. Further, blocking IFN-γ binding/signaling in astrocytes alleviated both Th1- and Th17-mediated adoptive EAE, indicating an important role for IFN-γ signaling in astrocytes in autoimmune CNS inflammation. Thus, our study defines novel mechanisms of action of IFN-γ in EAE pathogenesis, and also highlights an opportunity for development of multiple sclerosis therapies directed at CNS cells. The Journal of Immunology, 2015, 194: 4251–4264.

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are autoimmune diseases of the CNS (1, 2). It is thought that, in the initial stage of MS, myelin-reactive CD4+ T cells activated in the periphery infiltrate into the CNS, followed closely by a broad range of other immune cells, including T cells, macrophages, dendritic cells (DCs), B cells, and neutrophils (1, 2). Once in the CNS, myelin-reactive T cells become reactivated and produce proinflammatory mediators, which induce chemokine secretion by CNS resident cells, thus recruiting more inflammatory cells from the periphery and triggering an immunologic cascade that results in myelin damage (3). Although it appears that both IFN-γ–producing Th1 and IL-17–producing Th17 cells play important roles in EAE pathogenesis (4), the mechanisms by which autoreactive Th cells initiate the disease process remain only partially understood (5).

IFN-γ, a hallmark cytokine of Th1 cells, has traditionally been regarded as a proinflammatory factor (6). IFN-γ stimulates the differentiation of Th1 cells whereas inhibiting differentiation of Th2 cells (7); activates macrophages, cytotoxic T cells, and NK cells; and stimulates B cells to secrete IgG2a (6). Initially, it was believed that IFN-γ plays a pathogenic role in EAE, based on positive correlation between IFN-γ levels and EAE severity (8), and on the observation that altering signaling pathway in myelin-specific T cells, which results in decreased IFN-γ production, also decreases their encephalitogenicity (5, 9). However, IFN-γ– and IFN-γR–deficient mice, or wild-type (WT) mice treated with neutralizing anti–IFN-γ, developed more severe disease (10–12), demonstrating an overall anti-inflammatory role of IFN-γ in EAE.

More recent studies have shown that IFN-γ may suppress Th1 cell development by inhibiting IL-23R expression (13) and production of GM-CSF (14), a cytokine necessary for the encephalitogenicity of both Th1 and Th17 cells (15). IFN-γ treatment also converts CD4+ T cells to regulatory T cells with the capacity to suppress adoptively transferred EAE (16). Further, IFN-γ can limit the expansion of activated T cells by inducing their apoptosis (17). These findings provide the mechanistic basis for the protective effect of IFN-γ in EAE.

IFN-γR is expressed by most CNS cell types, including astrocytes, microglia, and oligodendrocytes (18–20). IFN-γ has primarily proinflammatory effects on CNS cells, for example, it

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Abbreviations used in this article: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; i.e.c.v., intracerebroventricular; MBP, myelin basic protein; MMP, matrix metalloproteinase; MNC, mononuclear cell; MS, multiple sclerosis; p.i., postimmunization; shRNA, short hairpin RNA; WT, wild-type.

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enables resident brain DCs to become effective APCs, which induce myelin-specific naïve CD4+ T cells to proliferate and secrete Th1/Th17 cytokines (21). IFN-γ promotes oligodendrocyte death, thus inducing demyelination (22, 23). The addition of IFN-γ to cultures of astrocytes and microglia stimulates these cells to produce proinflammatory cytokines and chemokines, and to express MHC class II (24). However, the net effect of IFN-γ signaling in the CNS is, similar to that in the periphery, also anti-inflammatory. Convincing proof can be found in an elegant bone marrow chimeric model, which showed that adoptive transfer of Ag-activated lymphoid cells from MOG_{35–55}-immunized IFN-γ−/− donor mice induced severe EAE in chimeric mice that lacked IFN-γR either in CNS cells or in the periphery (25, 26). The mechanism underlying the anti-inflammatory effects of IFN-γ signaling in CNS cells is not known. A recent study showed enhanced EAE severity in a transgenic mouse model, in which IFN-γ can bind to dominant-negative IFN-γR1 but cannot initiate downstream IFN-γ signaling pathways in astrocytes (27), whereas the role of IFN-γ responsiveness in microglia in MS/EAE has not been defined. Elucidating IFN-γ binding/signaling in individual CNS cells, for example, astrocytes and microglia, is crucial not only for our understanding of the mechanism underlying the development of MS/EAE, but also for designing a CNS-targeted therapy.

In this study, we generated two novel lentiviral vectors to specifically knock down IFN-γR expression and block IFN-γ binding/signaling in astrocytes or microglia. We demonstrated that IFN-γ signaling in these cells plays significant, but opposite, roles in EAE. Silencing IFN-γ signaling in astrocytes alleviated EAE by suppressing chemokine production and reducing infiltration of inflammatory cells into the CNS, whereas blocking IFN-γ signaling in microglia enhanced disease severity through augmented proliferation of microglia. Further, silencing of IFN-γ signaling in astrocytes effectively suppressed ongoing disease and alleviated both Th1- and Th17-mediated adaptive EAE without affecting the peripheral immune system, highlighting an opportunity for development of CNS cell-specific therapeutic approaches for MS.

Materials and Methods

Mice

Female C57BL/6 mice, 8–10 wk of age, 2D2 (TCR^{ROG}) transgenic mice (C57BL6-Tg [Tcr2D2, Tcrb2D2]Kuch/J, stock number: 006912), and IFN-γR1−/− deficient mice (B6.129S7-Ifngr1tm1Agt/J, stock number: 003288) were purchased from Jackson Laboratory (Bar Harbor, ME). All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Committee of Thomas Jefferson University.

