IFN-γ-dependent activation of the brain’s choroid plexus for CNS immune surveillance and repair

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Infiltrating T cells and monocyte-derived macrophages support central nervous system repair. Although infiltration of leucocytes to the injured central nervous system has recently been shown to be orchestrated by the brain’s choroid plexus, the immunological mechanism that maintains this barrier and regulates its activity as a selective gate is poorly understood. Here, we hypothesized that CD4+ effector memory T cells, recently shown to reside at the choroid plexus stroma, regulate leucocyte trafficking through this portal through their interactions with the choroid plexus epithelium. We found that the naïve choroid plexus is populated by T helper 1, T helper 2 and regulatory T cells, but not by encephalitogenic T cells. In vitro findings revealed that the expression of immune cell trafficking determinants by the choroid plexus epithelium is specifically induced by interferon-γ. Tumour necrosis factor-α and interferon-γ reciprocally controlled the expression of their receptors by the choroid plexus epithelium, and had a synergistic effect in inducing the epithelial expression of trafficking molecules. In vivo, interferon-γ-dependent signalling controlled trafficking through the choroid plexus; interferon-γ receptor knockout mice exhibited reduced levels of T cells and monocyte entry to the cerebrospinal fluid and impaired recovery following spinal cord injury. Moreover, reduced expression of trafficking molecules by the choroid plexus was correlated with reduced CD4+ T cells in the choroid plexus and cerebrospinal fluid of interferon-γ receptor knockout mice. Similar effect on the expression of trafficking molecules by the choroid plexus was found in bone-marrow chimeric mice lacking interferon-γ receptor in the central nervous system, or reciprocally, lacking interferon-γ in the circulation. Collectively, our findings attribute a novel immunological plasticity to the choroid plexus epithelium, allowing it to serve, through interferon-γ signalling, as a tightly regulated entry gate into the central nervous system for circulating leucocytes immune surveillance under physiological conditions, and for repair following acute injury.

Keywords: choroid plexus; blood–CSF barrier; CNS injury; trafficking; immune surveillance; IFN-γ

Abbreviations: CP = choroid plexus; EAE = experimental autoimmune encephalomyelitis; GM-CSF = granulocyte-macrophage colony stimulating factor; IFN = interferon; IL = interleukin; SCI = spinal cord injury; TNF = tumour necrosis factor
Introduction

The healthy mammalian central nervous system (CNS) is an immune privileged site, residing behind the blood–brain barrier and the blood–CSF barrier; these barriers, among their other roles, have long been viewed as protective against the potential devastating consequences of circulating immune cell entry to the CNS parenchyma (Engelhardt and Ransohoff, 2005; Wilson et al., 2010; Engelhardt and Coisne, 2011). Nevertheless, when stressed or injured, the CNS has the ability to mount a well-regulated immune response, as part of its repair (Moalem et al., 1999; Hauben et al., 2000; Olsson et al., 2003; Trivedi et al., 2006; Lewitus et al., 2008).

Our group has recently shown that following spinal cord injury (SCI), ‘healing’ monocyte-derived macrophages (M2) are recruited to the site of injury without breakdown of the blood–brain barrier (Shechter et al., 2013). Immune cell entry to the CNS was shown to take place through the blood–CSF barrier (Shechter et al., 2013), which is formed by the epithelial layer of the brain ventricular choroid plexus. Importantly, entry of leukocytes to the non-inflamed CNS, through the choroid plexus, has been proposed to explain both physiological immune surveillance (Kivisakk et al., 2003; Engelhardt and Ransohoff, 2005), and the early steps of T cell induced experimental autoimmune encephalomyelitis (EAE) (Petito and Adkins, 2005; Ransohoff, 2009; Reboldi et al., 2009). Therefore, the entry of leukocytes through the choroid plexus, which potentially contributes to the repair process following ‘sterile’ CNS injury, raises several key questions as to how the CNS orchestrates entry of circulating immune cells through the choroid plexus in response to parenchymal damage, without evoking an autoimmune disease.

We recently showed that CNS-specific immune cells reside within the choroid plexus stroma (Baruch et al., 2013). This observation supports our current working hypothesis that CNS-specific immune cells residing within the brain’s perimeter, and specifically within its epithelial borders, are activated through their cognate antigens, presented to them at these compartments, and may be further activated upon need by signals emitted from the brain parenchyma, when it is threatened or damaged (Schwartz and Baruch, 2012; Baruch and Schwartz, 2013).

In this study, we show that the choroid plexus epithelium is endowed with functional plasticity that reflects its immunological milieu. Specifically, we identified interferon-γ (IFN-γ) signalling as a selective key regulator of immune cell trafficking across the choroid plexus epithelium, under physiological conditions of CNS immune surveillance and following CNS trauma, with functional implications to the recovery of the injured CNS.

Materials and methods

Animals

Adult male and female wild-type and CX3CR1GFP/+ mice on a C57BL/6J background, and IL-4-ires-eGFP reporter mouse model on a NOD 6J background were purchased from the Jackson Laboratory. All experiments conformed to the regulations formulated by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Spinal cord injury and assessment of functional recovery

The spinal cords of deeply anaesthetized mice were exposed by laminectomy at T12, and moderate contusive centralized injury (1.3 mm tip; 2 mm height; 2 mm/s) was performed using the Infinite Horizon spinal cord impactor (Precision Systems), as previously described (Hauben et al., 2000; Ziv et al., 2006), causing bilateral degeneration without completely severing the spinal cord. The animals were maintained on twice-daily bladder expression. Recovery was evaluated by hind-limb locomotor performance, assessed according to the open-field Basso Mouse Scale (Basso et al., 2006). In this scale, a non-linear score ranging from 0 (complete paralysis) to 9 (normal mobility) is assigned, in which each possible score represents a distinct motor functional state. Blinded scoring ensured that observers were not aware of the identity of tested animals.

