RESEARCH ARTICLE

Type-I Interferons Suppress Microglial Production of the Lymphoid Chemokine, CXCL13

Nilufer Esen, Emily K. Rainey-Barger, Amanda K. Huber, Pennelope K. Blakely, and David N. Irani

Lymphoid chemokines are crucial for the development and maintenance of lymphoid organs, but their ectopic expression in non-lymphoid tissues is implicated in both local response to infection and chronic organ-specific autoimmunity. Production of one such chemokine, C-X-C motif ligand 13 (CXCL13), within the central nervous system (CNS) has been linked to the pathogenesis of multiple sclerosis (MS), although little is known about factors controlling its expression in different neural cell types and across a range of disease states. We provoked acute neuroinflammation in experimental animals without causing any associated demyelination using neuroadapted Sindbis virus (NSV) to better understand the sources and regulators of this chemokine in the CNS. We found that mice genetically deficient in the transcription factor, interferon (IFN) regulatory factor-7 (IRF7), made significantly higher CXCL13 protein levels in the CNS compared with wild-type (WT) controls. Microglia proved to be the main producer of CXCL13 in the brain during infection of both WT and IRF7$^{-/-}$ mice, and primary microglia cultured *in vitro* generated CXCL13 following stimulation with either virus particles or synthetic Toll-like receptor (TLR) ligands. Microglia cultured from IRF7$^{-/-}$ mice selectively overproduced CXCL13, and manipulation of extracellular type-I IFN levels demonstrated the existence of a negative feedback loop whereby type-I IFN receptor signaling specifically suppressed microglial CXCL13 release. Since IFN-β is used to treat patients with relapsing-remitting MS and yet acts through unknown mechanisms, we speculate that suppressed lymphoid chemokine production by microglia could contribute to its therapeutic effects.

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Key words: microglia, CXCL13, type-I interferon, IRF7

Introduction

Lymphoid chemokines are molecules constitutively expressed in lymphoid organs that recruit and compartmentalize lymphocytes and antigen presenting cells into and within these highly specialized structures (Allen et al., 2004; Ansel et al., 2000; Campbell et al., 2003; Forster et al., 1996; Fu and Chaplin, 1999; Legler et al., 1998; Mackay and Browning, 1998; Moser et al., 2002; Muller et al., 2003; Ngo et al., 1999; Yoshie et al., 1997; Zlotnik et al., 1999). C-C motif ligand (CCL) 19 and CCL21 bind to C-C motif receptor (CCR) 7 and recruit T cells and dendritic cells (DC) to T cell areas of secondary lymphoid tissues (Legler et al., 1998; Yoshie et al., 1997). C-X-C motif ligand (CXCL) 12 binds to C-X-C motif receptor (CXCR) 4 and attracts multiple immune cell types to lymph nodes and spleen, and along with CXCL13, drives the formation of germinal centers (Allen et al., 2004; Campbell et al., 2003). CXCL13 is made by stromal cells in B cell follicles, and recruits both B cells and T follicular helper (Tfh) cells to these compartments by signaling through its cognate receptor, CXCR5 (Allen et al., 2004; Ansel et al., 2000; Campbell et al., 2003; Forster et al., 1996; Legler et al., 1998; Moser et al., 2002; Muller et al., 2003). The expression of all lymphoid chemokines in both T and B cell areas of lymphoid organs depends on lymphotoxin (LT)-β and tumor necrosis factor (TNF)-α signaling in stromal cells that normally produce these chemoattractant

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molecules within these specialized tissues (Fu and Chaplin, 1999; Mackay and Browning, 1998; Ngo et al., 1999).

Beyond its role in the development and maintenance of lymphoid tissues, CXCL13 has also been implicated in the propagation of non-lymphoid tissue inflammation. Chronic gastrointestinal infection of humans with *Helicobacter pylori* causes the formation of mucosa-associated lymphoid structures that contain CXCL13 (Mazzucchelli et al., 1999), and antibody-mediated neutralization of CXCL13 in experimental animals directly inhibits this process (Yamamoto et al., 2014). Data generated in other infection models also point to a role for CXCL13 in recruiting B cells to organs such as the lung or the liver where it helps organize local immune responses against influenza and hepatitis B, respectively (Publicover et al., 2013; Rangel-Moreno et al., 2007). On the other hand, ectopic structures resembling T and B cell follicles are found in the synovia of patients with rheumatoid arthritis and in the salivary glands of patients with Sjögren's disease (Hjelmstrom, 2001), and transgenic expression of CXCL13 in an organ such as the pancreas is sufficient to cause the formation of ectopic lymphoid structures and produce laboratory evidence of diabetes (Luther et al., 2000). CXCL13 is also found in B cell aggregates that develop in the inflamed meninges of mice with experimental autoimmune encephalomyelitis (EAE) and humans with progressive multiple sclerosis (MS) (Aloisi et al., 2008; Magliozzi et al., 2004, 2007; Peters et al., 2011; Serafini et al., 2004). These data imply that the CXCL13-driven formation of ectopic lymphoid structures may be a natural feature of both organ-specific antimicrobial and autoimmune inflammation (Hjelmstrom, 2001; Lalor and Segal, 2010; Magliozzi et al., 2007; Serafini et al., 2004).

Within the central nervous system (CNS), recent reports describe elevated CXCL13 concentrations in the cerebrospinal fluid (CSF) of patients with either relapsing-remitting multiple sclerosis (RRMS) or neuromyelitis optica (NMO) compared with controls, and that levels are higher during active disease relapses and decline with successful therapy (Brettschneider et al., 2010; Khademi et al., 2011; Krumbholz et al., 2006; Piccio et al., 2010; Ragheb et al., 2011; Sellebjerg et al., 2009; Zhong et al., 2011). Quantitative PCR analysis of autopsy brain tissue from MS patients demonstrates that *cxcl13* gets selectively induced in active *versus* inactive MS lesions, and immunostaining of tissue sections shows CXCL13 protein within and around active MS plaques (Krumbholz et al., 2006). In tissue samples from patients with late-stage progressive MS, some specimens also demonstrate CXCL13 staining in B cell-containing follicles that develop along the meninges and deep within the sulci of the brain (Magliozzi et al., 2007; Serafini et al., 2004). These meningeal lymphoid follicles are hypothesized to be sites where myelin-specific T and B cells get reactivated in response to their cognate antigens thus propagating autoimmune damage to the brain and spinal cord (Lalor and Segal, 2010). Little, however, is known about factors controlling the local expression of CXCL13 in the CNS during a wide range of disease states.

