IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Interferon- γ Safeguards Blood-Brain Barrier during Experimental Autoimmune Encephalomyelitis

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The function of blood-brain barrier is often disrupted during the progression of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). However, the molecular mechanism of blood-brain barrier modulation during neuroinflammation remains unclear. Herein, we show that the expression of interferon- γ (IFN γ) receptor on endothelial cells (ECs) protected mice from the brain inflammation during EAE. IFN γ stabilized the integrity of the cerebral endothelium and prevented the infiltration of leukocytes into the brain. Further analysis revealed that IFN γ increased the expression of tight junction proteins zonula occludens protein 1 and occludin, as well as membranous distribution of claudin-5, in brain ECs. Silencing claudin-5 abolished the IFN γ -mediated improvement of EC integrity. Taken together, our results show that IFN γ , a pleiotropic proinflammatory cytokine, stabilizes blood-brain barrier integrity and, therefore, prevents brain inflammation during EAE. (*Am J Pathol* 2014, 184: 3308–3320; <http://dx.doi.org/10.1016/j.ajpath.2014.08.019>)

Experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (MS), is an autoimmune demyelination disease of the central nervous system (CNS).¹ It is initiated with peripheral activation and expansion of myelin-specific T cells. The autoimmune T cells first overcome the blood-brain barrier (BBB), and enter subsequently into the CNS. After reactivation in the CNS, the T cells produce a spectrum of cytokines that further affects BBB function. This process enables the entry of many leukocytes, ultimately resulting in demyelization and neuronal damage.^{2,3}

Mice with EAE often display ascending flaccid paralysis due to spinal cord inflammation, a phenotype described as a classic symptom. In other EAE models, brain inflammation—associated atypical symptoms, such as leaning or circling, ataxia, hyperreflexivity, and proprioception defects, have been observed.^{4,5} In such atypical EAE disease, brain inflammation, accompanied by infiltration of CD4⁺ T cells, macrophages, and neutrophils, mainly occurs in the cerebellum and brainstem.^{4,5}

The BBB is composed mainly of brain capillary endothelial cells (ECs), which are interconnected by tight

junctions (TJs). These junctions are composed of a complex of transmembrane proteins, including occludin (OCLN) and various claudins, as well as cytoplasmic scaffolding proteins, such as zonula occludens protein 1 (ZO-1).^{6,7} During EAE, the integrity of BBB is tightly regulated by a plethora of inflammatory cytokines.⁸ For example, tumor necrosis factor- α (TNF α) increases the permeability of the BBB by reducing the expression of OCLN and claudin-5,⁹ whereas vascular endothelial growth factor affects the BBB integrity by down-regulating claudin-5.¹⁰ In addition, interleukin-17 (IL-17) also diminishes the expression of ZO-1 and OCLN.¹¹ It has been demonstrated that the BBB disruption

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is an early key event during the development of brain inflammation in both EAE and MS.^{2,12} Pharmacological normalization of BBB might lead to amelioration of neuroinflammation.^{12–14} Until now, the molecular mechanism governing the modulation of BBB remains to be elucidated.

It is well accepted that interferon γ (IFN γ)-secreting CD4⁺ T cells are primary mediators of EAE^{4,15}; however, IFN γ ^{−/−} or IFN γ receptor (IFN γ R)^{−/−} mice develop more severe EAE than their wild-type (WT) counterparts.^{16–18} Especially, brain inflammation—associated atypical symptoms, which are not common in WT mice, are often observed in IFN γ ^{−/−} or IFN γ R^{−/−} mice during EAE.^{19–21} This is accompanied with a massive infiltration of leukocytes, including neutrophils, in the brain. The molecular mechanism governing how IFN γ regulates atypical EAE is still not clear.

Herein, by reconstitution of IFN γ R on ECs in IFN γ R^{−/−} mice, we show that IFN γ prevents brain inflammation in EAE by improving BBB function. The action of IFN γ on ECs represents a novel mechanism for immune quiescence in the brain during inflammation, and this may have some implications for the treatment of neuroinflammation.

Materials and Methods

Mice

C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China). The enhanced green fluorescent protein (eGFP) and IFN γ R^{−/−} mice congenic to C57BL/6 were originally obtained from Jackson Laboratories (Bar Harbor, ME). Tie2-IFN γ R mice have been described in our previous study.²² The genome of Tie2-IFN γ R mice is composed of a transgene containing IFN γ R cDNA under the control of *Tie2* promoter with an endogenous *IFN γ R*-deficient genomic background. Three lines of Tie2-IFN γ R mice (W16, W25, and W41) were established. Two lines (W16 and W25) were used in this study. Most results presented herein are from experiments using W25. All mice were housed in a specific pathogen-free facility of the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China), and the animal studies were conducted under protocols approved by the institutional animal care and use committee.

EAE Model

To induce active EAE, mice aged 8 to 12 weeks were immunized s.c. with 300 μ g MOG_{35–55} peptide (MEVG-WYRSPFSRVVHLYRNGK; ChinaPeptides, Shanghai, China), emulsified in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) on day 0. Pertussis toxin (200 ng per mouse; Sigma-Aldrich) was i.p. injected on days 0 and 2.

Passive EAE was induced by adoptive transfer of 3×10^7 spleen cells from MOG_{35–55} immunized Tie2-IFN γ R mice into sublethally irradiated (3.5-Gy) recipient mice. The proportion of MOG_{35–55} reactive T cells in these spleen cells was evaluated by mouse IFN γ ELISPOT (BD Biosciences,

Franklin Lakes, NJ) and approximately 20 MOG_{35–55} reactive T cells could be detected in 1×10^5 spleen cells. To induce passive EAE, 1×10^7 spleen cells from MOG_{35–55} immunized mice were suspended per milliliter of culture medium and stimulated with 10 μ g/mL MOG_{35–55} for 3 days. After removing dead cells by Ficoll-Paque (GE Healthcare, Shanghai, China) gradient, 3×10^7 spleen cells were i.p. transferred to irradiated mice. Pertussis toxin (200 ng per mouse) was i.p. injected on days 0 and 2. Mice were examined daily for clinical symptoms.