Reagents and Abs

Anti-CD3 (145-2C11) and anti-CD28 (37.51) Abs were purchased from BD Biosciences (San Jose, CA). The following Abs for flow cytometry were from Biolegend (San Diego, CA): FITC-conjugated anti-CD11b (M1/70) and allophycocyanin-conjugated anti–IFN-γ (XMG1.2), and PE-conjugated anti–IL-17A (TC11-18H10). The following Abs for cell culture were purchased from Jackson Laboratory (Bar Harbor, ME). Several lentiviral vectors were used in this study: pLenti-CD11b-EGFP-mi-shIFN-γR, pLenti-CD11b-EGFP-mi-shIFN-γR, and control lentiviral vectors. Vectors were constructed as previously described (30). In brief, for pLenti-CD11b-EGFP-mi-shIFN-γR vector construction, the shAct1 cassette in pLenti-EGFP-EGFP-mi-shAct1 was replaced by a fragment containing miR-30 based shIFN-γR vector cassette (cat. no. RMM4431-9820699, Open Biosystems). For pLenti-CD11b-EGFP-mi-shIFN-γR vector construction, the GFAP promoter in pLenti-EGFP-EGFP-mi-shIFN-γR vector was replaced by CD11b promoter sequence, which was composed of bp −1704 to bp +83 of the 5′ untranslated region of human CD11b gene simplified from human genomic DNA (31). The constructed vector sequence was verified by sequencing. The vector without insertion of mi-shIFN-γR was used as control. Primers used for vectors construction are listed in Supplemental Table I.

Isolation of primary astrocytes and microglia

The whole brain of mice embryos (E16) was harvested and dissociated with Neural Tissue Dissociation Kit (Miltenyi Biotech, Auburn, CA) following the manufacturer’s instructions. Astrocytes were purified with anti–ASCA-2” microbeads (Miltenyi Biotech) following the manufacturer’s MACS instructions (30). The purified astrocytes were centrifuged at 300 × g for 10 min and then resuspended with D-MEM/10% FBS plus 5 ng/ml M-CSF (Peprotech), and seeded on 6-well plates. The following Abs for flow cytometry were from Biolegend (San Diego, CA): FITC-conjugated anti-CD11b (M1/70) and allophycocyanin-conjugated anti–IFN-γ (XMG1.2), and PE-conjugated anti–IL-17A (TC11-18H10). The following Abs for cell culture were purchased from Jackson Laboratory (Bar Harbor, ME). All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Committee of Thomas Jefferson University.

Induction and assessment of EAE

For active EAE, mice were immunized s.c. on the back with 200 μg MOG_{35–55} (MEGVWYRSPFSR VHLYRNGK) emulsified in CFA (Difco Lab, Detroit, MI) containing 4 mg/ml Mycobacterium tuberculosis H37Ra (Difco). Two hundred nanograms of pertussis toxin (List Biological Lab, Campbell, CA) was given i.p. on days 0 and 2 postimmunization (p.i.). For passive EAE, short hairpin RNA (shRNA) GFAP-shIFN-γR or GFAP-shVec lentivirus injected mice were transferred with 3.0 × 10^{7} polarized MOG_{35–55}-specific Th1 or Th7 cells/mouse 4 h after sublethal irradiation (550 rad). To prepare MOG-specific polarized Th cell populations, we prepared draining lymph nodes and spleen cells from mice immunized as described earlier at day 9 p.i. Cells were cultured for 4 d with MOG_{35–55} at a concentration of 25 μg/ml under Th1-polarizing (20 ng/ml recombinant mouse IL-12 [Peprotech], 2 μg/ml anti–IL-23p9 [eBioscience]) or Th17-polarizing (20 ng/ml recombinant mouse IL-23 [Peprotech]) conditions (28). Mice were scored daily for appearance of clinical signs of EAE on a scale from 0 to 5 as described previously (29): 0, no clinical signs; 1, fully limp tail; 2, paralysis of one hind limb; 3, paralysis of both hind limbs; 4, paralysis of trunk; 5, moribund or death.


For in vivo virus infection, purified astrocytes or microglia were seeded in poly-lysine-coated 6-well plates at a concentration of 5 × 10^{5} cells/well. Two days later, culture medium was replaced by fresh complete DMEM medium supplemented with 1 × 10^{5} IU/well of different lentiviruses and 8 μg/ml polybrene, and then incubated for 16 h at 37˚C. After incubation, the medium with virus soap was replaced by fresh medium and cultured for further use. For in vivo injection, mice were anesthetized and fitted with intracerebroventricular (i.c.v.) cannula for virus microinjection. A microsyringe was inserted into 2.0 mm lateral, 1.0 mm caudal to bregma, and 2.5 mm below the skull surface. A total of 1 × 10^{5} IU/mouse GFAP-shIFN-γR, CD11b-shIFN-γR, or their control virus (in 20 μl volume) was given to the mice. Injection speed was maintained at 1 μl/min to prevent leaking.

Astrocyte and microglia treatment in vitro

Primary astrocytes and microglia cells were seeded on poly-lysine–coated 60-mm dishes (for microglia, without coating the dishes, 5 ng/ml M-CSF was supplied in the medium) at a density of 1 × 10^{5} dishes and cultured in D-MEM/10% FBS medium except when otherwise specified in the figure legends. Cells were treated with IFN-γ (10 ng/ml), IL-17A (50 ng/ml), and/or TNF-α (10 ng/ml). Cells were harvested at 24 h for RNA purification experiments. Although Jackson Lab IFN-γ and IL-17A treatments were used as a surrogate for Th1 and Th17 stimulation, respectively, TNF-α is included because this cytokine, together with IFN-γ, enhances microglia/astrocyte activation (24).

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Migration assay

Astrocytes were purified from C57BL/6 WT and IFN-γR−/− (B6.129S7-Ifngr1tm1Agt/J, stock number: 003288) mice and then stimulated with IFN-γ plus TNF for 24 h. Migration assays were performed as previously described, with some modifications (32). In brief, prestimulated astrocytes, with or without IFN-γR, were cultured in 24-well plates. Then 2D2 spleen cells that had been stimulated with 20 μg/ml MOG35-55 for 3 d were loaded onto the luminal side of Transwell inserts (3-μm pore size; Corning) and incubated at 37°C/5% CO2. Twenty-four hours later, 50 μl of 0.5 M EDTA was added to the abluminal side and the plates were placed on a shaker for 15 min to dislodge cells. Cells on the abluminal side were then harvested and counted under light microscopy.