We used neuroadapted Sindbis virus (NSV) to provoke acute neuroinflammation without any associated demyelination in experimental animals in order to investigate the sources and factors regulating CNS expression of CXCL13 in response to a biologically relevant stimulus. We found that mice genetically deficient in the transcription factor, interferon (IFN) regulatory factor-7 (IRF7), a critical intermediate in the type-I IFN response, made significantly higher CNS levels of CXCL13 protein compared with wild-type (WT) controls. Microglia were the main source of CXCL13 produced in the CNS during this infection, and primary microglia cultured *in vitro* secreted CXCL13 following stimulation with virus particles or particular Toll-like receptor (TLR) ligands. Microglia cultured from the brains of IRF7$^{-/-}$ mice selectively overproduced CXCL13 *in vitro*, and manipulation of extracellular type-I IFN levels demonstrated the existence of a negative feedback loop whereby type-I IFN receptor (IFNAR) signaling specifically suppressed microglial CXCL13 release. We speculate this feedback loop could impact host responses against certain viral pathogens that invade the CNS. It could also be relevant to the pathogenesis of MS, since exogenous IFN-β is a well-established treatment for patients with the relapsing-remitting form of this disease.

Materials and Methods

Animals

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice rendered genetically deficient in IRF7, CXCL13, and IFNAR were generous gifts from the laboratories of Dr. Gabriel Nunez, Dr. Benjamin Segal and Dr. Steven Kunkel, respectively. IRF7/CXCL13 double deficient mice were generated *de novo* and bred on site. Animals were maintained and used under specific pathogen-free conditions in strict accordance with guidelines set by the National Institutes of Health and protocols approved by our University Committee on the Use and Care of Animals. Mice were housed on a 10 h light/14 h dark cycle in ventilated cages, each containing no more than five animals. Food and water were available *ad libitum*.

Induction of Viral Encephalitis and Manipulation of Virus-Infected Animals

To induce encephalomyelitis, 6-week-old mice were anesthetized with isoflurane and 1000 plaque forming units (PFU) of NSV, suspended in 10 μL of phosphate-buffered saline (PBS), was inoculated directly into the right cerebral hemisphere of each animal. Most cohorts of infected mice were then monitored daily for survival in accordance with approved animal protocols. Some groups of live

animals were euthanized at defined intervals postinfection to collect brain and spinal cord tissue for further analysis, including moribund IRF7$^{-/-}$ mice on day 5 postinfection. Here, CNS tissues were collected following intracardiac perfusion with ice-cold PBS, weighed, snap frozen at −80°C, and used to generate tissue homogenates for virus titration assays or enzyme linked immunosorbent assays (ELISAs) (see below). In other cohorts, PBS-perfused brain tissue was used to isolate individual cell populations via a flow cytometry-based cell sorting protocol (see below).

Flow Cytometry-Based Separation of Discrete Immune and Glial Cell Populations from the CNS of Virus-Infected Mice

Perfused brains taken from virus-infected WT or IRF7$^{-/-}$ animals were minced into small fragments and pressed through a 70 μM stainless steel mesh sieve into Hank's balanced salt solution (HBSS) containing 10% fetal bovine serum (FBS). The resultant tissue homogenate was digested with 0.2 mg/mL collagenase (Worthington Biochemical Corp., Lakewood, NJ) and 28 units/mL DNase (Sigma–Aldrich, St. Louis, MO) for 40 min at 37°C. Mononuclear cells were then isolated from the digested tissue slurry via centrifugation over 30%/70% Percoll gradients (GE Healthcare Life Sciences, Pittsburgh, PA), and cells recovered from gradient interfaces were counted and washed thoroughly with HBSS. Cells pooled from 10 WT or 10 IRF7$^{-/-}$ mice on day 4 postinfection were then suspended in PBS containing 2% FBS and stained using a combination of fluorescently tagged anti-CD45 and anti-CD11b antibodies (BD Biosciences, San Jose, CA). After gating on live intact cells, three easily defined and discrete mononuclear cell populations were identified and sorted using a MoFlo XDP High-Speed Cell Sorter (Beckman-Coulter, Indianapolis, IN): CD45^{high}/CD11b- (infiltrating lymphoid) cells, CD45^{high}/CD11b+ (infiltrating myeloid) cells, and CD45^{dim}/CD11b+ (endogenous microglial) cells. Individual cell populations were sorted, washed in PBS and stored at −20°C in PrepProtect RNA stabilization solution (Miltenyi Biotec, Auburn, CA) until final RNA isolations were performed.

Preparation and Use of Primary Microglial Cultures

Primary microglia were isolated and cultured from the cortices of 2- to 3-day-old WT or IRF7$^{-/-}$ mice as described (Esen and Kielian, 2005). When mixed glial cultures reached confluency after 7 to 10 days, flasks were shaken overnight at 200 rpm at 37°C to detach microglia from the more firmly adherent astrocytes. Cells in suspension (>95% pure CD11b+ microglia) were collected and 1×10^5 cells plated into each well of 96-well plates. The next day, microglia were stimulated either with NSV or a known TLR ligand for 24 h in a total volume of 200 μL. To generate inactivated NSV particles, virus was exposed to ultraviolet (UV) light generated by a germicidal lamp (wavelength = 254 nm) at a distance of 5 cm for 1 h at 25°C. Virus inactivation was confirmed by standard plaque titration assays (described below). Stimuli included: PBS (referred to as control in all our *in vitro* assays), 1×10^6, 1×10^7, or 1×10^8 pfu NSV (corresponding to virus particle-to-cell ratios of 10:1, 100:1 or 1,000:1, respectively), 1×10^6, 1×10^7, or 1×10^8 pfu UV-inactivated NSV, 100 ng/mL *Escherichia coli* LPS (List Biological Laboratories, Campbell, CA) as a TLR4 stimulus, 25 mg/mL polyinosinic:polycytidylic acid (poly(I:C), Invivogen) as a TLR3 stimulus, 1 mM loxoribine (Invivogen, San Diego, CA) as a TLR7 stimulus or 3 μM unmethylated DNA oligodeoxynucleotides bearing CpG motifs (CpG-ODN) (Invivogen) as a TLR9 stimulus. Some cultures were also performed in the presence of exogenous recombinant mouse IFN-α (PBL Interferon Source, Piscataway, NJ) at described doses. At the end of the stimulation period, culture supernatants were collected and stored at −80°C until cytokine and chemokine ELISAs could be performed. Microglial viability was assayed using the well-established 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Infectious virus was assayed in culture supernatants to screen for productive infection of microglial cells using a standard plaque titration assay as described (Irani and Prow, 2007; Liang et al., 1999; Tucker et al., 1996). Some cells were washed in PBS and stored at −20°C in PrepProtect RNA stabilization solution (Miltenyi Biotec) until final RNA isolations were performed.