Clinical signs of classic symptoms were assessed according to the following scores²³: 0, no signs of disease; 0.5, partial loss of tone in the tail; 1, complete loss of tone in the tail; 2, hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; and 5, moribund. Atypical disease was assigned if symptoms of head tilt, spinning, axial rotation, or ataxia occurred.⁵

Bone Marrow Chimeric Mice

Bone marrow chimeric mice were constructed as described previously.^{22,24} Briefly, freshly prepared bone marrow cells of 6- to 8-week-old female IFN γ R^{−/−} or Tie2-IFN γ R mice were i.v. (retro-orbitally) injected into lethally irradiated (10-Gy) recipient mice (1×10^7 cells per mouse). The following groups of bone marrow chimera were established: IFN γ R^{−/−} $\rightarrow$ IFN γ R^{−/−}, Tie2-IFN γ R $\rightarrow$ IFN γ R^{−/−}, IFN γ R^{−/−} $\rightarrow$ Tie2-IFN γ R, Tie2-IFN γ R $\rightarrow$ Tie2-IFN γ R. Irradiated mice without bone marrow transplantation died within 2 weeks. Successful reconstitution of the hematopoietic system was also determined by PCR analysis of *IFN γ R* gene in peripheral blood mononuclear cells 8 weeks after transplantation.²² Eight weeks after bone marrow transplantation, mice were immunized to induce active EAE, as described above. To evaluate the degree of chimerism, eGFP $\rightarrow$ IFN γ R^{−/−} bone marrow transplantation was included and the mice were examined at 8 weeks after transplantation.

Neutralization of IFN γ in Tie2-IFN γ R Mice

To neutralize endogenous IFN γ , Tie2-IFN γ R mice were i.p. injected with rat anti-mouse IFN γ monoclonal antibody (300 μ g per mouse; R4-6A2, kept in our laboratory) or with an isotype-matched control monoclonal antibody (rat IgG).²⁵ The mice were divided into four groups with anti-IFN γ injection at the whole stage (days -1, 4, 14, and 24), at an early stage (days -1 and 4), and at a late stage (days 14 and 24), as well as with control antibody through the entire experiment (days -1, 4, 14, and 24). WT and IFN γ R^{−/−} mice were used as control. All mice were immunized to induce active EAE on day 0, as described above.

FACS Analysis

CNS mononuclear cells from perfused mice at peak EAE (day 20) were isolated. The following antibodies were used: CD11b, CD45, and CD4 (BD Biosciences). IFN γ R expression on the cell surface was determined using a

biotinylated rat anti-mouse IFN γ R monoclonal antibody (GR20; BD Pharmingen, Franklin Lakes, NJ). Allophycocyanin (APC)-conjugated streptavidin (BD Pharmingen) was used as secondary antibody. Samples were analyzed on fluorescence-activated cell sorter (FACS) Calibur (BD Pharmingen).

Transmission Electronic Microscopy

Tissues for electronic microscopy were fixed in 2.5% glutaraldehyde for 24 hours, followed by staining with 1% osmium tetroxide for 2 hours; and dehydrating in graded ethanol. Tissue was then embedded in plastics (Araldite based). Thin sections were made from plastic blocks and stained with uranyl acetate/lead citrate. Section slices were placed on copper grids, and images were taken using the transmission electronic microscope (Tecnai G2F20; FEI Company, Hillsboro, OR), which was operated at 220 kV, and mounted with a Gatan 2kx2k charge-coupled device camera (Gatan GmbH, München, Germany).

FITC-Dextran Injection and Carboxyfluorescein Succinimidyl Ester—Labeled Spleen Cell Transfer

To detect BBB disruption *in vivo*, WT, Tie2-IFN γ R, and IFN γ R^{-/-} mice at the peak of EAE (day 20) were i.v. (retro-orbitally) injected with 100 μ L fluorescein isothiocyanate (FITC)—dextran (1 mg/mL; 40 kDa; Sigma-Aldrich). After 2 hours, experimental mice were anesthetized and perfused with phosphate-buffered saline to pour out the vascular contents. Then, cerebellum was collected and embedded. The diffusion of FITC-dextran was detected on frozen sections.

To detect the migration of pathogenic inflammatory cells into the brain, 1×10^7 spleen cells isolated from MOG₃₅₋₅₅ immunized IFN γ R^{-/-} mice were labeled with carboxy-fluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA) and i.p. transferred into WT, Tie2-IFN γ R, and IFN γ R^{-/-} mice at the peak of EAE (day 20). After 48 hours, the mice were perfused and mononuclear cells were isolated from the whole brain. CD45⁺CD4⁺CFSE⁺ and CD45⁺CD4⁻CFSE⁺ cells were detected by FACS.

EC Culture

Mouse brain endothelial line bEND.3 was originally derived from ATCC (American Type Culture Collection, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (PAA, Pasching, Austria). The bEND.3 cells maintain the most important properties of brain ECs and are widely accepted in brain vascular research as a model to study BBB *in vitro*, according to many studies.^{26,27} For Western blot analysis, bEND.3 cells were seeded in a 6-well plate. On reaching confluency, the cells were serum starved in Dulbecco's modified Eagle's medium containing 2% fetal bovine serum for 2 hours, and then stimulated with 10 ng/mL IFN γ (PeproTech, Rocky Hill, NJ). Extracts were prepared

for both whole lysates and membrane fractions. Protein lysates were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and detected by immunoblotting using antibodies against ZO-1, OCLN, claudin-5 (Invitrogen), Akt, p-Akt (S473), β -catenin, p- β -catenin (S552; CST, Shanghai, China), and β -actin (Sigma-Aldrich).

