The Role of Interferon-γ in the determination of Central Nervous System Damage in Experimental Autoimmune Encephalomyelitis

By

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DEDICATION

I dedicate this thesis to my parents, without whom none of this was possible. I dedicate the rest of my life to my wife Quinn and the great adventures we will have together.

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ABSTRACT

In multiple Sclerosis (MS), a multifocal inflammatory demyelinating disease of the central nervous system (CNS), lesion distribution is highly variable between patients resulting in distinct patterns of disease progression and clinical deficits. The mechanisms that regulate immune cell migration to and subsequent lesion development in different areas of the CNS are not well understood.

Experimental Autoimmune Encephalomyelits (EAE), a critical model in developing effective therapies in MS, can also be used to examine mechanisms driving lesion localization. In the adoptive transfer EAE model, transfer of myelin-specific $CD4^+$ T cells into wild type (WT) mice results in an ascending paralysis due to inflammation predominantly in the spinal cord (SC) (conventional EAE), while transfer of these same T cells into mice lacking the interferon-γ receptor (IFNγR) results in balance deficits due to inflammation predominantly in the brainstem (BS) (atypical EAE). The reciprocal transfer of IFNγ KO myelin-specific T cells into WT mice results predominantly in atypical EAE. In addition to deficiencies in IFNγ signaling, increased IL-17 signaling has also been shown to promote brain inflammation and atypical EAE pathogenesis. However, the mechanisms by which these cytokines promote distinct lesion localization are incompletely characterized.

IFNγ and IL-17 play reciprocal roles in the recruitment of neutrophils to sites of inflammation. The absence of IFNγ or increased IL-17 signaling can promote neutrophil infiltration in multiple infection and autoimmune models of disease. Some early studies examined neutrophil infiltration in EAE but none have examined their absolute requirement in atypical EAE.

Using adoptive transfer models of EAE, we show that atypical EAE is driven by CXCR2-mediated recruitment of neutrophils to the brainstem, whereas conventional EAE is driven by CCR2-mediated recruitment of monocytes to the spinal cord. We also find that IFNγ signaling suppresses atypical EAE by directly inhibiting CXCL2-dependent neutrophil recruitment axis. IFNγ directly inhibits CXCL2 production by monocyte and microglia in the BS during EAE. Additionally, IFNγ suppresses neutrophil production of CXCL2 in response to CXCR2 binding by directly inhibiting expression of the CXCR2 receptor on neutrophils in the CNS. Overall,

these studies identify a distinct CXCR2-dependent recruitment pathway in CNS autoimmunity that is not currently targeted by current disease modifying therapies.

CHAPTER 1 - INTRODUCTION

Multiple Sclerosis (MS), an inflammatory demyelinating disease of the central nervous system (CNS), is one of the most common and costly autoimmune conditions in the world. There is a greater prevalence of MS in Western Europe and North America compared to the Middle East, Asia and Africa, however, the incidence of MS is increasing worldwide (1). The mean age of MS onset is 30 years of age with 70% of patients being diagnosed between the ages of 20 and 40 (2). The average cost of care for insured patients on current disease modifying therapies is approximately $27,000 dollars per year(3). Considering the early age of MS onset, increased prevalence of disease and longer overall life expectancy, these costs continue to escalate for patients as well as the healthcare system. Subsets of patients do not respond to the current disease modifying therapies, necessitating further study of MS in clinical studies and animal models.

1a. MS: Heterogeneity in disease progression and pathology

1a.i. Complexity in MS diagnosis and disease progression

Individuals with MS are generally classified into 3 subsets based on their clinical course. Relapsing-Remitting MS (RRMS), the most common form, is characterized by discrete attacks of neurological dysfunction that patients recover from, either partially or completely, early on. In the vast majority of RRMS patients the clinical course eventually transitions into a secondary progressive (SP) phase, during which relapses decrease in frequency and often disappear, only to be replaced by a slow indolent accumulation of disability. Approximately 50% of RRMS patients develop Secondary Progressive MS within 10 years of diagnosis. Primary Progressive

MS (PPMS), which occurs in 10-15% of cases, is characterized by a progressive decline without antecedent relapses. (Figure 1-1).