Cytokine and Chemokine Assays

Frozen tissue homogenates were thawed, minced, and homogenized in 0.5 mL of PBS containing a protease inhibitor cocktail and an RNase inhibitor (both from Sigma-Aldrich). After homogenates were centrifuged to pellet all the remaining tissue debris, total protein content was measured in each extract and supernatants were further diluted in PBS to a normalized total protein concentration. Microglial culture supernatants were used without further manipulation. Levels of individual cytokines and chemokines were measured directly in samples using commercial sandwich ELISA kits according to the manufacturers' instructions. Results from tissue samples reflect the mean ± SEM of the pg of cytokine/mg of tissue extracted protein derived from a minimum of three animals at each time point. Data from culture supernatants reflect the mean ± SEM pg of chemokine/mL of culture supernatant derived from triplicate cell culture wells. Quantification of TNF-α, interleukin (IL)−12p40, and CCL2 levels was performed using mouse OptEIA kits (BD Biosciences), CXCL12 and CXCL13 levels were measured using Duoset mouse ELISA kits (R&D Systems, Minneapolis, MN), IFN-α and IFN-β levels were determined using mouse VeriKine ELISA kits (PBL Interferon Source), CCL19 concentrations measured by a mouse ELISA kit (Abcam, Cambridge, MA), and CCL21 levels determined by a mouse ELISA kit (Sigma-Aldrich). The lower limit of detection for these assays was typically 15.6 pg/mL.

PCR Quantification of CXCL13 Transcripts in Distinct CNS-Derived Cell Populations

Samples prepared from each CNS flow sorted or cultured microglial cell population were carefully thawed and removed from the PrepProtect RNA stabilization solution. Total RNA was isolated from each sample using QIAshredder Kit and the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Germantown, MD). A cDNA template was then generated from each sample using the SuperScript® III First Strand Synthesis System for reverse transcriptase–PCR (Invitrogen, Carlsbad, CA). Semi-quantitative PCR was undertaken using the MyiQ Single Color Real-Time PCR

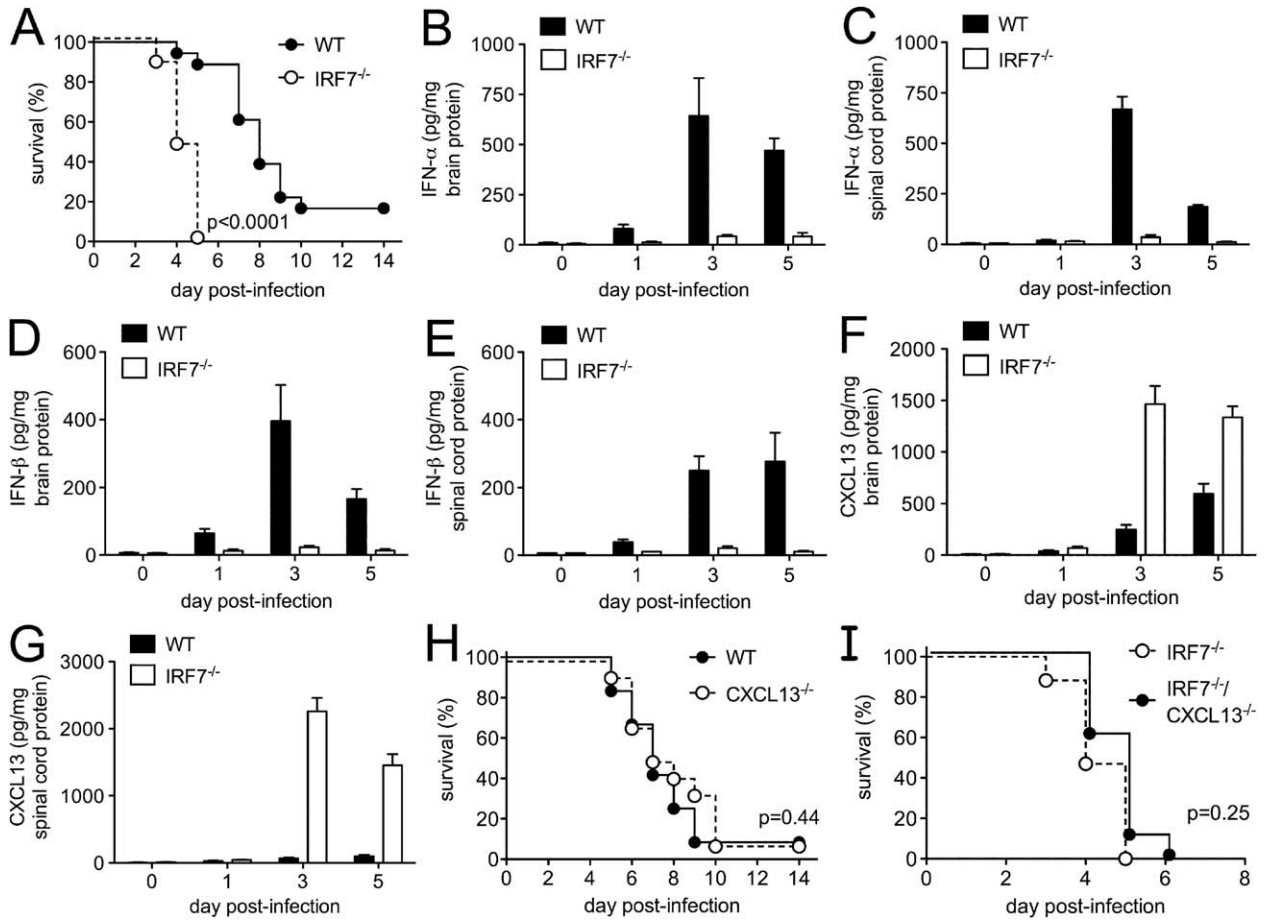


FIGURE 1: IRF7 controls both CXCL13 levels in the brain and spinal cord as well as disease outcome during lethal neuroadapted SV (NSV) encephalitis in mice. A. IRF7$^{-/-}$ mice are highly susceptible to a lethal NSV challenge compared with WT control animals (n = 16 per group). B–E. Neither IFN-α nor IFN-β is induced in the brains or spinal cords of IRF7$^{-/-}$ mice at any stage of infection prior to death. F, G. This heightened disease susceptibility in IRF7$^{-/-}$ mice is associated with unrestrained CXCL13 production in both CNS compartments of infected animals over time. H, I. CXCL13 is not pathogenic during NSV infection in either WT (n = 12 per group) or IRF7$^{-/-}$ mice (n = 10 per group) as its deletion from either host did not confer any protection against disease.