Migration Assays

Brain endothelial bEND.3 cells were placed on 3- μ m pore size Boyden chambers (Corning, Shanghai, China) at a density of 2×10^4 cells per well and were grown for 24 hours to reach confluence. Fresh 2×10^6 CFSE-labeled spleen cells from MOG₃₅₋₅₅ immunized IFN γ R^{-/-} mice were then added to the upper chambers. Meanwhile, 10 ng/mL IFN γ was added in the upper chambers. Twenty-four hours after spleen cell application, migration of immune cells was assessed by detecting and counting the CFSE⁺ cells in the lower chambers using flow cytometry.

Histopathological and Immunohistochemical Analyses

Brain and spinal cord tissues from perfused mice were collected for histopathological and immunochemical analyses. Paraffin transverse CNS sections (7 μ m thick) were stained with hematoxylin and eosin (H&E; Zhongshanjinqiao, Beijing, China) or luxol fast blue (Genmed, Shanghai, China). The severity of inflammation was scored according to the modified criteria, as published on H&E staining²⁸: 0, no infiltrate or focal meningeal infiltrates; 1, sparse infiltrates in the parenchyma; 2, severe perivascular infiltrate foci in the parenchyma with involvement of <5% of the white matter; 3, invasions involving 5% to 25% of the white matter; and 4, diffuse infiltration involving >25% of the white matter.

Frozen transverse sections (7 μ m thick) of the CNS were stained for CD45, CD4, CD11b, Gr1 (BD Pharmingen), Tie2 (R&D, Shanghai, China), and claudin-5 (Invitrogen), using anti-rat-IgG—horseradish peroxidase (Zhongshanjinqiao), anti-rat-IgG-Alexa Fluor-555, or anti-rabbit-IgG-Alexa Fluor-488 (Invitrogen) for detection. Images were created using an Olympus BX51 microscope (Olympus, Tokyo, Japan). To evaluate the amount of fluorescence content, the integrated optical density

$$(\text{IOD} = \text{area} \times \text{average density}) \quad (1)$$

was calculated.²⁹ Colocalization of Tie2 and claudin-5 signals was analyzed by measuring the ratio of claudin-5 signal in Tie2 signal. Integrated optical density and colocalization were both determined with Image-Pro Plus (Media Cybernetics, Rockville, MD).

Assessment of Paracellular Tightness

Paracellular tightness of the EC monolayer was determined by measuring the transendothelial electrical impedance using an xCELLigence System RTCA-SP instrument (ACEA

Biosciences Inc., San Diego, CA).³⁰ Briefly, 20,000 bEND.3 cells were seeded into the 96-well E-Plate (ACEA Biosciences Inc.) and monitored using the RTCA-SP instrument. Each group included at least triplicates. Impedance, indicated as cell index, was taken automatically every 15 minutes. Once cell index values reached their peak values, indicating that ECs have formed a monolayer, stimulators were added to the culture. The cell index value on stimulator application was adopted as a reference point. The impedance was analyzed as normalized cell index and presented as paracellular tightness. All experiments were repeated at least three times.

To assess the effect of IFN γ on paracellular tightness after knockdown of claudin-5, 1×10^4 bEND.3 cells were plated into a 96-well E-plate. The cell culture was permitted to reach approximately 90% confluence within 24 hours. bEND.3 cells were subsequently transfected with siRNA targeting claudin-5 (siClaudin-5; Santa Cruz Biotechnology, Dallas, TX) using Lipofectamine 2000 (Invitrogen). After 16 to 24 hours, culture medium was changed. When the trans-endothelial electrical impedance reached its peak value, IFN γ was added to the cell monolayer, together with siClaudin-5.

Knockdown of OCLN and Claudin-5

For OCLN knockdown, oligonucleotides against mouse *OCLN* gene were synthesized (Sangon Biotech, Shanghai, China) and cloned to pSUPER.retro.puro vectors (OligoEngine, Seattle, WA): shOCLN-1, 5'-GCACCAAGCAATGACATGT-3'; shOCLN-2, 5'-TGCGGAAAGAGTTGACAGT-3'. Brain endothelial bEND.3 cells were transfected with pSUPER-shOCLN. Cells were then selected for the stable expression of shOCLN using puromycin (Amresco, Solon, OH), and the resulting cells were then referred to as bEND.3-shOCLN. For claudin-5 knockdown, a pool of three target-specific 20- to 25-nucleotide siRNAs against mouse claudin-5 gene (Santa Cruz Biotechnology) was used for transfection of bEND.3 cells following the manufacturer's instruction.

Statistical Analysis

Data for percentage of atypical symptoms were analyzed by survival curve method (log-rank test) in GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA). Differences between classic score and paracellular tightness data sets were analyzed by two-way analysis of variance. Differences between groups were analyzed by one-way analysis of variance or Student's *t*-test.

Results

Reconstitution of IFN γ Expression on Tie2⁺ Cells Prevents Atypical Symptoms in IFN γ -Deficient Mice during EAE

To study the responsiveness of ECs to IFN γ during EAE, we used Tie2-IFN γ R mice, in which the expression of IFN γ R is

restricted to Tie2⁺ cells.²² In brain, the IFN γ R was expressed on ECs, and it was also functional in these mice (Supplemental Figure S1). A minimal expression of IFN γ R mRNA could be detected in Tie2⁺ bone marrow-derived cells in Tie2-IFN γ R mice; however, the expression of IFN γ R protein on the surface of hematopoietic cells is hardly detectable.²²

EAE was induced in Tie2-IFN γ R mice, together with WT and IFN γ R^{-/-} mice as control. Both Tie2-IFN γ R and IFN γ R^{-/-} mice developed more severe classic symptoms (i.e., ascending flaccid paralysis) than the control WT mice (Figure 1A). There was no significant difference between Tie2-IFN γ R and IFN γ R^{-/-} mice in terms of the incidence rate [28 of 38 (73.7%) versus 25 of 36 (69.4%)] and the severity of classic symptoms (Supplemental Table S1 and Figure 1A). However, atypical symptoms, including ataxia, decreased in Tie2-IFN γ R mice, similar to WT mice, when compared with IFN γ R^{-/-} mice (Figure 1B). Atypical EAE occurred only in 13.1% (5 out of 38) of Tie2-IFN γ R mice, but in 66.7% (24 out of 36) of IFN γ R^{-/-} mice (Supplemental Table S1). These results indicate that the reconstitution of IFN γ R on Tie2⁺ cells ameliorates atypical rather than classic EAE symptoms of IFN γ R-deficient mice.