Mononuclear cell preparation

Mice were sacrificed at days 12 (disease onset) and 20 p.i. (peak). Spleen lymphocytes were isolated from single-cell suspension by centrifugation with Ficol-Paque PLUS (Amersham Pharmacia Biotech AB, Piscataway, NJ). Lymphocyte layer was harvested and washed with cold PBS before in vitro stimulation. To isolate CNS cells, we mechanically dissociated spinal cords through a 70-μm cell strainer and washed with PBS. Washed cells were fractionated on a 60/30% Percoll gradient by centrifugation at 300 × g for 20 min. Infiltrating mononuclear cells (MNCs) were collected from the interface and washed with PBS for use.

Intracellular staining and flow cytometry

Cells isolated from spleen or spinal cord were stimulated with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and Golgi-Stop (1 μg/106 cells; BD Pharmingen, San Diego, CA) for 4 h at a density of 1 × 106 cells/ml in complete RPMI 1640 medium. Cells were harvested, washed in FACS buffer (PBS containing 1% FCS and 0.02% NaN3). After washing, cells were stained with fluorescent Abs to surface markers, followed by intracellular staining in accordance with the manufacturer’s instructions. In brief, cells were fixed and permeabilized using Fix/Perm cell permeabilization reagents (BD Biosciences), followed by incubation with fluorescently labeled Abs against intracellular cytokines. After the last wash, data were acquired by FACSaria (BD Biosciences). Data were analyzed using FlowJo Software (Tree Star, Ashland, OR).

Immunohistochemical staining

Mice treated with all lentiviral treatments were extensively perfused, and the brain and spinal cords were harvested. Immunohistochemical staining was performed as previously described, with some modifications (33). In brief, spinal cords were carefully excised from the brainstem to the lumbar region and cryoprotected with 30% sucrose in PBS. The lumbar enlargement was identified and then transected at the exact midpoint of the lumbar enlargement to standardize a site along the longitudinal axis of the cord, ensuring that the same lumbar spinal cord regions were analyzed for all conditions. Transverse sections of brain and spinal cord were cut, and immunohistochemistry was performed using different Abs. Immunofluorescence controls were routinely generated with irrelevant IgGs as first Abs. Finally, slides were covered with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA), containing 1 μM DAPI. Results were visualized by fluorescent microscopy (Eclipse 800; Nikon).

Statistical analysis

Differences for clinical scores of EAE were analyzed by using the two-way ANOVA test. Inflammation scores were analyzed using the Mann–Whitney U test. Differences for other parameters were analyzed by unpaired, two-tailed Student t test. A p value <0.05 was considered statistically significant.

Results

IFN-γ signaling in astrocytes and microglia has opposite roles in EAE development

To dissect the role of IFN-γ signaling in astrocytes versus microglia in EAE, we generated novel lentiviral vectors that express IFN-γR1–specific shRNA in either astrocytes or microglia. The schematics of these two vectors are shown in Fig. 1A. In both vectors, the shRNA cassette was inserted downstream of EGFP stop codon, allowing shIFN-γR to be transcribed simultaneously with EGFP, which served as a marker of transfection. Cultured primary astrocytes and microglia were infected with either GFAP-shIFN-γR or CD11b-shIFN-γR, or control lentivirus (GFAP-shVec or CD11b-shVec), and expression of IFN-γR was examined 3 d later. GFAP-shIFN-γR or CD11b-shIFN-γR lentivirus strongly reduced the levels of IFN-γR protein as determined by immunostaining (Fig. 1B) and mRNA expression as determined by real-time RT-PCR (Fig. 1C for astrocytes and Fig. 1D for microglia).

We then i.c.v. injected lentiviruses containing GFAP-shIFN-γR, CD11b-shIFN-γR, or their corresponding control vectors to naive mice and sacrificed them 3 d later to test cell specificity and in vivo knockdown efficiency. We have previously shown that GFAP promoter drives expression of EGFP and shRNA in an astrocyte-specific manner (30); we therefore tested in this study only the expression specificity in vivo of our CD11b promoter-driven lentiviruses. As shown in Supplemental Fig. 1, EGFP was only coexpressed with Iba-1+ cells in the parenchyma (microglia) and perivascular space (perivascular macrophages). More than 80% of Iba-1+ cells from brain and spinal cord expressed EGFP (data not shown), whereas other types of CNS-resident cells, including neurons, oligodendrocytes, astrocytes, and oligodendrocyte progenitor cells, did not express EGFP, thus confirming microglia-restricted expression of CD11b-shIFN-γR and CD11b-shVec.

To determine the role of astrocyte- or microglia-restricted IFN-γ signaling in EAE induction, we gave GFAP-shIFN-γR, CD11b-shIFN-γR, and control lentiviruses (1 × 10⁷ IU in 20 μl PBS/mouse) to mice by i.c.v. administration 3 d before immunization. This time point for i.c.v. injection was chosen based on a previous report that the brain-injected lentivirus would completely integrate into brain cells within 3 d (34), whereas peripheral macrophages infiltrated into the CNS >4 d after EAE induction (35). This approach therefore ensures that CD11b-shIFN-γR lentivirus and its control will only infect microglia but will be removed before peripheral macrophages infiltrate into the CNS. Mice injected with GFAP-shIFN-γR and GFAP-shVec were immunized with 200 μg/mouse MOG35-55 peptide. CD11b-shIFN-γR– and CD11b-shVec–injected mice were immunized with 100 μg/mouse of peptide to avoid death from enhanced EAE (based on our pilot experiments; data not shown). Silencing IFN-γ signaling in astrocytes significantly suppressed clinical EAE (Fig. 1E and 1F), whereas blocking its signaling in microglia enhanced disease severity (Fig. 1G and 1H). The majority of these mice experienced development of typical EAE, whereas a few (~10%) of CD11b-shIFN-γR–treated, but not GFAP-shIFN-γR–treated, mice exhibited signs of alternative EAE. These results demonstrate that IFN-γ signaling in astrocytes and microglia has opposite roles in EAE development.

The in vivo knockdown efficiency was then determined by real-time RT-PCR using cDNAs of purified GFAP⁺ astrocytes or CD11b⁺ microglia/macrophages from the spinal cords and brainstems of mice that were sacrificed at day 35 p.i. GFAP-shIFN-γR lentivirus effectively knocked down IFN-γR expression in astrocytes (Fig. 1I), whereas IFN-γR knockdown in CD11b⁺ cells was less efficient (Fig. 1J), likely because of unaffected IFN-γR expression in a part of infiltrating macrophages. Also, our results showed a relatively long-lasting knockdown effect of this approach.