Preparation of bone marrow chimeras

Bone marrow chimeras were prepared by subjecting gender-matched recipient mice to lethal whole-body γ-irradiation (950 rad) while shielding the head. The mice were then reconstituted with 5 × 10⁸ bone marrow cells. In this method, 70% chimerism is achieved. In order to attain full chimerism (99%), mice were subjected to a split-dose irradiation, with sub-lethal whole-body γ-irradiation (300 rad) without head shielding 3 days before the lethal (950 rad) irradiation. Chimeric mice were used 6–10 weeks after bone marrow transplantation.

Primary culture of choroid plexus cells

After perfusion with PBS, the choroid plexus was removed under a dissecting microscope (Stemi DV4; Zeiss) in PBS into tubes containing 0.25% trypsin, and kept on ice. After all choroid plexus samples were collected, the tubes were shaken for 20 min at 37°C, and were then dissociated by pipetting. The cell suspension was washed in culture medium for epithelial cells [Dulbecco’s modified Eagle medium/F12 (Invitrogen) supplemented with 10% foetal calf serum (FCS)] and dissociated by pipetting. The cell suspension was washed in culture medium for epithelial cells [Dulbecco’s modified Eagle medium/F12 (Invitrogen) supplemented with 10% foetal calf serum (FCS)] and the cells were either left untreated or incubated with indicated cytokines.

Epithelial permeability assay

For transepithelial electrical measurements, choroid plexus cells were isolated as above and plated on 5-μm pore size, 6.5 mm diameter
polycarbonate filters in 24-well Transwell chambers (Costar, Corning). After 5 days in culture transepithelial electrical resistance was measured using Epithelial VoltOhmmeter (World Precision Instruments); measurements expressed as Ω cm². Cytokines were added to both upper and lower chambers of the Transwells, and transepithelial electrical resistance was measured again after 24 and 72 h. Permeability to FITC-dextran was measured by replacing the medium in the upper chamber with 100 μl of medium containing 250 μg/ml FITC-dextran (molecular weight 40 kDa). After 0.5, 1 or 2 h, 100 μl of medium was collected from the lower chamber and fluorescence was measured using a SpectraMax Gemini (Molecular Devices) plate reader. The permeability of the monolayer was expressed as percentage of the permeability of filter alone following subtraction of the background fluorescence.

**Transepithelial migration assay**

For transepithelial migration assay, choroid plexus cells were isolated as above and plated on 5-μm pore size, 6.5 mm diameter polycarbonate filters in 24-well Transwell chambers (Costar, Corning) and grown for 7 days. The medium of the upper chamber was then replaced with 100 μl of freshly isolated CD115⁺ monocytes (1.5 x 10⁶ cells/ml in RPMI 1640 supplemented with 0.5% bovine serum albumin) and the inserts were transferred to new wells containing RPMI 1640 supplemented with 0.5% bovine serum albumin and 100 ng/ml CCL2 (PeproTech). The Transwells were then incubated at 37°C, 5% CO₂. After 24 h the migrating cells at the lower chamber were removed using cell scraper and CD11b⁺ cells were quantified by flow cytometry.

**RNA purification, complementary DNA synthesis and real-time quantitative polymerase chain reaction**

Total RNA of the choroid plexus was extracted using the ZR RNA MicroPrep kit (Zymo Research), and from the spinal cord using the RNeasy® Mini Kit (Qiagen). Messenger RNA (1 μg) was converted to complementary DNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The expression of specific messenger RNAs was assayed using fluorescence based real-time quantitative PCR. Quantitative PCR reactions were performed using Power SYBR® Green PCR Master Mix (Applied Biosystems). Quantification reactions were performed in triplicates for each sample using the standard curve method. Peptidylprolyl isomerase A (PPIA) was chosen as reference gene according to its stability in the target tissue. The amplification cycles were 95°C for 5 s, 60°C for 20 s, and 72°C for 15 s. At the end of the assay, a melting curve was constructed to evaluate the specificity of the reaction. All quantitative real-time PCR reactions were performed and analysed using the 7500 Real-Time PCR System (Applied Biosystems). The list of primers used is summarized in Supplementary Table 1.