To avoid the influence of the integration site of the transgene, two lines (W16 and W25) of Tie2-IFN γ R mice were used in this study. Although they express different levels of IFN γ R,²² similar results were obtained in both lines (Supplemental Table S1). This is also in agreement with the previous observation that the extent of IFN γ R expression appears to have no significant influences on the magnitude of IFN γ -induced responses.^{31,32}

IFN γ R Expression on Tie2⁺ Cells Is Sufficient to Prevent Brain Inflammation

The symptoms of EAE are closely associated with regional inflammation in the CNS.^{4,5} Classic symptoms are caused by leukocyte infiltration in the spinal cord, whereas atypical symptoms are caused by leukocyte infiltration in the brain.^{4,5} In IFN γ R^{-/-} mice, both brain and spinal cord inflammation was observed in EAE. In Tie2-IFN γ R mice, cerebellum and brainstem inflammation was drastically inhibited, whereas the severity of spinal cord inflammation was comparable to IFN γ R^{-/-} mice (Figure 2, A–D, and Supplemental Figure S2A). Accordingly, in Tie2-IFN γ R and WT mice, cerebellum and brainstem sections exhibited lower levels of infiltration of CD45⁺, CD4⁺, CD11b⁺, and Gr1⁺ inflammatory cells in comparison to that in IFN γ R^{-/-} mice (Supplemental Figure S2, B and C). In contrast, spinal cord sections of Tie2-IFN γ R and IFN γ R^{-/-} mice showed similar levels of infiltration of inflammatory cells, both being much higher than that of WT mice (Supplemental Figure S2D).

To characterize the nature of the inflammatory cells at the peak of EAE, mononuclear cells in the brain and spinal cord of diseased mice were isolated and analyzed by FACS. The numbers of CD45^{hi}, especially CD45^{hi}CD11b⁺ inflammatory cells, significantly decreased in the brain of

Figure 1 The reconstitution of IFN γ R expression on Tie2⁺ cells protects mice from atypical, rather than classic, symptoms in EAE. **A:** Mean clinical score of classic symptoms. Data expressed as means $\pm$ SEM. **B:** The percentage of mice with atypical symptoms determined in WT ($n = 16$), IFN γ R^{-/-} ($n = 12$), and Tie2-IFN γ R ($n = 14$) mice after induction of EAE with MOG₃₅₋₅₅ immunization. ** $P < 0.01$, *** $P < 0.001$.

Tie2-IFN γ R and WT mice, when compared with IFN γ R^{-/-} mice (Figure 2E). The demyelination in the cerebellum was also inhibited in both Tie2-IFN γ R and WT mice when compared with IFN γ R^{-/-} mice (Figure 2F). However, it is not the case in the spinal cord; high infiltration of CD45^{hi}CD11b⁺ cells was found in both Tie2-IFN γ R and IFN γ R^{-/-} mice, but not in WT mice (Supplemental Figure S2E).

To exclude the influence of developmental defects in Tie2-IFN γ R transgenic mice, IFN γ was neutralized by a specific anti-IFN γ monoclonal antibody at different stages of EAE. Neutralization of IFN γ at either early or whole stages of EAE totally eliminated the protection against atypical symptoms in Tie2-IFN γ R mice (Figure 2G). IFN γ neutralization at a late stage of EAE (days 14 and 24) elicited atypical symptoms to a lesser extent [2 of 7 (28.9%)], indicating that IFN γ acts mainly at the early stage of the

disease. Together, these results demonstrate that IFN γ R on Tie2⁺ cells efficiently inhibits brain, but not spinal cord, inflammation.

The Expression of IFN γ R by Tie2⁺ Bone Marrow-Derived Cells Is Not Responsible for the Prevention of Brain Inflammation in Tie2-IFN γ R Mice

Because the expression of IFN γ R mRNA by Tie2⁺ bone marrow-derived cells was also detected in addition to ECs,²² the potential role of IFN γ R expressed by Tie2⁺ bone marrow-derived cells in the progression of brain inflammation was evaluated. Lethally irradiated IFN γ R^{-/-} and Tie2-IFN γ R mice were reconstituted with bone marrow cells from either Tie2-IFN γ R or IFN γ R^{-/-} mice. To monitor the degree of chimerism of bone marrow chimeric mice, the eGFP transgenic mice were used. At 8 weeks after transplantation, almost all leukocytes of recipient mice have been substituted by those of donor mice (Supplemental Figure S3A). Indeed, the recruited leukocytes in both brain and spinal cord were mostly from the donor mice. For unknown reasons, approximately half of the microglia were from donor in both brain and spinal cord (Supplemental Figure S3B).

Eight weeks after bone marrow transplantation, the mice were immunized to induce EAE. Similar to IFN γ R^{-/-} $\rightarrow$ IFN γ R^{-/-} mice [5 of 8 (62%)], 6 of 10 Tie2-IFN γ R $\rightarrow$ IFN γ R^{-/-} mice (60%) developed atypical symptoms (Figure 3A and Supplemental Table S2). In contrast, neither IFN γ R^{-/-} $\rightarrow$ Tie2-IFN γ R (0/9) nor Tie2-IFN γ R $\rightarrow$ Tie2-IFN γ R (0/7) mice develop atypical symptoms. In addition, apart from atypical symptoms, these mice all had similar severity of classic symptoms (Figure 3B). Taken together, these results demonstrate that IFN γ R expression on Tie2⁺ ECs, rather than Tie2⁺ bone marrow-derived cells, is crucial to inhibit brain inflammation-associated atypical symptoms of EAE.