Silencing IFN-γR expression in astrocytes suppressed EAE by inhibiting inflammatory cell infiltration

To test how astrocytic IFN-γ signaling affects EAE development, we injected mice i.c.v. with 1 × 10⁷ IU GFAP-shIFN-γR or control virus 3 d before immunization, and brain, spinal cord, and spleen were harvested at day 20 p.i. We expected that the i.c.v. injection of the viruses would not affect development of MOG-specific immune response upon immunization. To test this, we isolated splenocytes of treated and untreated EAE mice, and their recall responses to MOG peptide were compared. There was no...
significant difference in spleen cell numbers between mice treated with GFAP-shIFN-γR and control mice (Supplemental Fig. 2A), and the numbers of Th1 and Th17 cells in spleens of the GFAP-shIFN-γR–treated group were the same as in the control group, as determined by intracellular staining for IFN-γ and IL-17 (Supplemental Fig. 2b). There was no significant difference in...
MOG_{35-55}-induced T cell proliferation between the two groups (Supplemental Fig. 2c). In addition, splenocytes from the GFAP-shIFN-γR- and GFAP-shVec–treated groups produced similar amounts of IFN-γ, IL-17, and IL-4 upon MOG stimulation (Supplemental Fig. 2d). Together, these results demonstrate that astrocyte-restricted knockdown of IFN-γR does not affect peripheral immune response during EAE.

Typical MNC infiltration foci were observed in the spinal cord white matter of control virus-treated mice, which were similar to untreated EAE mice (Fig. 2A). Consistent with reduced clinical severity, inflammatory cell infiltration in GFAP-shIFN-γR–treated mice was reduced (Fig. 2A). Similar results were observed in brainstem sections (data not shown). The remaining spinal cord and brainstem tissues were processed to count MNCs. There were ~3-fold more total MNCs in GFAP-shVec mice than in the GFAP-shIFN-γR–treated group in both brainstem and spinal cord (Fig. 2B). Isolated MNCs from spinal cords and brainstem were then analyzed by flow cytometry, and absolute numbers of various cell types were calculated. As shown in Fig. 2C, all infiltrating cell types tested, including CD4+ T cells, CD19+ B cells, CD11c+ DCs, F4/80+ macrophages/microglia, and Gr1+ neutrophil cells/monocytes, were significantly reduced in spinal cords and brainstems of GFAP-shIFN-γR–treated mice. Compared with the GFAP-shVec–treated control group, the frequencies of IL-17+ CD4+, IL-17+ IFN-γ CD4+, and IFN-γ CD4+ T cells were reduced in spinal cords of the GFAP-shIFN-γR–treated group (Fig. 2D), and absolute numbers of IL-17+ or IFN-γ+ cells (percentages × total number of MNCs) were significantly reduced in GFAP-shIFN-γR–treated mice (Fig. 2E). Similar results were observed in brainstem cells (data not shown). Staining for MBP expression in spinal cords showed that knockdown of IFN-γ in astrocytes significantly inhibited demyelination compared with control treatments (Fig. 2F and 2G). Similar results were obtained in brainstem tissues (data not shown). Given that there is no significant difference in total cell numbers and cell subsets between mice sacrificed at days 12 and 20 p.i., the results from day 20 p.i. are shown.

**Blocking IFN-γ signaling in astrocytes reduces chemokine expression in the CNS**

To investigate why interfering with IFN-γ signaling in astrocytes inhibits inflammatory cell infiltration, we determined cytokine and chemokine gene expression of purified astrocytes in the spinal cords of GFAP-shIFN-γR–treated and control shRNA-treated EAE mice. Expression of inflammatory cytokines such as IL-17A, IL-22, and IL-6 was reduced in GFAP-shIFN-γR–treated mice (≥2-fold), with IL-6 expression showing the greatest reduction (Fig. 3A). Expression of matrix metalloproteinase (MMP) 9 and of virtually all the chemokines we tested was also reduced (Fig. 3B). However, there was no difference in expression of NT-3 and BDNF, two important neurotrophic factors that are primarily produced by astrocytes (36, 37) (data not shown), suggesting that GFAP-shIFN-γR treatment influenced only the inflammatory response of astrocytes, but did not affect their capacity for neurotrophic factor expression.

To further confirm the in vivo finding that blocking IFN-γ signaling in astrocytes inhibited their chemokine production, we determined the profile of chemokine expression of primary astrocytes under different culturing conditions. Expression of MMP3 and chemokines CXCL-1, CXCL-2, CCL-20, CXCL-9, CXCL-10, and CXCL-11 was reduced in GFAP-shIFN-γR–treated astrocytes (Fig. 3C). A transmigration assay was performed to test the chemotactic capability of astrocytes deficient in IFN-γR. Compared with those from WT mice, astrocytes from IFN-γR-deficient mice attracted significantly fewer MOG-stimulated splenocytes from 2D2 mice (Fig. 3D). In addition, we found that none of the different combinations of cytokines altered the proliferation of primary cultured astrocytes either from WT or IFN-γR−/− mice (data not shown). Supernatants from different culture wells were collected for cytokotoxicity assay (LDH), and there was no difference among groups (data not shown). These results showed that lack of IFN-γ signaling did not affect the proliferation and survival of astrocytes under different inflammatory conditions, but changed the expression of chemokines in these cells.

**Silencing IFN-γR expression in microglia promotes CNS inflammatory infiltration and microglia expansion**

We next attempted to elucidate the phenomenon whereby knocking down IFN-γR in microglia resulted in enhanced EAE pathogenesis (Fig. 1G and 1H). Spinal cords and brainstems were harvested at day 20 p.i.; L3 of spinal cord was fresh-frozen and the remaining tissues were processed for MNC purification. Silencing IFN-γR expression in microglia did not alter the peripheral immune system as assayed by total numbers of spleen cells, immune cell populations, Th1 and Th17 cell frequencies, and regulatory T cell populations (Supplemental Fig. 3A–D). Due to suboptimal immunization, mice injected with the control virus had a few inflammatory cells in the CNS (Fig. 4A and 4B), consistent with mild EAE (Fig. 1G and 1H). In contrast, mice injected with CD11b-shIFN-γR had a significantly higher number of inflammatory cells in the CNS (Fig. 4A and 4B).