Detection System (Bio-Rad Laboratories, Hercules, CA) and the *cxcl13* primer/probe set in the TaqMan® Gene Expression Assay (Applied Biosystems Inc., Foster City, CA). Data were analyzed against a β-actin mRNA standard and are shown as relative *cxcl13* mRNA expression. For the flow-sorted cell populations, expression values were then normalized to the level present in CD45^{high}/CD11b+ cells isolated from WT mice assigned an arbitrary level of 1.0. For the cultured microglial cells, expression values were then normalized to the level present in vehicle-treated (control) WT cells assigned an arbitrary level of 1.0 (Fig. 3D) or to the level present in IRF7$^{-/-}$ cells assigned an arbitrary level of 1.0 (Fig. 4D).

Statistical Analyses

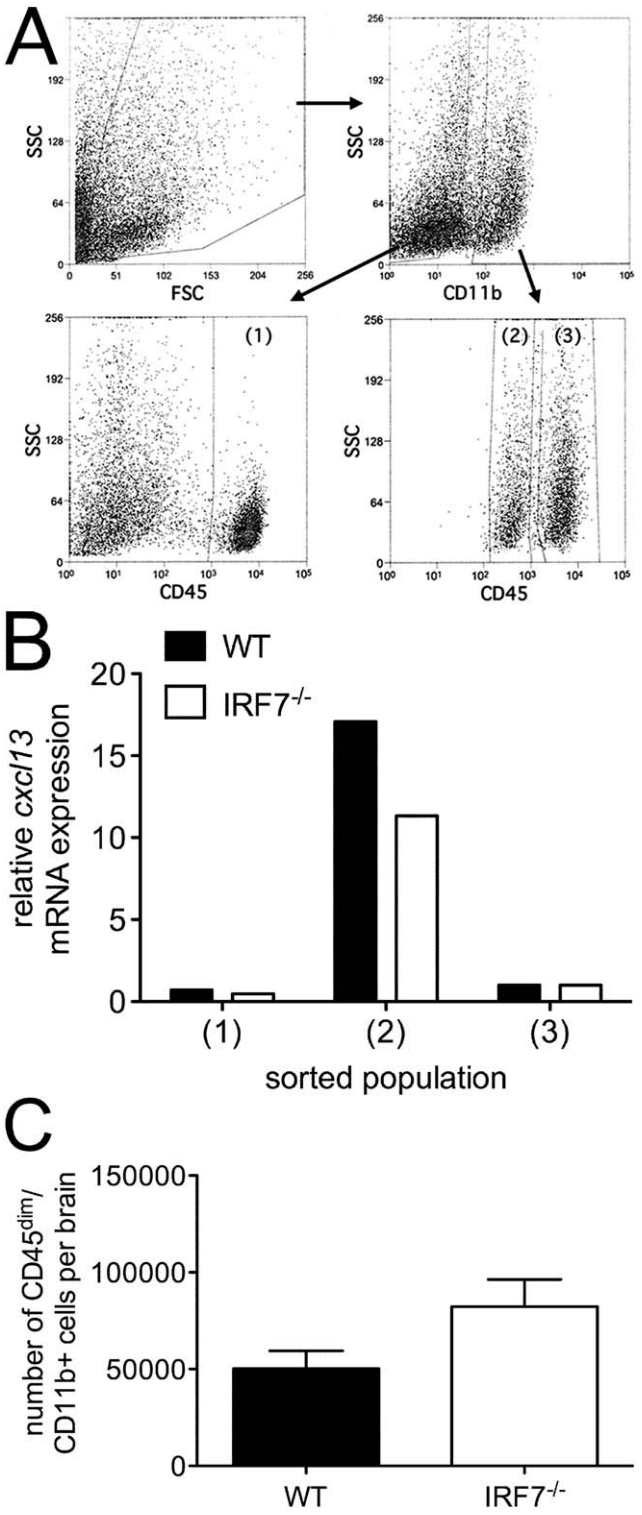
The Prism 5.0 software package (GraphPad Software Inc., La Jolla, CA) was used for all statistical analyses. Differences in survival among cohorts of mice were measured using a log-rank (Mantel-Cox) test. Unpaired Student's t test was used to assess differences between different experimental groups at a single time point. A two-way analysis of variance (ANOVA) test was applied when comparing different experimental groups over time. In all cases, differences at a $P < 0.05$ level were considered significant.

Results

High Levels of CXCL13 are Produced in the CNS of Mice who fail to Mount a Robust Type-I IFN Response During Virus-Induced Neuroinflammation

Host factors strongly influence disease outcome in mice with NSV encephalitis, and early innate immune responses are particularly important determinants of survival (Liang et al., 1999; Tucker et al., 1996). To investigate the role of IRF7 and type-I IFN in disease pathogenesis, WT and IRF7$^{-/-}$ mice were subjected to lethal NSV challenge. Survival assays showed heightened disease susceptibility among IRF7$^{-/-}$ mice compared with WT controls (Fig. 1A). Measurement of tissue viral titers showed evidence of unrestrained virus replication in the CNS of the IRF7$^{-/-}$ cohort (Supp. Info. Fig. S1), while analysis of tissue homogenates showed negligible

induction of either type-I IFN in the CNS of these animals (Fig. 1B–E). To better understand the downstream consequences of IRF7 and type-I IFN deficiency in this setting, multiple inflammatory mediators were measured in both brain and spinal cord tissues derived from these two hosts. One of the most notable differences related to tissue levels of the lymphoid chemokine, CXCL13; protein concentrations were up to 10-fold higher in the CNS of IRF7$^{-/-}$ animals over the course of acute infection (Fig. 1F,G). Nonetheless, a detrimental effect of CXCL13 on disease outcome could not be demonstrated, as deletion of *cxcl13* from either WT or IRF7$^{-/-}$ hosts had no impact on their survival following NSV challenge (Fig. 1H,I). Still, these *in vivo* data suggested that IRF7 and the type-I IFN it produces could be negative regulators of CXCL13 expression in the CNS.