Endothelial Expression of IFN γ R Is Necessary for Maintaining BBB Function

Because the opening of BBB is a dominant factor for the entry of inflammatory cells into the CNS, the effect of endothelial IFN γ R on BBB function was examined. When i.v. injected into EAE mice, FITC-dextran was found to massively diffuse into the granular layer and white matter of the cerebellum, in IFN γ R^{-/-} mice rather than in Tie2-IFN γ R mice (Figure 4A). Subsequently, CFSE-labeled spleen cells from MOG₃₅₋₅₅ immunized IFN γ R^{-/-} mice were adoptively transferred to different groups of mice with EAE. More CFSE⁺ cells (either CD4⁺ or CD4⁻) were recovered in the brain of IFN γ R^{-/-} mice in comparison to Tie2-IFN γ R and WT mice (Figure 4B). The proportion of CD4⁺ cells in total CFSE⁺ cells was higher in the brain than in the peripheral blood, implying that the CD4⁺ cells are more prone to cross the BBB (Figure 4C).

Figure 2 Brain inflammation is ameliorated in Tie2-IFN γ R mice in comparison to IFN γ R^{-/-} mice. Groups of mice ($n = 5$ to 8) were induced to develop EAE for 40 days, as indicated, or left untreated as control (naïve). H&E staining of cerebellum (**A**) or spinal cord (**C**) sections was used to determine inflammation scores (**B** or **D**). The **arrows** show infiltrating cells. **E**: The brain infiltrating cells from different groups of mice ($n = 3$), as described above, were isolated on day 20 on EAE induction and stained for CD45 and CD11b. The graphs show the proportion of inflammatory CD45^{hi}CD11b⁻ and CD45^{hi}CD11b⁺ in CD45⁺ cells. Data expressed as means $\pm$ SEM. **F**: Luxol fast blue staining of cerebellum sections was used to show the demyelination (**arrow**) of indicated mice on day 40 on EAE induction. **G**: Atypical symptoms developed in Tie2-IFN γ R mice without or with IFN γ neutralization at different stages of EAE. The WT and IFN γ R^{-/-} mice were used as control. Each group contained seven mice. **Gray arrows** indicate early-stage neutralization, on days -1 and 4. **White arrows** indicate late-stage neutralization, on days 14 and 24. **Black arrows** indicate whole-stage neutralization, on days -1, 4, 14, and 24. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 100 μ m (**A** and **C**); 500 μ m (**F**).

Whether the functional improvement of BBB by IFN γ R expression on ECs could retard the induction of brain inflammation by pathogenic T cells in healthy naïve mice was also examined (Supplemental Figure S4). Adoptive

transfer of spleen cells from MOG₃₅₋₅₅ immunized Tie2-IFN γ R mice induced atypical disease in 60% (3 out of 5) of the recipient IFN γ R^{-/-} mice, whereas no atypical disease was observed in Tie2-IFN γ R (0 out of 5) or WT (0 out of 5)

recipient mice (Figure 4D). Taken together, these results strongly suggest that endothelial IFN γ R signaling is necessary for the maintenance of BBB function.

IFN γ Strengthens the Paracellular Tightness of Brain ECs

The increased permeability of BBB to soluble factors and inflammatory cells implies the disruption of BBB during CNS inflammation in mice, whose ECs lack IFN γ R expression. To investigate the ultrastructure of endothelial TJs, transmission electronic microscope analysis was performed with the cerebellum tissues from mice with EAE. In tissues from IFN γ R^{-/-} mice with atypical symptoms, alterations and gaps between adjacent capillary ECs were developed, accompanied by myelin sheath disintegration in the parenchyma. Moreover, the basement membrane degraded and separated from brain capillary ECs, along with infiltration of inflammatory cells (Figure 4E and Supplemental Figure S5). In contrast, TJs were unaffected except for slight distortions of the luminal surface of the ECs in Tie2-IFN γ R and WT mice with EAE (Figure 4E).

To directly demonstrate that IFN γ could promote the junction of brain ECs, the paracellular tightness of the brain ECs, bEND.3, was used by measuring the transendothelial electrical impedance. Changes in the impedance reflect modification of paracellular tightness of a cell monolayer.³⁰ The paracellular tightness of the bEND.3 monolayer was increased by IFN γ stimulation in a dose-dependent manner *in vitro* (Figure 5A). Furthermore, IFN γ counteracted the disruptive effect of proinflammatory cytokines, such as TNF α or IL-17, on the integrity of the cell monolayer (Figure 5, B–D). To mimic the inflammatory cytokine milieu encountered by the BBB *in vivo*, supernatants of MOG₃₅₋₅₅-stimulated spleen cells from immunized IFN γ R^{-/-} mice were collected and added to the bEND.3 monolayer. The concentration of IFN γ , TNF α , and IL-17 increased in the supernatant of the spleen cell culture after MOG₃₅₋₅₅ stimulation (Figure 5E). When compared with the supernatant of MOG₃₅₋₅₅-stimulated spleen cells from naïve mice, the paracellular tightness of the monolayer was found to be enhanced by the supernatant from immunized mice (Figure 5F). Neutralization of IFN γ in the supernatant from immunized mice decreased the paracellular tightness of the cell monolayer, whereas addition of IFN γ to the supernatant enhanced it (Figure 5G). Moreover, the migration of spleen cells from immunized IFN γ R^{-/-} mice across the bEND.3 cell monolayer was also inhibited by the IFN γ treatment (Figure 5H). The above results suggest that IFN γ directly stabilizes the paracellular tightness of brain ECs.