**Immunohistochemistry and immunocytochemistry**

For whole mount staining of the choroid plexus, isolated tissues were fixed with 2.5% paraformaldehyde for 30 min, and subsequently transferred to PBS containing 0.05% sodium azide. Before staining, the dissected tissues were washed with PBS, blocked with M.O.M.™ immunodetection kit reagent (Vector Laboratories) containing 0.1% Triton™ X-100 (Sigma-Aldrich), and stained with the following primary antibodies: mouse anti-ZO-1 (1:100; Invitrogen), rat anti-CD31 (1:50; BD Pharmingen), and rabbit anti-CD3 (1:50; Dako). Secondary antibodies included Cy2-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-rabbit, and Cy5-conjugated donkey anti-rat (1:200; Jackson ImmunoResearch). Each step was followed by three washes in PBS. The tissues were mounted onto slides, using Immu-Mount™ (9990402, from Thermo), and sealed with coverslips. For staining of sectioned brains, two different tissue preparation protocols (paraffin embedded and frozen sections) were applied, as previously described (Ziv et al., 2006). Antibodies used for staining included rabbit anti-IFN-γ (1:100; Bioss), mouse anti-E-cadherin (1:100; Invitrogen), mouse anti-cytokeratin (Abcam), rabbit anti-TNF-R1 (1:100; Santa Cruz Biotechnology), chicken anti-vimentin (1:500, Millipore), rabbit anti-glial fibrillary acidic protein (GFAP, 1:200; Dako), and rabbit anti-CD3 (1:50; Dako). Secondary antibodies included Cy2-conjugated donkey anti mouse and Cy3-conjugated donkey anti rabbit (1:200; Jackson ImmunoResearch). For labelling of myeloid cells, FITC-conjugated Bandeiraea simplicifolia isoflectric B4 (IB 4, 1:50; Sigma-Aldrich) was used. For nuclear staining, Hoechst 33342 fluorochrome was used (Molecular Probes, Invitrogen). For immunocytochemistry, choroid plexus cells were isolated as described above and were grown on poly-γ-lysine coated cover slips for 7 days, replacing the medium every 3 days. Cytokines were added for the last 3 days of culture. Following 7 days of culture, the wells were washed with PBS and the cells were fixed either with 2.5% paraformaldehyde for 30 min or methanol-acetone (1:1) for 10 min at −20°C, followed by two washing steps with PBS. The cover slips of the cultured choroid plexus cells were blocked with M.O.M.™ immunodetection kit reagent (Vector Laboratories) containing 0.1% Triton™ X-100 (Sigma-Aldrich), and stained with the following antibodies: rabbit anti-cytokeratin (1:100; Covance), rat anti-ICAM1 (1:100; Abcam), rabbit anti-IFN-γ (1:100; Bioss), mouse anti-araninase-1 (1:100; BD Pharmingen), rabbit or mouse anti-ZO-1 (1:200; Invitrogen), rabbit anti-VCAM1 (1:100; Santa Cruz Biotechnology). Secondary antibodies included: Cy2/Cy3-conjugated donkey anti-rat, mouse or rabbit antibody (1:200; Jackson ImmunoResearch). The cover slips were exposed to Hoechst stain (1:2000; Invitrogen) for 1 min and mounted onto slides, using Immu-Mount™ (9990402, from Thermo). Two negative controls were routinely used in immunostaining procedures, staining with isotype control antibody followed by secondary antibody, and staining with secondary antibody alone. For quantification of staining intensity, cell borders (20–25 cells per picture) were marked, and the corrected total cell fluorescence was quantified using ImageJ software as described previously (Burgess et al., 2010).

**Cerebrospinal fluid collection**

CSF was collected by the cisterna magna puncture technique. In brief, mice were anaesthetized and placed on a stereotactic instrument so that the head formed a 135° angle with the body. A sagittal incision of the skin was made inferior to the occiput and the subcutaneous tissue and muscle were separated, and a capillary was inserted into the cisterna magna through the dura matter lateral to the arteria dorsalis spinalis. Approximately 15 μl of CSF could be aspirated from an individual mouse. The collected CSF was taken for analysis by flow cytometry.

**Flow cytometry sample preparation and analysis**

Before tissue collection, mice were intracardially perfused with PBS, and their blood was collected into heparin-containing tubes. Spleens were mashed with the plunger of a syringe and treated with ACK
order to isolate only those T cells that reside in the choroid plexus stroma, mice were intracardially perfused with PBS before the choroid plexus excision, as we recently described (Baruch et al., 2013). After this procedure, the T cells isolated were those that reside within the choroid plexus stroma (Fig. 1A), rather than circulating cells, which differed in their composition (Fig. 1). To determine the effector potential of these cells, we assessed their cytokine production levels by flow cytometry, in comparison to the T cell populations in the spleen and in the blood. Intracellular staining of the CD4+ T cell populations showed that IFN-γ-producing T cells were present in the naïve choroid plexus (Fig. 1B). To quantify the population of interleukin (IL)-4 producing cells, we took advantage of mice that express green fluorescent protein (GFP) under the IL-4 promoter (Mohrs et al., 2001), and found a population of CD4+ T cells in the choroid plexus that expresses IL-4 (Fig. 1C). Since inflammatory autoimmune diseases are associated with Th17 cells (Korn et al., 2009), among other cellular immune components, we also examined the frequencies of IL-17-producing CD4+ T cells in the choroid plexus of healthy naïve mice, relative to the blood and the spleen; we found these cells to be absent from the choroid plexus and the blood (Fig. 1D). Granulocyte-macrophage colony-stimulating factor (GM-CSF) secreting helper T cells were shown to be highly encephalitogenic (Codari et al., 2011), we therefore also examined whether this cell population is present in the choroid plexus, and found it to be absent in the naïve choroid plexus (Supplementary Fig. 1). Finally, to determine the frequency of regulatory T cells in the choroid plexus, we used Foxp3GFP mice (Fontenot et al., 2005), and found that ~15% of the CD4+ T cells in the choroid plexus were Foxp3-positive (Fig. 1E). Overall, the choroid plexus showed enrichment in CD4+ effector memory T cells expressing IFN-γ and IL-4, but not of cells expressing IL-17 or GM-CSF.