Microglia are the Main Source of CXCL13 Transcripts in the CNS During Virus-Induced Neuroinflammation

To identify the cellular source of CXCL13 in the CNS during NSV infection, candidate mononuclear cell populations derived from either WT or IRF7$^{-/-}$ animals were sorted from one another by flow cytometry based on different patterns of CD11b and CD45 expression (Fig. 2A). Total cellular RNA was then extracted from each population of cells that had been pooled from the brains of multiple WT or IRF7$^{-/-}$ mice on day 4 post-infection, and relative *cxcl13* mRNA levels were quantified compared with a *β-actin* mRNA standard. Pilot studies confirmed that CD45^{neg} cells isolated from the CNS of either host did not express *cxcl13* (data not shown), consistent with our previous *in vitro* data showing that non-hematopoietic neural cells such as astrocytes do not produce this mediator in response to virus challenge (Rainey-Barger et al., 2011). Amongst the three isolated CD45+ cell subsets, *cxcl13* transcripts predominated in CD45^{dim}/CD11b+ microglia (sorted population 2) derived from both WT and IRF7$^{-/-}$ mice (Fig. 2B). These CD45^{dim}/CD11b+ cells proved to be slightly more abundant in the brains of the IRF7$^{-/-}$ cohort, although the difference was not significant (Fig. 2C). Since CXCL13 was not likely being made by another CNS cell type in IRF7$^{-/-}$ mice, and

FIGURE 2: Microglia are the principal source of CXCL13 production in the CNS during NSV encephalitis in mice. A. Mononuclear cells were isolated from the brains of 10 WT and 10 IRF7$^{-/-}$ animals on day 4 post-infection as described in Materials and Methods. Using flow cytometry to gate on intact cells, individual mononuclear populations were separated based on CD11b expression, then on CD45 levels. Pooled CD45^{high}/CD11b- lymphoid cells (population 1), CD45^{dim}/CD11b+ microglia (population 2) and CD45^{high}/CD11b+ monocytes (population 3) were then used for RNA extraction and quantitative PCR as described in Materials and Methods. B. Relative *cxcl13* mRNA levels, first normalized to *β-actin* transcript levels and then to expression levels in CD45^{high}/CD11b+ myeloid cells from WT mice, were then determined for each of the three populations of CNS-derived cells from either WT or IRF7$^{-/-}$ animals. C. CD45^{dim}/CD11b+ microglial cell numbers present in the brains of WT and IRF7$^{-/-}$ mice at this stage of disease were not significantly different from one another ($P = 0.12$).