IFN γ Enhances the Expression and Membranous Distribution of TJ Components

To further define the molecular mechanism responsible for the IFN γ -mediated improvement of EC tightness, the

Figure 3 The expression of IFN γ on Tie2⁺ ECs, but not Tie2⁺ bone marrow (BM) cells, is essential for the prevention of atypical EAE. Bone marrow chimeric mice were established: IFN γ R^{-/-} → IFN γ R^{-/-} ($n = 8$), Tie2-IFN γ R → IFN γ R^{-/-} ($n = 10$), IFN γ R^{-/-} → Tie2-IFN γ R ($n = 9$), Tie2-IFN γ R → Tie2-IFN γ R ($n = 7$). Eight weeks later, mice were immunized with MOG₃₅₋₅₅ to induce EAE. **A:** The percentage of bone marrow chimeric mice with atypical symptoms. **B:** Mean clinical score of classic symptoms in bone marrow chimeric mice. Data expressed as means $\pm$ SEM.

expression of TJ proteins in IFN γ -stimulated bEND.3 cells was determined. The results showed that, on exposure to IFN γ , the expression of ZO-1 and OCLN was rapidly up-regulated, reaching maximum levels, within 4 and 12 hours, respectively. Thereupon, both ZO-1 and OCLN maintained high expression levels for at least 12 hours (Figure 6, A and B). In comparison, the expression of claudin-5 reached the maximum levels at approximately 8 hours, but decreased within 16 hours after IFN γ stimulation (Figure 6C). Interestingly, the membrane fractions of both OCLN and

Figure 4 The functional integrity of BBB is protected in Tie2-IFN γ R mice compared with IFN γ R $^{-/-}$ mice. **A:** Diffusion of fluorescence into the cerebellum of WT, IFN γ R $^{-/-}$, or Tie2-IFN γ R mice ($n = 3$). The mice with EAE (day 20) were i.v. injected with FITC-dextran and, 2 hours later, perfused with phosphate-buffered saline. Tissue cryosections were prepared to examine fluorescence diffusion. Fluorescence content, indicated by integrated optical density, was calculated according to the microscopic images (12 random images per group). **B:** CFSE-labeled spleen cells (1×10^7) from immunized IFN γ R $^{-/-}$ mice were adoptively transferred into WT, IFN γ R $^{-/-}$, or Tie2-IFN γ R mice with EAE (day 20), and the migration of the spleen cells into the brain was determined 48 hours after adoptive transfer. CD45 $^{+}$ brain mononuclear cells were gated and analyzed for CFSE/CD4. **C:** The proportion of CD4 $^{+}$ cells in CFSE $^{+}$ cells from the blood and brain of IFN γ R $^{-/-}$ mice. **D:** Atypical symptoms in WT, IFN γ R $^{-/-}$, or Tie2-IFN γ R mice ($n = 5$) after adoptive transfer of MOG₃₅₋₅₅ immunized and restimulated spleen cells from Tie2-IFN γ R mice (Tie2-IFN γ R versus IFN γ R $^{-/-}$). **E:** Electronic micrographs show the disrupted TJs between endothelial cells (EC) of cerebellum in diseased IFN γ R $^{-/-}$ mice (day 20). The WT, Tie2-IFN γ R mice with EAE, and naïve IFN γ R $^{-/-}$ mice without EAE were used as control. The dashed white lines indicate TJs with high electron density. Arrowheads indicate myelin sheath disintegration. Arrows indicate slight distortions of the luminal surface of the EC. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 100 μ m (A); 500 nm (E). G, granular layer; L, lumen; M, molecular layer; W, white matter.

claudin-5 were increased after IFN γ treatment (Figure 6, D and E). Accordingly, the up-regulation of ZO-1 and OCLN by IFN γ stimulation was also observed in primary brain ECs (Supplemental Figure S6).

To study the downstream signaling pathway by which IFN γ enhances TJ proteins, several related signaling mediators were examined. Akt and β -catenin were activated shortly after IFN γ stimulation, and the accumulation of β -catenin was observed (Figure 6F). The β -catenin may act as an upstream regulator of TJ components, and the accumulation of β -catenin induced by IFN γ is potentially responsible for the functional promotion of TJs.

Because claudin-5 is a major contributor to the TJ complex of BBB *in vivo*,³³ its expression at cerebral microvessels from diseased mice was carefully examined. The expression of claudin-5 was down-regulated on Tie2 $^{+}$ brain microvessels in IFN γ R $^{-/-}$ mice, when compared with Tie2-IFN γ R and WT mice (Figure 6G). Together, the results above demonstrate that IFN γ is involved in up-regulating

the expression of TJ components and stabilizing their membranous distribution on adjacent ECs.

Claudin-5 Is Essential for IFN γ -Mediated Improvement of Brain EC Integrity

The key membranous TJ proteins downstream of IFN γ responsiveness on brain ECs were studied. OCLN and claudin-5 were knocked down individually in bEND.3 cells, and the paracellular tightness of the cell monolayer was measured. Under regular culture conditions without IFN γ , the lack of OCLN had no significant impact on the paracellular tightness of the bEND.3 monolayer. On addition of IFN γ , the paracellular tightness was greatly enhanced despite the lack of OCLN (Figure 7, A and B). In contrast, silencing claudin-5 resulted in decreased paracellular tightness (Figure 7, C and D). Addition of IFN γ did not reverse, but instead even further reduced, the paracellular tightness of bEND.3 monolayer (Figure 7E). The results indicate that