Choroid plexus epithelial cells upregulate trafficking molecules in response to a specific cytokine milieu

As we found that the choroid plexus is constitutively exposed to T cell-derived cytokines, we hypothesized that such cytokines might have the potential to regulate the expression of trafficking molecules by the choroid plexus epithelium. We therefore established an in vitro model using primary cultures of murine choroid plexus cells. To verify the purity of the epithelial cultures, we isolated choroid plexus cells from mice that express GFP on their myeloid cells (CX3CR1GFP+/+ mice (Jung et al., 2000)), enabling us to detect any residual myeloid cell population within the epithelial cell cultures. Immunostaining of the choroid plexus cell cultures for the epithelial cytoskeletal marker, cytokeratin, revealed their uniformity (Fig. 2A), and quantitative analysis of the immunostained cells showed that 97.8 ± 0.4% of the cells expressed cytokeratin, and only 2.15 ± 0.3% of the cells were of myeloid origin, as determined by GFP expression (measured by direct counting). After 1 week in culture, the epithelial cells established tight junctions, as shown by immunostaining for the tight junction molecule, ZO-1 (Fig. 2B).
Figure 1  The resident T cell populations of the naïve choroid plexus. (A) Confocal imaging of whole mount choroid plexus from a cardially perfused wild-type mouse immunostained for the endothelial marker CD31, the epithelial tight-junction molecule, ZO-1, and for the T cell marker, CD3. Insets show separate channels of the marked area. Scale bar = 50 μm. (B–E) Flow cytometric analyses of CD4+ T cells from the spleen, blood and choroid plexus. (B) CD4+ T cells from wild-type mice were activated with para-methoxyamphetamine and ionomycin for 6 h, treated with Brefeldin-A for the last 4 h, and then stained for intracellular IFN-γ. (C) CD4+ T cells expressing IL-4 were quantified using the IL-4-IRES-eGFP mouse reporter model. (D) CD4+ T cells from wild-type mice were activated with para-methoxyamphetamine and ionomycin for 6 h, treated with Brefeldin-A for the last 4 h, and then stained for intracellular IL-17. (E) CD4+ regulatory T cells were quantified using the Foxp3-GFP reporter mouse model. Bar graphs represent mean frequencies ± SEM; *P < 0.05; ***P < 0.001; one-way ANOVA, followed by Student’s t-test post hoc analysis (n = 4–8 per group). CP = choroid plexus.
The choroid plexus was recently shown to be activated after remote SCI (Shechter et al., 2013), and pro-inflammatory cytokines, such as IL-6, IL-1β, and tumour necrosis factor (TNF)-α, are elevated at the lesion site (Pineau and Lacroix, 2007), rostral to it (Emmetsberger and Tsirka, 2012), and in the CSF (Harrington et al., 2005; Shechter et al., 2013); such cytokines can potentially affect the choroid plexus through CSF circulation. We therefore tested whether cytokines released at the injury site would affect the choroid plexus in addition to the T cell-derived cytokines.

We exposed the cultured choroid plexus cells to the characteristic Th1-, Th17-, Th2- and regulatory T cell-derived cytokines, IFN-γ, IL-17, IL-4, and IL-10, respectively, and to TNF-α and IL-6. After 24 h in culture, we examined the effect of these cytokines on the gene expression levels of specific trafficking molecules. Treatment with IFN-γ induced the upregulation of a wide array of trafficking molecules, such as intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1) (Fig. 2C), the chemokines CCL5, CXCL9 and CXCL10, and major histocompatibility

**Figure 2** Choroid plexus epithelial cells upregulate trafficking molecules in response to a specific cytokine milieu. (A) Representative microscopic images of cultured choroid plexus cells from CX3CR1GFP/+ mice stained for the epithelial marker, cytokeratin. Scale bar = 50 µm. (B) Representative microscopic images of cultured choroid plexus cells from wild-type mice stained for the tight-junction molecule, ZO-1, showing tight-junctions between epithelial cells after 7 days in culture. Scale bar = 50 µm. (C–G) Messenger RNA levels, measured by real time quantitative PCR, of cultured choroid plexus cells from wild-type mice 24 h after the addition of the cytokines IL-4 (10 ng/ml), IL-10 (10 ng/ml), IL-6 (10 ng/ml), IL-17 (100 ng/ml), IFN-γ (100 ng/ml), TNF-α (100 ng/ml) or the combination of IFN-γ and TNF-α, relative to untreated (UT) cells. Bars represent mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001 versus untreated cells; #P < 0.05; ##P < 0.01; ###P < 0.001 between indicated groups by one-way ANOVA followed by Tukey’s HSD post hoc analysis (n = 3 per group). Results of one of three independently performed experiments are presented. (H–J) Representative microscopic images of cultured choroid plexus cells from wild-type mice 3 days after the addition of IFN-γ (100 ng/ml) immunostained for the epithelial marker, cytokeratin, and for ICAM1 (H) or VCAM1 (I). IL-4 (10 ng/ml) treated cells were stained for ZO-1 and ARG1 (J). Scale bars = 100 µm. Quantitative analysis of the intensity (corrected total cell fluorescence) of staining per cell in arbitrary units is presented next to each image. Data are expressed as mean ± SEM; *P < 0.05; ***P < 0.001; Student’s t-test (n = 3 per group).
complex II (MHCII; Fig. 2D), which can contribute to T cell trafficking across epithelial barriers and to T cell activation (Porter and Hall, 2009; Murugesan et al., 2012). The chemokines CCL2, fractalkine (CX3CCL1) and macrophage colony-stimulating factor (M-CSF), which were shown to be involved in monocyte trafficking to the CNS (Szmydynger-Chodobska et al., 2012; Shechter et al., 2013), were also upregulated by the choroid plexus in response to IFN-\(\gamma\) (Fig. 2E). When IFN-\(\gamma\) was combined with TNF-\(\alpha\), a strong synergistic effect was observed with respect to most of the tested genes. Notably, TNF-\(\alpha\) was the only cytokine that upregulated, in an IFN-\(\gamma\)-independent manner, mucosal vascular addressin cell adhesion molecule 1 (MADCAM1) expression (Fig. 2C), as previously reported (Steffen et al., 1996). To further verify that the observed ability to induce trafficking molecules by the choroid plexus was unique to IFN-\(\gamma\), we repeated the in vitro experiments using escalating doses of the cytokines IL-4, IL-6, IL-10, GM-CSF and IFN-\(\gamma\). None of the tested cytokines, other than IFN-\(\gamma\), had any effect, whereas IFN-\(\gamma\) activated the choroid plexus in a dose dependent manner to express trafficking-related genes (Supplementary Fig. 2). Although IL-4 had no effect on trafficking molecules, it had a dramatic effect on the upregulation of arginase 1 (ARG1) (Fig. 2F), an enzyme classically associated with anti-inflammatory activity (Bronte et al., 2003).