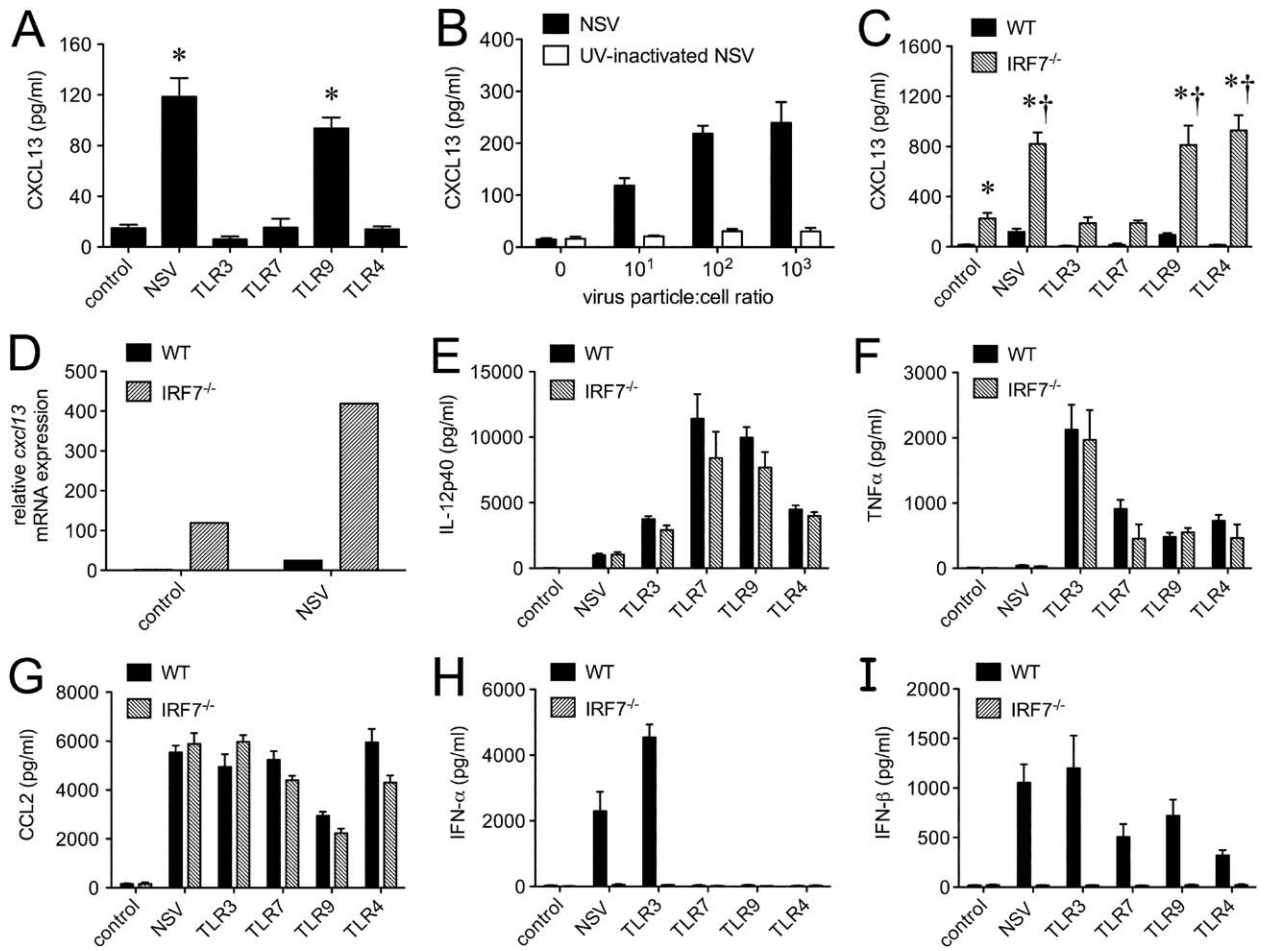


FIGURE 3: Specific ligands and signaling pathways control cytokine and chemokine production by primary microglial cells *in vitro*. A. Both NSV and a synthetic TLR9 agonist provoked microglial CXCL13 production that was not induced using agonists specific for TLR3, TLR7, or TLR4 (*$P<0.05$ *vs.* unstimulated control cultures). B. This NSV-induced release of CXCL13 by microglia varied approximately twofold across a 100-fold difference in the ratio of virus particles to target cells, and it was not elicited by UV-inactivated virus. C. CXCL13 release was significantly higher in IRF7$^{-/-}$ *versus* WT cells (*$P<0.05$ in IRF7$^{-/-}$ *vs.* WT cultures; †$P<0.05$ in stimulated *vs.* unstimulated IRF7$^{-/-}$ cultures). D. *cxcl13* transcripts were higher at baseline and following NSV stimulation in IRF7$^{-/-}$ *versus* WT cells. E–G. Stimulated production of selected other cytokines and chemokines was not different between WT and IRF7$^{-/-}$ microglia. H, I. Cells from IRF7$^{-/-}$ mice did not produce measurable amounts of either IFN-α or IFN-β in response to any of the stimuli examined.

because there was not a dramatic increase in the numbers of activated microglia in the brains of these hosts (Fig. 2C), these gene expression data infer that differences in CXCL13 protein levels present in CNS tissue homogenates from infected mice (Fig. 1F,G) derive from an effect of IRF7 or type-I IFN acting at a translational or post-translational level. They also clearly identify microglia as being the principal mononuclear cell source of CXCL13 in the CNS during NSV encephalitis.

Type-I IFN Regulates CXCL13 Production by Primary Microglia In Vitro

Microglial activation is an early neuropathological finding observed in mice infected with NSV (Irani and Prow, 2007, Prow and Irani, 2007). Nonetheless, the various receptors, signaling pathways, and soluble mediators either acting upon or produced by these innate immune cells during infection remain poorly defined. Using primary microglial cell cultures, we found that both NSV as well as a synthetic TLR9 ligand induced measurable CXCL13 production *in vitro* (Fig. 3A). Ligands specific for TLR3, TLR4, and TLR7 did not elicit this response (Fig. 3A), nor did any of these stimuli trigger microglia to produce other lymphoid chemokines, including CXCL12, CCL19, or CCL21 (Supp. Info. Fig. 2). At no point did exposure to the virus cause productive infection or diminished viability of these innate immune cells (Supp. Info. Fig. 3). Any residual astrocytes that might contaminate our microglial cultures were not considered to be a likely source of CXCL13 based on our prior data showing that these cells do not produce this mediator following virus exposure

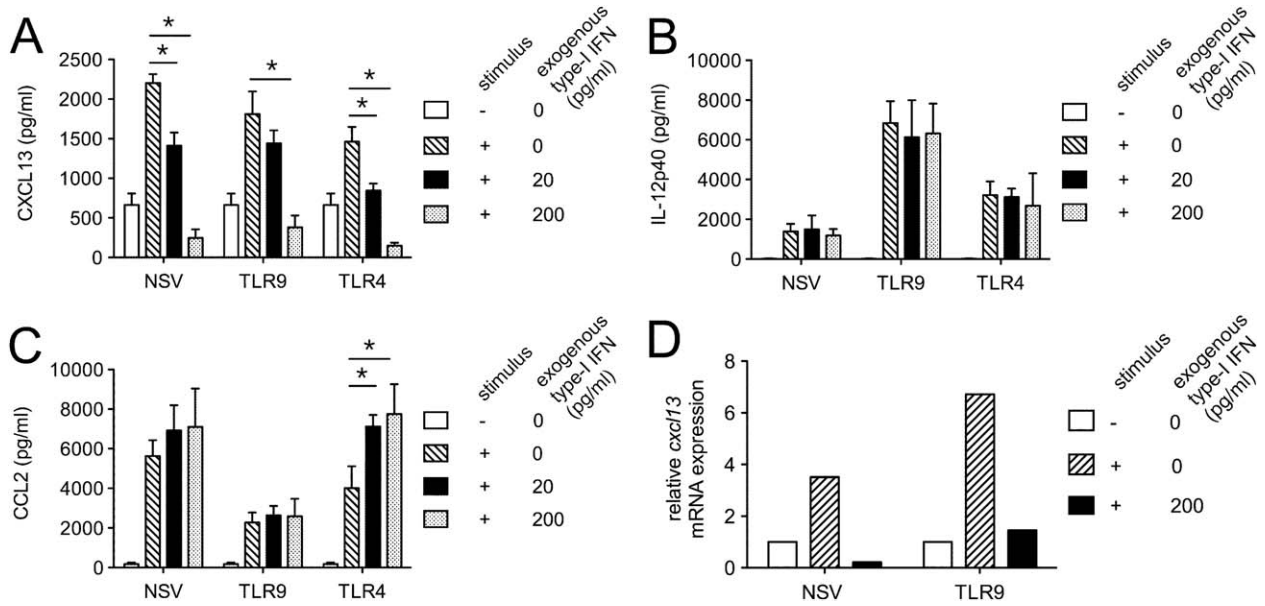


FIGURE 4: Exogenous type-I IFN suppresses CXCL13 production by IRF7$^{-/-}$ microglia in a dose-dependent manner. A. Both NSV as well as TLR9 and TLR4 ligands stimulated robust CXCL13 production by IRF7$^{-/-}$ microglia that was suppressed by the addition of increasing concentrations of recombinant IFN-α to the cultures (*$P<0.05$ in treated *vs.* untreated cultures). B, C. The same treatment had no effect on the stimulated production of the inflammatory cytokine, IL-12p40, and it had no effect or even the opposite effect on the stimulated production of the non-lymphoid chemokine, CCL2 (*$P<0.05$ in treated *vs.* untreated cultures). D. Exogenous type-I IFN directly suppressed the induction of *cxcl13* transcript levels following either NSV or TLR9 stimulation.