Figure 5 IFN γ promotes paracellular tightness of brain EC monolayer. Shown are paracellular tightness of confluent bEND.3 monolayer after stimulation with the following: different concentrations of IFN γ (A), IFN γ , TNF α , or IL-17 (B), TNF α or IFN γ + TNF α (C), and IL-17 or IFN γ + IL-17 (D). The cytokine concentrations used in B–D are as follows: IFN γ , 10 ng/mL; TNF α , 30 ng/mL; IL-17, 30 ng/mL. E: The concentration of IFN γ , TNF α , or IL-17 in the supernatant of spleen cell cultures. Spleen cells (1×10^7 /mL) were isolated from either naïve or MOG₃₅₋₅₅ immunized IFN γ R $^{-/-}$ mice and restimulated with or without MOG₃₅₋₅₅ (10 μ g/mL) for 3 days. F: Paracellular tightness of confluent bEND.3 monolayer after stimulation with control medium, supernatants of MOG₃₅₋₅₅ stimulated spleen cells from either naïve or immunized IFN γ R $^{-/-}$ mice. G: Paracellular tightness of confluent bEND.3 monolayer after stimulation with supernatants of MOG₃₅₋₅₅ restimulated immunized spleen cells alone, together with either 4 μ g/mL α -IFN γ antibody or 10 ng/mL IFN γ . H: Migration of spleen cells from immunized IFN γ R $^{-/-}$ mice through confluent bEND.3 monolayer. Cells were stimulated with 10 ng/mL IFN γ or without stimulation as control. Arrows indicate the start point of stimulation. * $P < 0.05$, *** $P < 0.001$.

the presence of claudin-5, but not OCLN, is essential for IFN γ -mediated improvement of brain EC integrity.

Discussion

Herein, we show, for the first time to our knowledge, that IFN γ safeguards the BBB by stabilizing endothelial TJs and, therefore, protects mice from brain inflammation during EAE. IFN γ could increase the paracellular tightness of brain ECs, enhance the expression of TJ-related proteins ZO-1 and OCLN, and promote the membranous distribution of claudin-5, whereas knockdown of claudin-5 impairs the barrier-protection effect of IFN γ . The finding that an inflammatory cytokine maintains BBB function during brain inflammation may have some implications for developing novel strategies to treat diseases resulting from neuroinflammation, such as MS.

The Tie2-IFN γ R mouse is a reliable model to study the effects of IFN γ on ECs during EAE. First, in these mice, IFN γ R is expressed specifically on the surface of ECs.²² Other cells, such as neurons, astrocytes, and fibroblasts,

are Tie2 $^{-}$ and, therefore, are deficient of IFN γ R.^{22,34} Second, the biological function of endothelial IFN γ R in Tie2-IFN γ R mice could recapitulate that in WT mice during EAE. It is widely accepted that there does not appear to be a direct correlation between the extent of IFN γ R expression and the magnitude of IFN γ -induced responses in cells.^{31,32} Three lines (W16, W25, and W41) of Tie2-IFN γ R mice express different levels of IFN γ R.²² However, two lines (W16 and W25) of Tie2-IFN γ R mice used in this study both received similar results with respect to the progression of EAE in this study. Third, although a subset of Tie2 $^{+}$ bone marrow–derived cells has been reported,^{35,36} the expression of IFN γ R on these cells is hardly detectable in Tie2-IFN γ R mice.²² As for microglia, the macrophage-like cells in the brain, we were unable to detect any expression of IFN γ R on these cells in Tie2-IFN γ R mice too (data not shown). Most important, experiments of bone marrow chimera did not support a role played by the IFN γ responsiveness of these cells during the progression of EAE. Reasons for the impaired expression of IFN γ R protein on these cells are still not clear. IFN γ has been reported to significantly down-regulate Tie2 mRNA level in IFN γ R competent Tie2 $^{+}$

Figure 6 IFN γ enhances expression and membranous distribution of TJ proteins in brain ECs. The expression of ZO-1 (A), OCLN (B), and claudin-5 (C) in whole cell lysates of IFN γ -stimulated confluent bEND.3 cells, as determined with Western blot analysis. The expression of OCLN (D) and claudin-5 (E) in the membrane fractions extracted from IFN γ -stimulated confluent bEND.3, as determined with Western blot analysis. F: The bEND.3 cells were stimulated with IFN γ , and the expression and phosphorylation of Akt and β -catenin were determined. G: Reduced expression of claudin-5 on Tie2⁺ microvessels in cerebellum of diseased IFN γ R^{-/-} mice when compared with WT or Tie2-IFN γ R mice on day 20 ($n = 3$). Colocalization of Tie2 and claudin-5 was calculated and expressed as the ratio of claudin-5 signal in Tie2 signal (six micrographs per group). *** $P < 0.001$. Scale bar = 50 μ m (G).

hematopoietic cells.²² Another possibility is that the transgene in bone marrow-derived cells of Tie2-IFN γ R mice may be severely inactivated because of the genetic/epigenetic mosaicism during development.³⁷ A previous study also reported that Toll-like receptor (TLR) 4 expression under the control of *Tie2* promoter was undetected in hematopoietic cells in the Endo^{TLR4} transgenic mice.³⁸

In this study, the molecular mechanism governing how IFN γ is involved in brain inflammation is further clarified. It has been reported that atypical EAE and brain inflammation developed in IFN γ ^{-/-} or RAG1^{-/-}IFN γ ^{-/-} mice, but not in IFN γ competent mice.^{19,20} By using a passive EAE model, Lees et al²¹ showed that adoptive transfer of WT CD4⁺ T cells to IFN γ R^{-/-} mice elicited brain, but not spinal cord, inflammation. However, the exact targets of IFN γ that affect atypical EAE are still elusive. The results herein demonstrate that the activation of IFN γ signaling in ECs is sufficient and necessary to prevent atypical EAE and brain inflammation. Although IFN γ R is expressed on all ECs in Tie2-IFN γ R mice, the responsiveness of local brain ECs, rather than peripheral and spinal ECs, to IFN γ appears to be responsible for the suppression of brain inflammation. There are no significant changes of peripheral immune responses and spinal cord inflammation between Tie2-IFN γ R and IFN γ R^{-/-} mice. In addition, in a passive EAE model in

which autoreactive T cells could be only reactivated and produce IFN γ in the CNS, the atypical EAE is still inhibited in Tie2-IFN γ R mice.