Although we found the frequency of IL-17 expressing T cells in the choroid plexus to be low (Fig. 1D), the association of Th17 cells with EAE (Korn et al., 2009) prompted us to test its potential effect on inducing trafficking molecules by the choroid plexus. Interestingly, we found that IL-17, but not IFN-\(\gamma\), elevated the expression of the chemokine CCL20 by the choroid plexus epithelial cells (Fig. 2G); CCL20 was proposed to participate in CCR6\(^+\) cell trafficking through the choroid plexus in the initiation of EAE (Reboldi et al., 2009).

The localization of the induced integrin receptors, ICAM1 and VCAM1, was tested by co-immunostaining the cultured cells for these molecules together with epithelial markers; the integrin receptors were co-localized with the choroid plexus epithelial cells, and were elevated following treatment with IFN-\(\gamma\) (Fig. 2H and I). We also immunostained IL-4-treated choroid plexus epithelial cells for ARG1, and found it to be higher relative to untreated cells (Fig. 2I).

**TNF-\(\alpha\) and IFN-\(\gamma\) reciprocally control the expression of their receptors by the choroid plexus**

To gain insight regarding the mechanism underlying the synergism, described above, between TNF-\(\alpha\) and IFN-\(\gamma\), we examined their reciprocal effects on the levels of their corresponding receptors in vitro. We found that the inflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\), but not IL-6, elevated the expression of IFN-\(\gamma\)-receptor (IFN-\(\gamma\)R) by the choroid plexus epithelial cells (Fig. 3A). Immunostaining of the cultured cells also showed elevation of IFN-\(\gamma\)R following TNF-\(\alpha\) treatment (Fig. 3B). Notably, TNF-\(\alpha\)-producing CD4\(^+\) T cells were not found in the naïve choroid plexus (Supplementary Fig. 3).

TNF-\(\alpha\) receptors (TNF-R) are differentially expressed in various tissues, and were shown to mediate both inflammation and repair of the CNS (Hohmann et al., 1989; Clark et al., 2010). We therefore used neutralizing antibodies directed against TNF-R1 or TNF-R2 to assess which TNF-R mediates the observed elevation of IFN-\(\gamma\)R by the choroid plexus. We found that the induction of IFN-\(\gamma\)R by the choroid plexus was mediated by TNF-R1 signalling, as only neutralizing antibodies directed against TNF-R1 impaired IFN-\(\gamma\)R expression (Fig. 3C). Indeed, immunostaining confirmed the expression of TNF-R1 on the epithelial cells (cytokeratin\(^*\)) of the choroid plexus of naïve mice (Fig. 3D). In addition to the elevated expression of IFN-\(\gamma\)R following treatment with TNF-\(\alpha\), IFN-\(\gamma\) elevated the expression of TNF-R1 (Fig. 3E), suggesting that the synergistic effect between TNF-\(\alpha\) and IFN-\(\gamma\) by the choroid plexus epithelium is bidirectional, with each cytokine inducing the expression of its reciprocal cytokine receptor.

To further support the observed synergy between the cytokines, we assessed the transepithelial electrical resistance of the choroid plexus cultures, a measure frequently used to determine epithelial barrier integrity (Schwerk et al., 2010), and found it to be reduced by either TNF-\(\alpha\) or IFN-\(\gamma\), with greater reduction when the cytokines are combined (Fig. 3F). Importantly, the reduction in transepithelial electrical resistance did not affect cell viability (Supplementary Fig. 4A), nor the permeability of the culture to FITC-Dextran (40 kDa) (Supplementary Fig. 4B).

**IFN-\(\gamma\) signalling is required to maintain immune surveillance of the healthy central nervous system**

To further substantiate the role of IFN-\(\gamma\) signalling in the expression of trafficking molecules by the choroid plexus epithelium in vivo, we examined the choroid plexus of IFN-\(\gamma\)R knockout (IFN-\(\gamma\)R-KO) mice. We found the basal expression levels of a wide array of trafficking molecules to be lower in the choroid plexus of IFN-\(\gamma\)R deficient mice, relative to wild-type animals (Fig. 4A). Notably, the basal expression level of ARG1, which was shown above to be induced by the choroid plexus epithelium in response to IL-4 (Fig. 2F and J), was significantly elevated in the choroid plexus of IFN-\(\gamma\)R-KO mice. To confirm that the observed results were not due to the deficiency of IFN-\(\gamma\)R on circulating immune cells, we created chimeric mice following total body irradiation while protecting the head (Shechter et al., 2009); in these mice, the bone marrow of the IFN-\(\gamma\)R-KO mice was replaced by wild-type bone marrow. The same pattern of downregulation of trafficking molecules by the choroid plexus was observed (Fig. 4B). We also created chimeric mice lacking expression of the cytokine IFN-\(\gamma\) in circulating immune cells. These chimeric mice were created by replacing the bone marrow of wild-type mice with IFN-\(\gamma\) KO bone marrow, following a split-dose irradiation protocol, to allow total depletion of the host hematopoietic cells (achieving 99% chimerism; see ‘Materials and methods’ section). Strikingly, after 2 months of bone marrow-chimerism, the choroid plexus of these animals showed reduced levels of choroid plexus-expressed trafficking molecules, relative to their controls that received wild-type bone marrow (Fig. 4C), further supporting the
need for IFN-γ signalling for the expression of trafficking molecules by the choroid plexus. These results prompted us to examine whether the outcome of poor trafficking molecule expression by the choroid plexus of IFN-γR-KO mice might result in reduced immune surveillance of the CNS. We therefore examined the numbers of CD4+ T cells in the choroid plexus and CSF of IFN-γR-KO mice, and found a significant reduction in their numbers relative to age matched wild-type controls (Fig. 4D). This reduction was specific for the choroid plexus and CSF and did not result from a systemic reduction in CD4+ T cells, as the numbers of these cells in the circulation and lymphoid organs were similar to the wild-type mice (Fig. 4D). These results demonstrated a pivotal role of IFN-γ signalling in maintaining CNS immune surveillance through the choroid plexus, and prompted us to examine the role of IFN-γ signalling in CNS repair.