(Rainey-Barger et al., 2011). Production of CXCL13 increased somewhat in proportion to the amount of NSV added to the cultures, while UV-inactivated virus failed to trigger microglial CXCL13 release (Fig. 3B). This finding is consistent with the need for an undamaged viral genome or the presence of some replication intermediate to trigger the pattern recognition receptor(s) that drive microglial production of this chemokine. To clarify a role for IRF7 in dampening this response, primary cells isolated from IRF7$^{-/-}$ mice were examined. In keeping with our *in vivo* findings, CXCL13 protein levels were significantly increased in the supernatants of stimulated microglia derived from IRF7$^{-/-}$ hosts (Fig. 3C). Thus, IRF7$^{-/-}$ microglia produced some CXCL13 without any exogenous stimulation, showed heightened CXCL13 production compared with WT cells following both NSV exposure or TLR9 ligation, and were now capable of eliciting CXCL13 release in response to a TLR4 ligand (Fig. 3C). Unlike our analysis of *ex vivo* microglia (Fig. 2B), however, the absence of IRF7 and/or type-I IFN resulted in higher *cxcl13* transcript levels in cultured microglia both at baseline and in response to NSV exposure (Fig. 3D). In contrast to CXCL13, IRF7$^{-/-}$ microglia did not overproduce other pro-inflammatory cytokines or chemokines following exposure to NSV or different TLR ligands (Fig. 3E–G, Supp. Info. Fig. 2). As predicted, IRF7$^{-/-}$ cells did not make type-I IFN in response to any of the different *in vitro* stimuli examined (Fig. 3H,I).

Since IRF7 is a central regulator of type-I IFN production by myeloid cells (Honda et al., 2005; Ning et al., 2011), and because stimulated IRF7$^{-/-}$ microglia did not produce measurable amounts of type-I IFN in response to a range of stimuli (Fig. 3H,I), we hypothesized the existence of a negative feedback loop whereby type-I IFN specifically suppresses microglial CXCL13 release. To investigate this possibility, IRF7$^{-/-}$ microglia were stimulated in the presence of increasing amounts of exogenous IFN-α. Treatment with this mediator caused CXCL13 protein levels to decline in a dose-dependent manner following stimulation with NSV as well as TLR4 or TLR9 ligation (Fig. 4A), while having no effect on cell viability over the doses and culture intervals examined (data not shown). Conversely, exogenous IFN-α had no inhibitory effect on the production of other cytokines or chemokines by these innate immune cells (Fig. 4B,C). When the effecte of exogenous IFN-α on stimulated CXCL13 production by IRF7$^{-/-}$ microglia were examined at a transcriptional level, treatment clearly suppressed the accumulation of *cxcl13* transcripts (Fig. 4D).

Finally, to confirm the existence of this negative feedback mechanism, microglia prepared from IFNAR-deficient mice were examined. These cells overproduced CXCL13 at baseline, and they also made higher levels of CXCL13 compared with WT cells in response to either NSV or a TLR9 ligand (Fig. 5). The addition of exogenous IFN-α had no inhibitory effect on CXCL13 production in these cultures

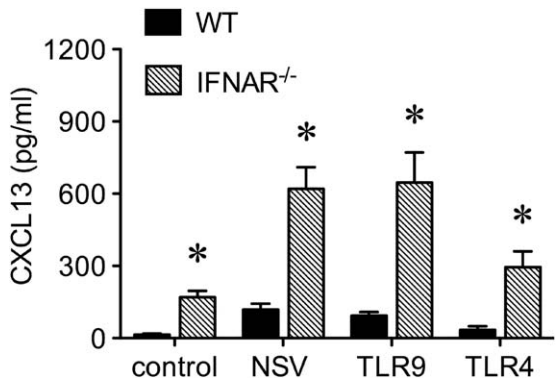


FIGURE 5: Genetic deletion of IFNAR enhances CXCL13 production compared with WT microglia. Microglia cultured from IFNAR-deficient mice made significantly higher amounts of CXCL13 compared with WT cells under all culture conditions examined (*$P < 0.05$ in IFNAR$^{-/-}$ *vs.* WT cultures).

(data not shown). Taken together, these data show that local production of type-I IFN by microglial cells specifically inhibits release of the homeostatic chemokine, CXCL13, in an autocrine and/or paracrine manner via IFNAR signaling by acting to inhibit *cxcl13* gene transcription or transcript stability.

Discussion

The stromal cells found in B cell follicles of lymphoid organs normally produce CXCL13 that signals through its cognate receptor, CXCR5, to recruit B cells and Tfh cells to these specialized structures (Allen et al., 2004; Ansel et al., 2000; Campbell et al., 2003; Forster et al., 1996; Legler et al., 1998; Moser et al., 2002; Muller et al., 2003). Outside of maintaining homeostasis in lymphoid tissues, however, other functions for CXCL13 remain incompletely defined. Data generated in two infection models point to a role for CXCL13 in recruiting B cells to non-lymphoid organs such as the lung or liver where it helps organize local immune responses against influenza or hepatitis B, respectively (Publicover et al., 2013; Rangel-Moreno et al., 2007). Other evidence suggests that ectopic expression of CXCL13 may instead propagate destructive tissue inflammation; when transgenic mice are forced to express CXCL13 in pancreatic islets, B cells accumulate abnormally, structures reminiscent of lymphoid follicles appear, and animals develop diabetes (Luther et al., 2000). Understanding whether and how CXCL13 contributes to the pathogenesis of CNS diseases remains obscure, in part because little has been published describing either its source or the factors controlling its expression in the brain. In experiments reported here, we find that microglia are the main producers of CXCL13 in the CNS of mice with virus-induced neuroinflammation, and that type-I IFNs inhibit production of this chemokine by these cells. We speculate that such a system may have evolved to limit any destructive potential associated with chronic tissue expression of this chemokine. It could also explain the therapeutic benefit of exogenous type-I IFN (in this case, IFN-β) in chronic CNS autoimmune diseases such as MS.