Interestingly, silencing IFN-γR in microglia significantly increased the absolute number of CD4+ T cells in the CNS and the absolute numbers of IL-17+ and IFN-γ+ cells among CD4+ T cells (Fig. 4C–E). Absolute numbers of CD4+, CD11b+, and CD11c+ cells, except for CD19+ B cells, were increased in both spinal cords (Fig. 4D) and brainstems (Fig. 4E) of CD11b-shIFN-γR–treated mice compared with the control group. Although there was no difference in the Gr-1<sup>hi</sup> monocytes between the two groups, the number of Gr-1<sup>hi</sup> neutrophils was significantly higher after CD11b-shIFN-γR treatment. Thus, in contrast with astrocytes, knocking down IFN-γ signaling in microglia significantly enhanced CNS inflammatory infiltration.

Unlike in astrocytes, silencing IFN-γR expression in microglia did not decrease expression of chemokines in the spinal cord (Fig. 4F); instead, a slight increase in CXCL11, IL-6, and IFN-γ and a decrease of IL-10 (Fig. 4F and 4G) were observed in these mice. Dramatically increased CD11b+ cells were also observed in spinal cord sections of CD11b-shIFN-γR–treated mice as determined by immunohistology (Fig. 4H and 4I). This result was further confirmed by the significantly increased frequency and absolute numbers of the CD11b<sup>+</sup>CD45<sup>+</sup> and CD11b<sup>+</sup>CD45<sup>+</sup> populations in spinal cord of CD11b-shIFN-γR–treated mice by flow cytometry (Fig. 4J and 4K). Together, these results show that IFN-γ signaling in microglia plays an important role in controlling numbers of infiltrating macrophages and activated microglia during CNS inflammation.

We further addressed whether MNCs in the CNS that originate from peripheral CD11b<sup>+</sup> myeloid cells express GFP in mice treated with CD11b-shVec-eGFP 3 d before EAE induction. GFP was expressed in a large portion of CD11b<sup>+</sup> cells, but in only a few of the other cell types, for example, infiltrating Gr-1<sup>+</sup> cells in the CNS (both spinal cord and brainstem; Fig. 5A). In contrast, GFP was expressed on up to 92.4% of CD45<sup>+</sup> (microglia) and 71.8% of CD45<sup>+</sup>, whereas the uninfected (GFP<sup>−</sup>; 28.2%) CD45<sup>+</sup>CD11b<sup>+</sup> cells might have been infiltrating macrophages from the periphery (Fig. 5B), with significantly lower numbers of CD45<sup>+</sup>CD11b<sup>+</sup> GFP<sup>+</sup> cells compared with total numbers of the CD45<sup>+</sup>CD11b<sup>+</sup>
FIGURE 2. Knocking down IFN-γR expression in astrocytes suppressed inflammatory infiltration in the CNS. Spinal cords and brainstems of mice described in Fig. 1E were harvested at day 20 p.i. (A) Inflammatory infiltration was assayed by H&E staining at the L3 level. Original magnification ×200. (B) Similar amounts of the remaining spinal cords and brainstems were processed for isolation of MNCs. Total MNC numbers in spinal cord and brainstems were counted under light microscopy (n = 5 each group). (C) Percentages of MNC subtypes were determined by flow cytometry, and absolute numbers of these subtypes were calculated by multiplying the percentages of these cells and total MNCs in each spinal cord and brainstem tissue; comparison between GFAP-shVec– and GFAP-shIFN-γR–treated groups (n = 5 each group). (D) MNCs isolated from spinal cord were briefly stimulated with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and GolgiStop (1 μg/10⁶ cells; BD Pharmingen) for 4 h. Frequencies of Th1 and Th17 cells were assayed by intracellular staining and analyzed in gated CD4+ cells by flow cytometry. Absolute numbers of Th1 and Th17 cells (E) were calculated by multiplying the percentage of these cells in (D) and total MNC numbers in each mouse. Demyelination in each group was assessed by MBP staining (F), quantified with National Institutes of Health ImageJ software, and presented as the percentage of light pixels (G). Original magnification ×200. Results are shown as mean ± SD (n = 10 each group). *p < 0.05, **p < 0.01.
FIGURE 3. Blocking IFN-γ signaling in astrocytes impaired CNS expression of cytokines and chemokines, and reduced chemoattractant ability of astrocytes. Total RNA was prepared from purified astrocytes at the end of the experiment from a fraction of spinal cords and brainstems at day 20 p.i. cDNA synthesis was done separately in each mouse (n = 10 each group) using the same amount of total RNA for the following real-time RT-PCR analysis. (A) Expression of an array of cytokine genes was determined by real-time RT-PCR. More than 2-fold changes were considered significant between groups. (B) Fold change in expression of an array of chemokine genes was analyzed by real-time RT-PCR. More than 2-fold changes were considered significant between groups. (C) Purified astrocytes of naive WT C57BL/6 mice were infected with GFAP-shVec or GFAP-shIFN-γR lentivirus, and 24 h later, these cells were stimulated with different cytokines for 24 h. Chemokine expression was determined by real-time RT-PCR. (D) Primary astrocytes from C57BL/6 WT and IFN-γR−/− mice were stimulated with IFN-γ plus TNF for 16 h. MOG35–55–stimulated 2D2 spleen cells were loaded onto the luminal side of Transwell inserts with a pore size of 3 μm and incubated for 24 h. Numbers of cells in the abluminal side were quantified. Data are shown as mean ± SEM of three independent experiments. Relative expression was calculated by −ΔΔCt values from triplicate PCR. *p < 0.05, **p < 0.01.
Blocking IFN-γ signaling in microglia enhanced inflammatory infiltration and microglia proliferation. (A) Spinal cords and brainstems of mice described in Fig. 1G were harvested at day 20 p.i. and fresh-frozen. Inflammatory infiltration was assayed by H&E staining. Representative sections at L3 level of spinal cords are shown. Original magnification ×200. (B) Similar amounts of the remaining spinal cords and brainstems were processed for MNC suspension, and total MNC numbers were counted under light microscopy (n = 5 each group, *p < 0.05 compared with untreated EAE control group). Frequencies of Th1 and Th17 cells from the spinal cord and brainstem of these mice were assayed by intracellular staining and analyzed in gated CD4+ T cells by flow cytometry. A representative figure is shown in (C), and absolute numbers of various subtypes of CNS-infiltrating cells were calculated by multiplying the percentages of these cells with the total number of MNCs in each spinal cord (D) and brainstem (E). *p < 0.05, **p < 0.01, comparison between CD11b-shVec and CD11b-shIFN-γR-treated groups (n = 5 each group). Total RNA was prepared from purified CD11b+ cells at the end of the experiment from a fraction of spinal cords of CD11b-shIFN-γR-treated or CD11b-shVec–treated EAE mice. Real-time PCR (Figure legend continues)
cell population (Fig. 5C). Given that at EAE peak, the CD45hi CD11b+ population contained both activated microglia and infiltrated peripheral macrophages (35), a portion of the CD45hi CD11b+GFP+ cells could have been infiltrated macrophages. In contrast, all splenic CD11b+ cells were GFP− (data not shown), indicating that i.c.v.-injected lentiviral vectors did not leak into the periphery.