IFN-γR-KO mice have impaired recovery associated with failure of choroid plexus activation for leucocyte trafficking after spinal cord injury

The reduced trafficking of leucocytes across the choroid plexus to the CSF in IFN-γR-KO animals under physiological conditions, led us to consider that such a defect might have a much more pronounced effect following injury. We subjected wild-type mice to a severe, well-calibrated contusive SCI at the level of T-12 (Ziv et al., 2006), and first tested whether such injury affected the expression of IFN-γR by the choroid plexus. We found IFN-γR expression to be upregulated by the choroid plexus epithelium on Day 1 and Day 7 post-SCI (Fig. 5A and B), corresponding to previously described
Figure 4  IFN-γ signalling is needed to maintain immune surveillance of the healthy CNS. (A) Messenger RNA levels of various adhesion molecules, chemokines and immunoregulatory molecules, measured by real time quantitative PCR in the choroid plexus of IFN-γR-KO mice. Bars represent mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001 versus wild-type (WT); Student’s t-test (n = 5–6 per group). (B) Chimeric IFN-γR-KO or wild-type mice were reconstituted with wild-type bone marrow cells. Messenger RNA levels for the indicated genes were measured by real time quantitative PCR in their choroid plexus. Bars represent mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001; Student’s t-test (n = 5 per group). (C) Chimeric wild-type mice were reconstituted with either wild-type or IFN-γR-KO bone marrow cells. Messenger RNA levels for the indicated genes were measured by real time quantitative PCR in their choroid plexus. Bars represent mean ± SEM; *P < 0.05; **P < 0.01; one-way ANOVA, followed by Student’s t-test post hoc analysis (n = 5 per group). (D) The number of CD4+ T cells in the spleen, blood, choroid plexus and CSF of wild-type and IFN-γR-KO mice was quantified using flow cytometry. Quantitative analysis is shown below the representative dot plots. Bars represent mean ± SEM; *P < 0.05 by Student’s t-test (n = 5–7 per group). CP = choroid plexus.
Figure 5  IFN-γ-R-KO mice have impaired recovery after SCI, which is associated with failure of choroid plexus activation for leukocyte trafficking. (A) Wild-type mice (WT) were subjected to SCI and messenger RNA levels of IFN-γ-R in their choroid plexus were measured by real time quantitative PCR on Days 1, 3 and 7 post-injury. Bars represent mean ± SEM; ***P < 0.001 versus non-injured; one-way ANOVA, followed by Student’s t-test post hoc analysis (n = 4–6 per group). (B) Representative micrographs of choroid plexus from non-injured wild-type mice or mice at 1 and 3 days post-injury, immunostained for the epithelial tight-junction molecule, E-cadherin, and for IFN-γ-R. Scale bar = 50 µm. (C) IFN-γ-R-KO and wild-type mice were similarly injured and messenger RNA levels of ICAM1, CXCL9 and CXCL10 in their choroid plexus were measured by real time qPCR at Day 7 post-injury. Bars represent mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001 versus wild-type mice; #P < 0.05; ##P < 0.01; ###P < 0.001 between indicated groups by one-way ANOVA followed by Student’s t-test post hoc analysis (n = 5–12 per group). (D) IFN-γ-R-KO and wild-type mice were subjected to a well-calibrated SCI, and were followed for hind-limb locomotor activity assessed according to the Basso Mouse Scale. Bars represent mean ± SEM; *P < 0.05; **P < 0.01; repeated measures ANOVA, followed by Student’s t-test post hoc analysis (n = 11–12 per group). (E) Messenger RNA levels of TNF-α, IL-1β and IL-6 at the site of injury were measured by real time quantitative PCR 24 h after the injury in IFN-γ-R-KO and wild-type mice. Bars represent mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001 versus wild-type mice by one-way ANOVA, followed by Student’s t-test post hoc analysis (n = 6 per group). (F) The numbers of CD4⁺ T cells and monocytes (CD11b⁺) in the CSF of wild-type and IFN-γ-R-KO mice were quantified using flow cytometry at 1 day post SCI, and were compared to non-injured wild-type and IFN-γ-R-KO mice. Bars represent mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001 versus wild-type mice; ##P < 0.01; ###P < 0.001 between indicated groups by one-way ANOVA, followed by Student’s t-test post hoc analysis (n = 6–8 per group). (G) Staining for the mesenchymal cell marker, vimentin, and for CD3, showing T cells at the mesenchymal layer of the central canal, rostral to the site of injury. Scale bar = 200 µm. (H) Representative microscopic images of spinal cord from wild-type mouse 7 days post SCI, stained for IB4 and CD3 showing presence of T cells at the epicentre of the injury site. Scale bar = 200 µm. (I) Quantitative analysis of the number of T cells at the epicentre of SCI in wild-type and IFN-γ-R-KO mice 7 days post SCI and IFN-γ-R KO mice 7 days post SCI, showing significant reduction in the numbers of T cells in the IFN-γ-R-KO mice. Bars represent mean ± SEM; **P < 0.01 by Student’s t-test (n = 5–6 per group). (J) The numbers of CD4⁺ T cells and monocytes (CD11b⁺/CD45.2high) in the spinal cord of wild-type and IFN-γ-R-KO mice were quantified using flow cytometry at day 7 post SCI. Bars represent mean ± SEM; *P < 0.05; ***P < 0.001 by Student’s t-test (n = 6–7 per group).
waves of immune cell trafficking to the CNS following acute injury to the spinal cord (Trivedi et al., 2006). Notably, although the expression of IFN-γ peaked in two waves, immunostaining for the tight junction molecule E-cadherin revealed its reduction in the choroid plexus epithelium for several days after the injury (Fig. 5B). Next we determined whether in the absence of IFN-γR, the choroid plexus would lose its capacity to be activated for expression of trafficking molecules after injury. We inflicted SCI in wild-type and IFN-γR-KO mice and found that although in the wild-type animals the choroid plexus was activated to enable leukocyte trafficking, IFN-γR-KO mice failed to elevate expression of the adhesion molecule ICAM1, and the chemokines CXCL9 and CXCL10 (Fig. 5C). The lack of activation of the choroid plexus of the IFN-γR-KO mice for trafficking prompted us to test their functional recovery from the injury. A follow-up of hind-limb locomotor performance, assessed according to the open-field Basso Mouse Scale (in which a score of 0 represents complete paralysis and score of 9 complete mobility) (Basso et al., 2006), revealed that IFN-γR-KO mice had a worse recovery following the injury relative to wild-type mice (Fig. 5D). To rule out the possibility that the different activation of the choroid plexus for expression of trafficking molecules resulted from differences in the injury-induced inflammatory response at the lesion site, we subjected wild-type and IFN-γR-KO mice to SCI, and examined the lesion site for levels of the pro-inflammatory cytokines, TNF-α, IL-1β and IL-6. Both wild-type and IFN-γR-KO mice showed similar increase of local levels of the tested pro-inflammatory cytokines 24 h after the injury (the hyper-acute phase) (Fig. 5E). In addition, immunohistochemical analysis revealed that microglia and astrocytes were activated at the site of injury, in both wild-type and IFN-γR-KO mice (Supplementary Fig. 5).