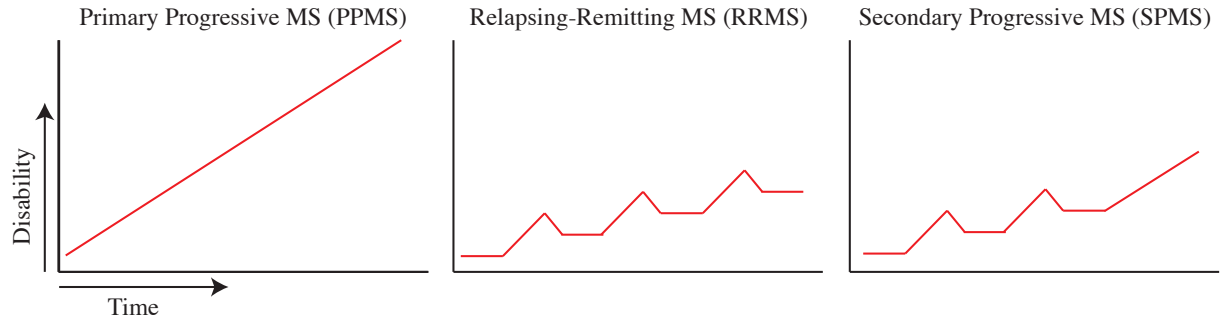

Figure 1-1: Patterns of disability progression over time in PPMS, RRMS and SPMS.

1a.ii. Disease Pathogenesis

Clinical and neuropathological studies have implicated dysregulation of the immune system, particularly T cells and myeloid subsets, in the pathogenesis of MS. Hallmark features of MS plaques include perivascular infiltrates, primarily composed of T cells and monocyte/macrophages(4-6). Genome-wide association studies (GWAS) have shown that most susceptibility loci in MS involve immune system-associated genes. The strongest heritable risk factors have been found in major histocompatibility complex (MHC) loci (7, 8), and in MHC Class II genes in particular. Additionally, single nucleotide polymorphisms of the IL-2 receptor (*IL2RA*) and IL-7 receptor alpha chains (*IL7RA)* have been associated with MS risk (9). Collectively, the genetics of MS supports an autoimmune etiology and a role of pathogenic CD4+ T cell responses.

1a.iii. Complexity in lesion location and type

Inflammation, demyelination, remyelination, neurodegeneration and glial scarring occur in the white and grey matter in the brain, spinal cord (SC) and optic nerves in all forms of MS(10, 11). However, there is a great deal of heterogeneity in the location and microscopic appearance of lesions. The majority of patients have prominent periventricular, subcortical and brainstem (BS) white matter lesions. Some individuals have lesions predominantly in the SC and optic nerves(12, 13). An opticospinal form of MS is more prevalent in Asian populations(14). In addition to a skewed distribution of lesions, opticospinal MS differs from the conventional form MS (that is common in the Western Hemisphere) due to a greater prominence of neutrophils in

parenchymal infiltrates. Cerebrospinal fluid (CSF) levels of the neutrophil recruitment-promoting cytokine IL-17(15, 16) and the neutrophil chemoattractant IL-8 are higher in patients with opitcospinal compared to conventional MS(17). Another CNS autoimmune condition, neuromyelitis optica (NMO), is also characterized by a predominance of lesions in the optic nerves and spinal cord and neutrophil rich infiltrates(18). However, 70% of NMO patients test positive for the presence of anti-aquaporin 4 antibodies in the sera(19) whereas only a small percent of individuals with opticospinal(20) and conventional MS(21) are positive for the antibody. While NMO and opticospinal MS are thought to be distinct conditions there may have overlapping pathological pathways.

Among patients with conventional MS there is also heterogeneity in the histopathological features of lesions. An extensive neuropathological study compared 51 biopsy and 31 autopsy samples of actively demyelinating lesions and classified them into 4 distinct patterns(22). Pattern I, the most common form, was characterized by focal demyelination and perivascular cuffs populated with T cells and macrophages. Pattern II lesions resembled Pattern I lesions but were also notable for the presence of IgG and complement deposits. Unlike Patterns I and II, Pattern III lesions were not centered around veins or venules. There was more diffuse demyelination, significant apoptosis of oligodendrocytes (23) and disproportionately decreased staining for myelin-associated glycoprotein (MAG). Pattern IV lesions were rare, found only in PPMS patients. They differed from pattern III lesions in that oligodendrocyte loss did not appear to be due to apoptosis. While diffuse demyelination was present, the MAG protein was not preferentially depleted. The complexity in the clinical manifestations and pathological findings in multiple sclerosis make it a difficult disease to study using only clinical assessments, patient samples and post-mortem pathology. Fortunately, an animal model, Experimental Autoimmune Encephalomyelitis (EAE), simulates many of the clinical and pathological features of MS and has provided insights into the mechanisms that drive autoimmune demyelination.