**IFN-γ signaling reduces microglia numbers in vitro**

CD11b-shIFN-γR lentiviral treatment increased the number of CD11b+ cells in vivo, but it was not known whether this was the cause or result of enhanced CNS inflammation. We therefore tested the effect of IFN-γ on proliferation of primary microglia in vitro. Interestingly, both IFN-γ and IFN-γ + TNF treatments inhibited microglial proliferation (Fig. 6A). In addition, inhibition of microglia proliferation by IFN-γ did not occur in IFN-γR−/− microglia (Fig. 6A).

Given that IFN-γ can activate microglia (38, 39), we tested the effect of IFN-γ on microglial chemokine expression. As shown in Fig. 6B, IFN-γ plus TNF enhanced expression of chemokines that are inducible by IFN-γ signaling (e.g., CXCL9, CXCL10, CCL11) (28, 30), whereas reducing expression of those that are inducible by IL-17 signaling (e.g., CXCL1, CXCL2, CCL20) (28, 30). IFN-γ + TNF treatment also enhanced microglial expression of TNF and iNOS. IFN-γ expression was highly enhanced when microglia were treated with TNF alone, whereas IFN-γ + TNF treatment had the opposite effect.

We then tested whether silencing IFN-γ signaling had cytotoxic and/or proapoptotic effects on microglia. IFN-γ treatment did not induce microglia apoptosis or microglia death as determined by LDH cytotoxicity assay (data not shown). Together, these results show that, during EAE pathogenesis, IFN-γ signaling limits the number of microglia by inhibiting their proliferation but without inducing apoptosis or cell death.

It has been reported that IFN-γ can inhibit macrophage/microglia proliferation by arresting the cell cycle at the G1/S boundary (40). We found that expression of p21, a cyclin-dependent kinase inhibitor, which inhibits activity of the cyclin E-CDK2 complex, thereby arresting the cell cycle at the G1/S phase (41), was significantly induced in cultured microglia. In contrast, expression of c-myc, which controls G1/S transition by activating cyclin-CDK complexes and inducing transcription of...
FIGURE 6. IFN-γ effectively restricts microglia proliferation. (A) Microglia isolated from naive adult WT and IFN-γR−/− mice were stimulated with different combinations of cytokines for 3 d, and their proliferation was measured by [3H]Tdr incorporation. (B) Microglia isolated from naive adult WT mice were stimulated with different combinations of cytokines for 24 h, and expression of several chemokines and cytokines of interest, as well as iNOS, was assessed by real-time RT-PCR. (C) Expression of cell-cycle–related genes P21 and c-myc was tested in primary microglia and astrocytes treated with IFN-γ or IFN-γ + TNF. The entire experiment in this figure was repeated three times. (A) One representative of three independent experiments; (B and C) mean ± SEM of three repeated experiments. **p < 0.01.
genes required for the S phase (42), was decreased after stimulation either with IFN-γ alone or IFN-γ + TNF (Fig. 6C). On the contrary, expression of both of p21 and c-myc was not changed in astrocytes, whether stimulated by IFN-γ alone or IFN-γ + TNF (Fig. 6C). These results suggest that IFN-γ signaling restricted microglia proliferation by regulating cell-cycle–related genes p21 and c-myc.

Blocking IFN-γ signaling in astrocytes alleviates ongoing active EAE and both Th1 and Th17 cell–transferred EAE

Given that IFN-γ signaling in astrocytes promotes EAE development by recruiting inflammatory cells into the CNS, targeting this pathway in astrocytes may provide a CNS-targeted therapy for EAE. Our results showed that, similar to administering virus before immunization (Fig. 1G), silencing IFN-γR expression in astrocytes after disease onset (Fig. 7A) or at disease peak (Fig. 7B) significantly alleviated EAE, demonstrating its therapeutic effects. Further, unlike blocking IL-17 signaling in astrocytes, which suppressed only Th17 cell–mediated EAE but had no effect on Th1 cell–mediated EAE (28, 30), blocking IFN-γ signaling in astrocytes decreased disease severity in both Th1 and Th17 cell–mediated adoptive EAE (Fig. 7C and 7D). These results are consistent with the decreased expression of chemokines responsive to both IFN-γ and IL-17 in active EAE and in in vitro–cultured astrocytes when IFN-γR was silenced (Fig. 3).