Importantly, the lack of activation of the choroid plexus was correlated with impaired trafficking of both CD4+ T cells and monocytes to the CSF of the injured IFN-γR-KO mice relative to wild-type mice after SCI (Fig. 5F). The IFN-γ-dependent trafficking of monocytes through the choroid plexus was further verified in vitro using a transepithelial migration assay; TNF-α in combination with IFN-γ stimulated the choroid plexus to mediate monocyte trafficking towards CCL2 (Supplementary Fig. 6).

Leucocyte trafficking across the choroid plexus to the CNS parenchyma, was further supported by the appearance of T cells in the ependymal layer of the central canal, rostral to the lesion site (Fig. 5G), indicating their migration to the injury site through this route. Their numbers were significantly reduced in the lesion site epicentre in IFN-γR-KO mice following the injury, relative to wild-type mice (Fig. 5H and I). In addition, flow cytometry analysis of the spinal cord of IFN-γR-KO mice on Day 7 post-injury showed reduced numbers of CNS-recruited CD4+ T cells and monocytes relative to their numbers in injured wild-type mice (Fig. 5J). Taken together, these results demonstrate the role of IFN-γ-signalling in CNS repair process, through its essential role in regulating leucocyte trafficking through the choroid plexus.

Discussion

In this study we identified IFN-γ as a key regulatory cytokine for leucocyte trafficking through the choroid plexus to the CNS both under physiological conditions and after acute CNS damage. The absence of IFN-γR/IFN-γR signalling in the choroid plexus resulted in reduced expression of immune cell trafficking molecules by the choroid plexus epithelium and reduced numbers of CD4+ T cells in the choroid plexus and CSF of naïve mice. After SCI, IFN-γR-KO mice failed to activate the choroid plexus to express leucocyte trafficking molecules, had reduced numbers of infiltrating T cells and monocytes in the CSF and spinal cord parenchyma, and showed worse recovery from the injury.

As an immune privileged site, the CNS has a poor tolerance for immune cell infiltration to its parenchyma, and much of what we know about immune infiltration to the CNS was studied in pathological states (Engelhardt and Ransohoff, 2005). Moreover, little attention has been devoted to the distinction between CNS pathologies that are inflammatory in their aetiology, manifested by pathogenic immune cell infiltration, and for which immune suppression is beneficial (Vaknin et al., 2011), versus pathologies that have a non-inflammatory basis; in the latter situation, infiltrating monocyte-derived macrophages and T lymphocytes are essential for repair (Moalem et al., 1999; Hauben et al., 2000; Simard et al., 2006; Beers et al., 2008; Butchi Banerjee et al., 2008; Shechter et al., 2009; Derecki et al., 2012). The choroid plexus was recently identified as a site through which circulating monocytes, which locally become resolving macrophages (Ly6C<sup>low</sup>/CX<sub>3</sub>CR1<sup>high</sup>), are preferentially recruited to the CNS after SCI (Shechter et al., 2013).