1b. EAE

1b.i. Origins of the EAE model

The EAE model was largely discovered when Dr. Thomas M Rivers was studying rabies vaccinations at the Rockefeller Institute in the 1930s(24). At the time, rabies vaccines were produced by infecting animals with the rabies virus and pulling their CNS tissue during the end stages of the infection, inactivating the virus via ether-phenol incubation for use in rabies

vaccination in humans(25). Dr. Rivers hypothesized that small amounts of active rabies virus present in such vaccines was responsible for neurological symptoms patients experienced following vaccination. When he tested this theory, animals that had been immunized with uninfected CNS tissue also developed neurological deficits. When CNS extracts were mixed with Complete Freund's Adjuvant (CFA), invented in the 1940s, animals developed the neurological deficits at a higher frequency(26). Subsequent studies detailing the neuropathology of EAE revealed similar features to MS including primary damage to the white matter, focal lesions associated with blood vessels, and variability in lesion and symptom patterns(27). Subsequent whole CNS fractionation studies showed that immunization with fractions enriched for myelin basic protein were the most encephalitogenic(28).

1b.ii. EAE pathology induced by myelin-reactive T cells

To identify immunological mechanisms of EAE pathogenesis, investigators sought to identify an immune cell type isolated from myelin protein-immunized donors capable of transferring EAE to naive mice. Dr. Paterson at NYU showed that transferring whole lymph nodes from rats immunized with myelin peptide in CFA could induce EAE in naïve recipients(29). Subsequently, it was shown that incubating lymphoblasts from myelin protein/CFA-immunized rats with myelin protein and (MHC)-expressing, syngenic accessory cells improved the efficiency of adoptive transfer experiments, as fewer cells had to be transferred per animal(30). Subsequent studies demonstrated that CD4+ T cells isolated from the cultured cells were encephalitogenic.

1b.iii. EAE model recapitulates variability in MS disease progression

Following the introduction of the EAE model, there was a rapid expansion into different mouse strains using different myelin peptides as the autoantigen. Initial studies found B10.PL, PL/J and SJL mouse strains developed EAE in response to immunization with MHC II-restricted Myelin Basic Protein (MBP) epitopes(31), but the clinical course varied between strains. B10.Pl and PL/J mice had acute monophasic encephalomyelitis while SJL mice had a clinical course that resembled RRMS(32, 33). A major breakthrough came when the peptide myelin oligodendrocyte glycoprotein residues 35-55 (MOG_{35-55}) was found to be an encephalitogenic epitope in the C57BL/6 strain of mice(34). This discovery, coupled with the rapid expansion of genetic alterations made on the C57BL/6 background, facilitated more detailed mechanistic studies.

1b.iv. Modeling variable lesion localization in MS using the EAE model

EAE models can differ regarding predominant lesion location and clinical phenotype. In the majority of EAE models, the spinal cord is preferentially targeted, resulting in an ascending paralysis. These mice progress from a limp tail, to hind limb weakness, to paralysis of the hind limbs; in rare, severe cases there can be full paralysis or death(35). The clinical phenotype is referred to as conventional EAE (cEAE). In other models, for example C3H/Fej mice primed with the myelin peptide MOG_{97-114} generate Th17 effector cells that target the cerebellum, resulting in balance deficits. Conversely, C3H/Fej mice primed with the alternative peptide MOG_{35-55}, generate Th1 effector cells that target the spinal cord, resulting in an ascending paralysis(36). Hence, there are intrinsic differences between inbred strains, as well as in T cells of different lineages and antigenic specificities, that may predispose towards either cEAE or aEAE.

A prominent regulator of lesion location across multiple mouse backgrounds is the cytokine IFNγ. In the absence of IFNγ signaling, mice develop an axial rotary or atypical form of EAE (aEAE) that is characterized by a progressive loss of balance due to primary infiltration of the vestibular nucleus in the BS (37-41). In subsequent sections we will explore what is known about IFNγ signaling in immune responses, MS and EAE and how it may regulate regional infiltration in CNS autoimmunity.