The recruitment and retention of antibody-secreting cells (ASCs) to the meninges or the brain is an important adaptive host response that occurs with many experimental viral infections of the CNS. Here, locally generated antiviral antibodies can neutralize extracellular virus particles or block viral budding from infected cells to promote non-cytolytic clearance of these pathogens (Phares et al., 2013). Where they have been examined, B cell chemoattractant, survival and differentiation factors are induced in the CNS during these infections, although a specific role for CXCL13 in acute viral clearance and recovery has not been confirmed in any of these systems (Phares et al., 2013; Rainey-Barger et al., 2011). Nonetheless, mice that recover from acute alphavirus encephalitis because of early CNS viral clearance generate virus-specific ASCs that reside long-term in the brain (Tyor et al., 1992). These cells guard against late viral reactivation, and it is likely that CXCL13 contributes to this chronic host immune response (Rainey-Barger et al., 2011). Thus, the ongoing actions of CXCL13 in the CNS may be adaptive for the host in this recovery setting, and it may occur efficiently since local type-I IFN levels have long since returned to baseline following the acute infection.

Experimental evidence in animals also paradoxically confirms an important role for CXCL13 in propagating aberrant CNS inflammation. Thus, ectopic lymphoid follicles have also been described in the meninges of mice with EAE, the principal animal model of MS (Magliozzi et al., 2004; Peters et al., 2011). In this experimental system, however, CXCL13 prolongs disease by sustaining myelin-specific CD4+ T cell responses rather than by recruiting CXCR5+ B cells to the acutely inflamed CNS (Bagaeva et al., 2006; Rainey-Barger et al., 2011). While CXCL13$^{-/-}$ mice are susceptible to EAE induction and develop early symptoms that parallel WT animals, CNS inflammation subsides in CXCL13$^{-/-}$ hosts who recover back to baseline instead of showing persistent deficits that typifies WT disease (Bagaeva et al., 2006). How CXCL13 actually sustains pathogenic CD4+ T cells in this setting remains unclear, but since it brings lymphocytes into close physical proximity with DC in lymphoid organs (Ansel et al., 2000; Forster et al., 1996; Legler et al., 1998), it may serve a similar function in the CNS. Regardless, if the main role of CXCL13 during EAE is to sustain myelin-specific CD4+ T cells once they have been generated, then this chemokine becomes an excellent therapeutic target during more established stages of CNS autoimmunity.

Only a small handful of studies have reported microglial production of CXCL13. In a unique *ex vivo* slice culture model using primate brain tissue, acute exposure to the Lyme disease spirochete, *Borrelia bergdorferi*, induced CXCL13 staining of Iba-1+microglia along with histochemical evidence of apoptosis in both neurons and oligodendroglia (Ramesh et al., 2008). Likewise, recent transcriptional profiling of mouse microglia prepared and/or obtained from multiple sources showed a gene expression pattern that was distinct from other macrophage lineage cell types and included *cxcl13* (Beutner et al., 2013). In our hands, NSV infection triggered the generation of *cxcl13* transcripts almost exclusively in $CD45^{dim}$/CD11b+ microglia when purified CNS mononuclear cells were studied directly *ex vivo* (Fig. 2A,B). Although *cxcl13* transcript levels present in WT and $IRF7^{-/-}$ microglia (Fig. 2B) did not account for differences in CXCL13 protein concentrations directly in CNS tissues (Fig. 1F,G), our *in vitro* studies confirmed that the absence of IRF7 and/or type-I IFN enhances *cxcl13* transcript abundance in cultured microglia (Fig. 2D) and that exogenous type-I IFN clearly suppresses stimulated transcription of this gene in $IRF7^{-/-}$ cells (Fig. 4D). Ongoing studies aim to clarify the molecular mechanisms underlying this transcriptional control.

Primary microglia clearly respond to extracellular IFN-β (McManus et al., 2000), and emerging evidence suggests that both type-I and type-II IFN can influence the expression of CXCL13 outside the CNS. Thus, IFN-γ is known to transiently inhibit CXCL13 expression in lymph nodes in a way that helps orchestrate local immune responses (Mueller et al., 2007), while IFN-β can alter CXCL13 production by epithelial cells in the thymus to influence germinal center formation and autoantibody production in myasthenia gravis (Cufi et al., 2014). Interestingly, type-I IFN signaling in DC actually stimulates the generation of CXCR5+ Tfh within lymph nodes that, in turn, promote B cell immunity (Cucak et al., 2009). Thus, while type-I IFN suppresses the generation of a signal that recruits B cells into tissues and forms germinal centers, it also promotes the development of a distinct T cell subset that plays an important role to support these same processes. Our data are the first to identify a similar regulatory process controlling CXCL13 production in the CNS, even as it is clear that B cells find CXCL13-independent pathways into the CNS (Rainey-Barger et al., 2011), and that induction of this particular mediator has no direct impact on the outcome of the acute infection being studied here (Figs. 1H,I).

Whether and how microglial production of CXCL13 might relate to the formation of ectopic lymphoid follicles that specifically localize to the meninges during either infectious or autoimmune CNS disorders remains unknown. NSV infection, particularly in the $IRF7^{-/-}$ host, is simply too rapid to allow these organized structures to form prior to death. Our data do not exclude the possibility that a stromal-like cell present in the meninges could also produce CXCL13 under the appropriate circumstances, and in doing so would drive the focal accumulation and organization of lymphoid cells at that site. On the other hand, there are also examples of where meningeal cells clearly influence brain parenchymal structure and function (Derecki et al., 2010), so it is not inconceivable that local CXCL13 production by parenchymal microglia could influence events in the overlying meninges. Finally, construction of a transgenic mouse where inducible expression of CXCL13 will lie under control of the transthyretin promoter, thus causing selective chemokine expression in the meninges and choroid plexus (Schreiber, 2002), is underway and could be another way to illuminate the roles of parenchymal- *versus* meningeal-derived CXCL13. One hypothesis to be explored in such mice is that the induction of EAE could potentially result in the formation of meningeal lymphoid follicles, thereby allowing investigation of their role in more chronic progressive forms of CNS demyelinating disease.

In conclusion, we demonstrate the microglia are an important source of CXCL13 production in the acutely inflamed brain, and *in vitro* experiments confirm that type-I IFN selectively inhibits microglial production of CXCL13 in a dose-dependent manner. Our results confirm the existence of a novel regulatory pathway controlling expression of this lymphoid chemokine within the CNS. Further elucidation of these events could have important implications for understanding chronic B cell immunity within the CNS and they could even shed light on how exogenous IFN-β functions in the clinical management of patients with MS.

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