Here we demonstrate that both IFN-γ and TNF-α signalling can independently and synergistically activate the choroid plexus epithelium to express trafficking molecules in vitro. Our results are consistent with the reported in vitro synergistic effect of TNF-α and IFN-γ stimulation on the expression of trafficking molecules by both lung (Barrett et al., 1998) and colon (Fish et al., 1999) epithelial cells, suggesting that similar mechanisms for leucocyte trafficking are shared between the choroid plexus and other epithelial barriers.

The choroid plexus stromal T cell population was recently found by us to be enriched with effector memory CD4+ T cells specific for CNS antigens (Baruch et al., 2013). Here, we focused on IFN-γ signalling by the choroid plexus epithelium, as IFN-γ producing cells were found to be abundant in this compartment. Our in vivo studies using IFN-γR-KO and IFN-γR-KO transgenic and chimeric mice showed that expression of trafficking molecules by the choroid plexus and leucocyte trafficking to the CSF during physiological immune surveillance of the CNS, are dependent on IFN-γR/IFN-γR signalling by the choroid plexus epithelium. Our in vitro results further suggest that under inflammatory conditions this effect is amplified by the induction of IFN-γR expression on the choroid plexus epithelial cells in response to TNF-α, and suggest that the observed synergistic effect between TNF-α and IFN-γ on the choroid plexus results from their reciprocal induction of IFN-γR and TNF-R1, respectively. Importantly, after SCI, although TNF-α levels were upregulated to the same extent at the site of injury in the spinal cords of both wild-type and IFN-γR-KO mice, the latter failed to elevate the choroid plexus expression of trafficking molecules and showed reduced trafficking of leucocytes to the CSF and spinal cord, further supporting the need for IFN-γ signalling by the choroid plexus. These results are in line with the
findings that TNF-KO mice develop EAE to the same extent as wild-type (Kassiotis et al., 1999) whereas in mice lacking IFN-γR in the CNS, leucocytes do not enter the spinal cord following EAE induction, resulting in an atypical form of the disease (Lees et al., 2008). Our results, thus, identify IFN-γ signalling as the predominant signalling pathway controlling leucocyte trafficking to the CNS. Further studies are needed to achieve a detailed understanding of the relationships between the post-injury choroid plexus activation and the dynamics of the cell populations that traffic through this portal to the CNS.

The trafficking of monocytes through the choroid plexus after SCI was recently found to be VCAM1 and ICAM1 dependent (Shechter et al., 2013). Although we found that both IFN-γ and TNF-α can upregulate ICAM1 and VCAM1 expression by the choroid plexus epithelium in vitro, in the absence of IFN-γ signalling, the choroid plexus failed to upregulate ICAM1 expression following the injury. As previously reported, ICAM1 is constitutively expressed at the apical side of the choroid plexus epithelium, and is elevated following EAE induction (Wolburg et al., 1999); similarly, induction of ICAM1 was found here following SCI, and was localized to the apical side of the choroid plexus (data not shown). Apical expression of ICAM1 was suggested to serve as a foothold against the shear forces at the luminal side of the epithelial tissue in the final stages of the migration process (Zen and Parkos, 2003), in contrast to transendothelial migration in which ICAM1 is needed for arresting leucocytes on blood vessels against the shear flow of the blood circulation (Shulman et al., 2012). In line with this notion, in vitro studies of basal-to-apical T cell egress through the lung epithelium showed that ICAM1 expression is not essential for the adhesion of T cells to the epithelial cells but rather for their consequent transmigration (Porter et al., 2008). It was further shown that ICAM1 is upregulated when the epithelium is stimulated by IFN-γ, in the presence or absence of TNF-α (Miller and Butcher, 1998; Taguchi et al., 1998; Porter et al., 2008, Porter and Hall, 2009).

Crossing the epithelial monolayers involves, in addition to adhesion, the expression of chemokines by the epithelial cells in order to create a chemical gradient (Taguchi et al., 1998; Porter et al., 2008). We found the expression levels of CXCL9 and CXCL10 by the choroid plexus to be significantly lower in the CNS compared to the spinal cord. CXCL10 was upregulated in vitro to be significantly lower in the choroid plexus to be significantly lower in the CNS compared to the spinal cord. CXCL10 was upregulated in vitro by the choroid plexus epithelium, a chemokine reported to be involved in monocyte recruitment to the CNS (Simard et al., 2006; D’Mello et al., 2009; Szmydynger-Chodob ska et al., 2012). The abundance of IL-4 producing T cells in the choroid plexus substantiate a role for this cytokine in local immunomodulation, possibly through its induction of ARG1 (Gordon, 2003; Mantovani et al., 2004; Baruch et al., 2013), which is known to induce differentiation of myeloid cells to M2 macrophages (Gordon, 2003), which are recruited to the injured CNS through the choroid plexus (Shechter et al., 2013). Other molecules that were tested such as IL-10, IL-6 and GM-CSF did not have any effect on the choroid plexus expression of any of the examined genes.

In summary, in this study we found the choroid plexus to be a tightly regulated gateway for leucocyte trafficking to the CNS, determining physiological immune surveillance and recovery following CNS injury. This regulation is dependent on IFN-γ expression by the choroid plexus, and IFN-γ production by immune cells. It is therefore possible that modulating IFN-γ/IFN-γR signalling by the choroid plexus, to boost the recruitment of inflammation-resolving cells, may serve as a novel approach for treatment of CNS trauma or neurodegenerative conditions, and conversely, blocking this signalling may be beneficial in autoimmune diseases such as multiple sclerosis.

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Supplementary material

Supplementary material is available at Brain online.

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