1c. IFNγ: pleiotropic cytokine in CNS autoimmunity

1c.i. IFNγ: functionally distinct member of the Interferon family

Interferons (IFNs) were initially characterized as the secreted factors which interfere with viral replication(42, 43). Subsequent studies determined they comprise a diverse family of factors with distinct signaling pathways and roles in immune responses. The IFNs have been grouped into 2 distinct ligand types based on their receptor specificity. Type I IFNs, the most common of which are IFNα and IFNβ, bind the heterodimeric receptor consisting of IFNAR1 and IFNAR2. IFNγ falls into its own category in the Type II IFN family, which binds the heterodimeric receptor consisting of IFNγR1 and IFNγR2(44). These receptors mediate distinct signaling pathways, Type I IFN receptors phosphorylate STAT 1 and 2, which heterodimerize and translocate to the nucleus and bind Interferon Response Elements (IREs) to mediate downstream signaling. Type II IFN receptors predominantly signal through STAT1 homodimers

and bind Gamma Interferon Activation Sites (GAS) in promoter regions of target genes(45-47). Additionally, Type I Interferons are primarily produced by myeloid cells, particularly dendritic cells, and are critical effector molecules in direct anti-viral immunity. IFNγ is produced by a variety of cell types, predominantly NK cells and T cells, and plays a much more prominent role in regulation of leukocyte trafficking, regulatory actions and defense against intracellular bacteria than direct anti-viral immunity(48, 49). While both Type I and Type II IFNs upregulate MHC molecule expression and can induce antibody class switching, IFNγ plays a non-redundant role in Th1 cell differentiation and effector function(50, 51). Much like their functions within immune responses, Type I and Type II IFNs appear to have distinct effects on MS progression. IFNγ is not an effective therapeutic in MS patients while Type I IFN administration is one of the more common treatments for RRMS. The role of IFNγ in MS remains a complicated question with few answers and the mechanisms by which it influences CNS inflammation continue to be studied in the EAE model.

1c.ii. IFNγ role in controlling EAE severity

Multiple early studies showed IFNγ signaling in EAE models could dampen CNS autoimmunity. Intrathecal administration of anti-IFNγ antibody increased morbidity and mortality of EAE, while systemic administration of IFNγ decreased disease severity, in mice(52). Additionally, in rats, intrathecal injection of IFNγ decreased disease severity(53). Loss of IFNγ signaling in IFNγRKO mice renders normally EAE resistant strains susceptible(54, 55) and induces more severe disease in mouse strains that were already susceptible to EAE(56). Studies with reciprocal bone marrow chimeric mice showed that loss of IFNγR signaling in both the hematopoietic and non-hematopoietic compartments contribute to severity of disease(55). In addition to attenuating EAE severity, IFNγ signaling also determines the primary site of inflammatory demyelination within the CNS and subsequent EAE clinical phenotype .

1c.iii. IFNγ role in suppressing aEAE phenotype

A variety of model systems have shown that IFNγ suppresses aEAE, but there is not a clear consensus on the mechanism by which this occurs. aEAE clinical symptoms were initially described as an axial rotation of the head and trunk due to primary infiltration of the BS and cerebellum(57). This is in contrast to the more common form, cEAE, which is characterized by an ascending paralysis due to primary infiltration of the SC. Multiple model systems have shown

greater incidence of aEAE, but none more consistently than those involving loss of IFNγ signaling. In an EAE model where MBP-specific TCR-transgenic Rag KO mice spontaneously develop cEAE, researchers found that crossing the mice onto an IFNγ KO background converted spontaneous cEAE to aEAE. Since T cell subsets are major producers of IFNγ, subsequent studies of IFNγ's role on aEAE phenotype focused on the encephalitogenic CD4 T cells.