Discussion

IFN-γ, a hallmark cytokine of Th1 cells, is reported to be at higher levels in the sera and cerebrospinal fluid of MS patients (5). It has been shown that administration of IFN-γ to MS patients enhanced disease severity (43), whereas its neutralization had a therapeutic effect in MS patients (44). In EAE, modifying signaling pathways, which results in decreased IFN-γ production in myelin-specific T cells, decreases the encephalitogenic capacity of these cells (9, 45). However, these observations are contradicted by the findings that both IFN-γ−/− and IFN-γR−/− mice experience more severe clinical EAE, associated with pronounced inflammatory infiltrates into the brain parenchyma and even into gray matter (11, 12). Similarly, treating EAE mice with anti–IFN-γ exacerbates EAE (12). It has been speculated that IFN-γ regulates EAE by suppressing proliferation and driving activation-induced cell death of CNS-infiltrating lymphocytes (6, 41, 46). IFN-γ also plays a role in induction of Foxp3 expression and conversion of CD4+CD25+ T cells to CD4+ regulatory T cells thus modulating immune responses (16). Thus far, the majority of studies on the role of IFN-γ in the pathogenesis of EAE have focused on peripheral immune regulation; however, its action on CNS cells has yet to be defined (25). Data from other groups (18–20) and our own show that IFN-γR is highly expressed in astrocytes, microglia, and oligodendrocytes, and its expression is greatly increased in the CNS during EAE. Although IFN-γ induces apoptosis in human oligodendrocytes (22, 23), the role of microglia–or astrocyte–restricted IFN-γ signaling in EAE remains unknown.

In this study, we developed a novel approach to silence IFN-γ signaling in astrocytes and microglia. Although ablation of IFN-γ signaling in all CNS aggranulated disease severity, as shown by bone marrow chimeric mice and the adoptive EAE model (25, 47), blocking IFN-γ signaling only in astrocytes significantly alleviated EAE, with fewer infiltrating inflammatory cells. This result could most likely be attributed to significantly decreased expression of chemokines and proinflammatory cytokines in the CNS of treated mice. Indeed, unlike blocking IL-17 signaling in astro-
cytes, which inhibited only chemokines induced by IL-17 signaling, but not by IFN-γ signaling (28, 30), blocking IFN-γ signaling in astrocytes not only inhibited expression of MMP3 and chemokines induced by IFN-γ (e.g., CXCL9, CXCL10, and CXCL11), but, surprisingly, also inhibited expression of chemokines induced by IL-17 signaling (e.g., CXCL1, CXCL2, and CCL20). The mechanism underlying this phenomenon may be that the IFN-γ–IFN-γR pathway in astrocytes is a common pathway at the effector stage for other proinflammatory cytokines, via endogenous IFN-γ expression and secretion in these cells. Consistent with this, we found that IL-17 induced endogenous expression of IFN-γ and IL-17 in astrocytes, an effect that was diminished in cells lacking IFN-γ signaling (data not shown). These results also explain our observation that silencing IFN-γ signaling in astrocytes significantly inhibited IL-17– and TNF-induced chemokine expression in vitro. It can be concluded from these findings that IFN-γ signaling in astrocytes plays an important role in triggering expression/secretion of a broad range of chemokines by these cells, thus attracting inflammatory cells into the CNS during EAE. Mayo et al. (48) recently demonstrated that astrocytes play an important pathogenic role in progression of EAE by recruiting inflammatory monocytes into the inflamed CNS and by activating resident microglia via secretion of proinflammatory factors, such as CCL2 and GM-CSF (49). The proinflammatory phenotype of astrocytes in mice with EAE was recapitulated in vitro by stimulation of quiescent astrocytes with LPS + IFN-γ, which is consistent with our findings that IFN-γ signaling in astrocytes has a detrimental role in EAE. Therefore, silencing IFN-γ signaling in astrocytes diminishes a secondary wave of inflammatory infiltration and ensuing disease development. A recent report showed that IFN-γ signaling in astrocytes protects from autoimmune-mediated neurologic disability (27), and we believe that the difference in results in our study and this report is mainly due to the different knockdown approaches used. In the report by Hindinger et al. (27), IFN-γ signaling in astrocytes was blocked through transgenic overexpression of dominant-negative IFN-γR1 in astrocytes. IFN-γ can still bind to this dominant-negative IFN-γR1 but cannot start downstream signaling pathways. Because the large number of astrocytes in the CNS overexpressed dominant-negative IFN-γR1, a large amount of IFN-γ will bind it, and only a low level of free IFN-γ will be left to act on other cell types in the CNS, which may be immunoregulatory (e.g., microglia as we show in this study). In contrast, our approach knocked down IFN-γR itself, thus blocking astrocytic IFN-γ binding and leaving a large amount of free IFN-γ in the CNS. This free IFN-γ can exert immunoregulatory function by acting on other CNS cells such as microglia and suppress EAE. Based on this reasoning, we believe that our approach is novel and more specifically dissects the effects and mechanisms underlying IFN-γ binding/signaling in astrocytes/microglia in EAE.

Why does silencing IFN-γ signaling block not only Th1, but also Th17 cell responses in the CNS in EAE? Three mechanisms may contribute to this phenomenon. First, due to the plasticity of Th17 cells, IFN-γ has been found to be the major cytokine produced by Th17 cells after they have migrated into the CNS. By using IL-17A fate reporter mice, it has been demonstrated that IL-23–induced Th17 cells rapidly lose IL-17A production when they reside in the inflamed spinal cord (50). These “ex-Th17 cells” no longer express IL-17A protein, and a considerable proportion produce IFN-γ. Interestingly, IFN-γ and other Th1-type cytokines in the spinal cord are produced almost exclusively by these “ex-Th17 cells.” Although the molecular mechanism underlying this phenotype plasticity of Th17 cells is not clear, this result provides strong evidence that IFN-γ is the key effector molecule for both Th1 and Th17 cells in the inflamed CNS; therefore, blocking IFN-γ signaling could diminish not only Th1, but also Th17, cell-induced EAE. Second, IL-23–induced Th17 cells from WT donors were able to transfer EAE to mice that lacked IL-17R, whereas those Th17 cells from IFN-γ−/− donor mice failed to do so (51), indicating the key role of IFN-γ in facilitating development of Th17 cell–induced EAE. Third, this study provides evidence that, unlike blocking IL-17 signaling in astrocytes, which reduced only IL-17–induced chemokines (30), silencing IFN-γ signaling reduced CNS production of both IFN-γ– and IL-17–induced chemokines. Together, these results strongly indicate that IFN-γ signaling is the common pathway in both Th1 and Th17 cell–mediated EAE.