IFN γ R expression on ECs influences the brain inflammation both quantitatively and qualitatively. IFN γ R deficiency on ECs allows many inflammatory cells, including CD45⁺, CD11b⁺, CD4⁺, and Gr1⁺ cells, to enter into the brain parenchyma during EAE. Notably, the permission of infiltration through the BBB seems to be selective. Adoptively transferred CD4⁺ T cells are more potent than CD4⁻ cells in their ability to penetrate the BBB. This selectivity might be accomplished by orchestrating adhesion molecule expression and BBB opening by IFN γ .³⁹ Key adhesion molecules, such as intercellular adhesion molecule and vascular cell adhesion molecule, expressed by brain ECs could be also affected by IFN γ , which further regulates the adhesion of immune cells.⁴⁰ However, when IFN γ R is present on brain ECs, the adhered inflammatory cells mostly cannot cross the BBB, because of the improvement effects of IFN γ on the integrity of BBB endothelium, as demonstrated by our results. This is further supported by earlier reports demonstrating that TJs between ECs limit the access of soluble factors and circulating cells into the CNS, and the disruption of TJs benefits the entry of inflammatory cells into the CNS.^{12,41,42} As a fundamental proinflammatory

Figure 7 Knockdown of claudin-5, but not OCLN, alters IFN γ -mediated improvement effect on the paracellular tightness of brain EC monolayer. **A:** The expression of OCLN is stably knocked down by a shRNA targeting OCLN in bEND.3 cells (shOCLN). **B:** Paracellular tightness of confluent bEND.3-shControl and bEND.3-shOCLN monolayers stimulated with 100 ng/mL IFN γ . The **arrow** indicates the start of stimulation. **C:** The expression of claudin-5 by bEND.3 cells is knocked down by a siRNA targeting claudin-5 (siClaudin-5). **D:** Paracellular tightness of confluent bEND.3 monolayer after knockdown of claudin-5 (**black arrow** indicates transfection with siRNA targeting claudin-5; **grey arrow** indicates change new medium). **E:** After knockdown of claudin-5, the confluent bEND.3-siClaudin-5 monolayer at plateau was stimulated with or without IFN γ . Paracellular tightness of the cell monolayer was measured. The **arrow** indicates the start of stimulation. Data expressed as means $\pm$ SEM. *** $P < 0.001$.

cytokine, IFN γ -mediated defense of BBB integrity might dedicate to the critical guarding of immune quiescence of brain during inflammation.

An important finding of this work is that IFN γ regulates TJ proteins in several ways. The increased expression of ZO-1, OCLN, and claudin-5 by IFN γ subsequently contributes to their membrane association or membrane distribution. At the same time, knockdown of claudin-5, but not OCLN, is essential for maintaining the paracellular tightness of brain ECs by IFN γ . This is in agreement with the fact that claudin-5 is tightening the paracellular cleft,⁴³ and the observation that the deficiency of claudin-5,³³ but not OCLN,⁴⁴ causes opening of the BBB. Lack of claudin-5 reverses the beneficial effects of IFN γ to destructive. Previous observations also showed that IFN γ disrupted TJs in human umbilical vein ECs.^{45,46} Expression level of claudin-5 might act as a switch between stabilizing and disrupting effects mediated by IFN γ on different cell types.⁷ However, the underlying mechanism is still elusive. As to the downstream signaling mediators, IFN γ activates Akt and downstream β -catenin in brain ECs. The activation of Akt could also induce claudin-5 up-regulation by removing its transcriptional inhibition mediated by FoxO1.⁴⁷ The activation of β -catenin is a crucial CNS-specific signaling for barrier genesis.^{48,49} EC-specific stabilization of β -catenin both *in vivo* and *in vitro* enhances BBB maturation, whereas inactivation of β -catenin in ECs causes BBB breakdown.^{50,51} It is clear that plasma accumulation of β -catenin

promotes the membranous amount of claudin-5.^{47,52} Therefore, accumulation of β -catenin induced by IFN γ is potentially responsible for the functional promotion of TJs. On the other side, nuclear β -catenin binds to FoxO1 to suppress the transcription of claudin-5.⁴⁷ The claudin-5, ZO-1, and β -catenin could bind together to pin up TJ strands.^{52,53} When claudin-5 is absent from the plasma membrane, we speculate that the retention of β -catenin on plasma membrane might decrease, and it enters into the nucleus to inhibit the transcription of alternative claudins that form the TJs.

Another remaining unclear question is why the responsiveness of brain and spinal cord ECs to IFN γ has different outcomes on local tissue inflammation. According to the comparative studies, the BBB and blood-spinal cord barrier (BSCB) have significant intrinsic differences.⁵⁴ The pericyte coverage on BSCB is reduced in comparison to the BBB, and the permeability of BSCB for cytokines increased.⁵⁵ The expression of ZO-1, OCLN, and β -catenin by spinal cord ECs is lower than brain ECs.^{55,56} These distinctions may render BSCB more sensitive to toxic factors, subsequently becoming susceptible to inflammation. Moreover, because of the intrinsic differences between brain and spinal cord ECs, we conjecture that the initiation of downstream signaling mediators might be variable when they respond to IFN γ stimulation.

In clinical practice, IFN γ is closely associated with the progression of MS⁵⁷; however, the effort to explore IFN γ

signaling to treat MS is still not successful.^{58,59} It is common that many inflammatory cytokines play paradoxical roles, especially when considering their exogenous or endogenous origins, their sites and time of action, and their quantitative differences in dosage.^{57,60,61} IFN γ activates inflammatory cells, such as T cells, macrophages, and neutrophils, to expand the immune responses during MS. On the other side, IFN γ also stimulates the parenchyma cells, such as ECs, to quench inflammation. Although increasing evidence indicates that pharmacological correction of the BBB alteration is a potential therapeutic lead in MS,^{12–14} selective activation of the IFN γ R signaling pathway in brain ECs may present a novel avenue for future treatment of MS.

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Supplemental Data

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