Given that IFN-γ plays an important role in astrocyte-mediated inflammatory cell infiltration and IFN-γ induced oligodendrocyte apoptosis, hence demyelination (22, 23), it seemed that blocking the IFN-γ signaling pathway in CNS should prevent EAE. However, the net effect of IFN-γ signaling in the CNS has been an anti-inflammatory one in EAE development, as shown by the adoptive transfer model or active EAE in bone marrow chimeric mice (25, 47). Thus, IFN-γ signaling in certain CNS resident cells, for example, microglia, rather than in oligodendrocytes or astrocytes, may protect mice from EAE. Indeed, i.c.v. injection of CD11b-shIFN-γR significantly exacerbated the severity of EAE, with increased total MNC numbers, particularly CD11c+ cells and Th1 and Th17 cells. Although there are no specific cell-surface markers to identify infiltrating macrophages versus resident microglia, the level of CD45 expression has been widely considered as a marker for that purpose, that is, CD45+CD11b+ for resting microglia, and CD45−CD11b− for infiltrating macrophages and activated microglia (35). In addition to more CD45+CD11b+ cells, we found that the number of CD45−CD11b− microglia was significantly increased after CD11b-shIFN-γ treatment, indicating a negative regulatory mechanism of IFN-γ signaling in microglia proliferation. This is consistent with our in vitro experiment in which IFN-γ or IFN-γ + TNF inhibited microglia proliferation but did not induce apoptosis or cell death. It has been reported that IFN-γ can inhibit macrophage/microglia proliferation by arresting the cell cycle at the G1/S boundary (40). We found that expression of p21, a cyclin-dependent kinase inhibitor that inhibits activity of the cyclin E–CDK2 complex, thereby arresting the cell cycle at the G1/S phase (41), was significantly increased in vitro-cultured microglia, whereas expression of c-myc, which controls G1/S transition by activating cyclin–CDK complexes and inducing transcription of genes required for S phase (42), was greatly decreased. The increased p21 and decreased c-myc may contribute to the IFN-γ–induced inhibitory effect on the proliferation of microglia through cell-cycle arrest at the G1/S stage. These results indicate that the dominant effect of IFN-γ signaling on microglia is to restrict cell numbers, and lacking IFN-γ signaling results in uncontrolled microglial proliferation, thus promoting disease severity. In addition to the known mechanisms of IFN-γ signaling in promoting regulatory T cells (16, 52) and suppressing Th17 cells (13) in the peripheral immune system, our study provides a novel, CNS-restricted mechanism underlying the enhanced CNS inflammatory demyelination in mice lacking IFN-γ or the IFN-γR (10–12).

We have found that lacking IFN-γ binding/signaling in astrocytes alone resulted in reduced EAE, whereas lacking this binding/signaling in microglia alone resulted in enhanced EAE. When IFN-γ signaling in all CNS cells is lacking (i.e., in IFN-γR−/− mice), however, the disease is more severe (10–12). We believe that the mechanism underlying this phenomenon is that, whereas IFN-γ signaling exerts a differential effect in astrocytes and microglia, lacking IFN-γ signaling in microglia (enhanced EAE) may exert a predominant effect over its signaling in astrocytes (reduced EAE), which would overcome the effect of the latter and...
result in a more severe EAE. Although we have tried to define certain mechanism underlying our findings, the fine mechanisms of IFN-γ signaling in these cells are worthy of follow-up in future studies.

In summary, our data demonstrate that IFN-γ signaling in astrocytes versus microglia cells plays opposite roles in EAE development. Blocking IFN-γ binding/signaling in astrocytes alleviates EAE by decreasing chemokine expression, whereas silencing IFN-γ binding/signaling in microglia, and likely in some infiltrating macrophages, promotes disease severity via enhanced proliferation of these cells. Further, silencing IFN-γ signaling in astrocytes effectively suppressed ongoing EAE, and this approach neither altered the peripheral immune system nor diminished the beneficial effect of astrocytes (e.g., production of BDNF and NT-3). Together, the results obtained in this study will not only help us understand the complex role of IFN-γ in the development of EAE, but will also provide a promising CNS-specific therapeutic approach for MS/EAE treatment by blocking the encephalitogenicity of both myelin-reactive Th1 and Th17 cells, the two major pathogenic mechanisms in CNS autoimmunity.

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Disclosures
The authors have no financial conflicts of interest.

References

Fig. S1. pLenti-CD11b-EGFP-MCS lentiviral EGFP expression in CNS microglia. 1×10^7 IU/mouse of CD11b-shvec and control lentiviruses were given to the CNS of naïve C57Bl/6 mice through i.c.v. injection. Three days later, the mice were perfused and the spinal cords were removed and fresh frozen. Sections of the spinal cord were costained with different CNS-resident cell markers and EGFP. Iba-1: microglia; NG2: oligodendrocyte progenitor cells; MBP: oligodendrocyte; β-tubulin III: neuron; GFAP: astrocyte.
Fig. S2 Peripheral immune system was not impacted by i.c.v. injection of GFAP-shIFN-γR lentivirus. Peripheral immune system was not impacted by i.c.v. injection of GFAP-shIFN-γR lentivirus. Splenocytes were harvested at the end point of the experiment shown in Figure 1g. (a) Total spleen cell numbers were counted. (b) The frequency of IFN-γ+ Th1 cells and IL-17+ Th17 cells in gated CD4+ T cells was measured by flow cytometry. (c) T cell proliferation determined by 3H-incorporation. Results are shown as mean ± SD (n=10 each group). One representative of three experiments is shown. (d) After 3 days’ culture, cytokine production in culture supernatants was determined by ELISA. n.s., not significant.
Fig. S3 Peripheral immune system was not impacted by i.c.v. injection of CD11b-shIFN-γR lentivirus. Splenocytes were harvested at the end point of the experiment shown in Figure 1H. (a) Total spleen cell numbers were counted. (b) The frequency of different inflammatory cells in spleen were measured by flow cytometry. (c) The frequency of IFN-γ+ Th1 cells and IL-17+ Th17 cells in gated CD4+ T cells were measured by flow cytometry. (d) The regulatory T cells population was determined by CD4+CD25+FoxP3+ cell populations between groups. One representative of three independent experiments was shown.
### Table S1. Primers used for vector construction

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