Multiple theories about how IFNγ suppresses aEAE emerged in the literature. In adoptive transfer experiments, hosts deficient in IFNγR signaling developed aEAE following adoptive transfer of MOG_{35-55}-specific WT C57BL/6 CD4 Th1 polarized cells. Transfer of the same cells into WT C57BL/6 mice resulted in cEAE. Similarly, transferring MOG_{35-55}-specific IFNγ KO CD4 T cells into WT C57BL/6 mice produced an aEAE phenotype. Interestingly MOG-specific IFNγ KO CD4 cells can be regulated by MOG-specific WT CD4 T cells. Hence in co-transfer experiments the incidence of aEAE declines in direct correlation with the ratio of WT to KO cells. Collectively, these data indicate that myelin-specific T cell production of, and host response to, IFNγ determine lesion localization in EAE(39). In another adoptive transfer paradigm, the specific myelin epitope used to immunize donor mice determines the Th lineage of encephalitogenic effector cells and the clinical phenotype of EAE that they induce . Hence, C3Heb/Fej mice immunized with peptide MOG_{97-114}, mount Th17 responses. These Th17 cells induce aEAE in naïve recipients. In contrast, immunization of C3Heb/Fej mice with the alternative peptide MOG_{35-55}, triggers the generation of Th1 cells that induce cEAE. If MOG_{94-114}-reactive CD4 T cells are polarized into Th1 cells via polarization with IL-12, they begin to induce cEAE. Conversely, polarization of MOG_{35-55}-reactive CD4 T cells with IL-23 to drive Th17 differentiation makes them more likely to induce aEAE. (36).

The observations that IFNγ deficiency and IL-17 production are predisposing factors for the development of aEAE are not incompatible. IFNγ can negatively regulate Th17 cell differentiation and suppress IL-17 production (58, 59). Subsequent experiments with mice on the C57BL/6 background showed that encephalitogenic Th1-polarized IFNγ KO CD4 T Cells, which induce aEAE when transferred to WT mice, induce cEAE when transferred to IL-17 Receptor subunit A (IL-17RA) KO recipients(40). These experiments demonstrate that IFNγ signaling may directly suppress IL-17 production and subsequent signaling that is critical for aEAE pathogenesis. However, the absolute requirement of IL-17 signaling for aEAE

pathogenesis in the absence of functional IFNγ signaling has not been demonstrated as IL-17RA KO cells can still produce IFNγ and both transferred IFNγ KO T cells and host cells can respond to IFNγ. The absence of IFNγ signaling may be required for aEAE, and IL-17 simply enhances the aEAE phenotype. Current experiments analyzing mechanisms by which IFNγ affects lesion localization have focused on CD4 T cells and have not examined direct effects of IFNγ on myeloid cell recruitment and behavior subsequent to CD4 T cell infiltration of the CNS.

1d. Neutrophils

1d.i. Neutrophil role in inflammation and autoimmunity

Neutrophils are some of the first responders at sites of injury and infection. They are often overlooked, as they enter tissue and are cleared early in an immune response. However, they comprise 20% of mouse and 80% of human blood leukocytes(60), are crucial mediators of anti-bacterial and anti-fungal immunity, and are potent cells in resolution of inflammation and promotion of wound repair (61, 62). Conversely, neutrophils also play critical roles in overactive immune responses, contributing to pulmonary inflammation in asthma(63) as well as joint damage in rheumatoid arthritis(64).

1d.ii. Granulocyte colony stimulating factor (G-CSF) and CXCR2: major players in neutrophil mobilization and migration into inflamed tissue

Mobilization of neutrophils to the site of inflammation is a rapid process with a few critical drivers. During an infection or damage to tissue, G-CSF levels are rapidly upregulated in the serum(65), which promotes the proliferation and differentiation of neutrophil progenitor cells in the bone marrow(66). Part of the neutrophil differentiation process that promotes their release out of the bone marrow is the upregulation of the chemokine receptor CXCR2, which is mediated by G-CSF-dependent signaling(67). CXCR2 is, also, a critical mediator of neutrophil infiltration into inflamed tissue (68). The major ligands for CXCR2 in the mouse are CXCL1 and CXCL2 (69). Both ligands can bind the receptor and promote neutrophil chemotaxis as well as production of CXCL2(70). Multiple studies have identified tissue resident cells, such as endothelial cells and fibroblasts, as producers of CXCL1(70), while infiltrating immune cells

produce more CXCL2(70). The regulation of neutrophil recruitment factors is also relevant in the MS literature.

1d.iii. Neutrophils in MS

Neutrophils are not often found in neuropathological studies of MS lesions. However, MS lesions in biopsy and autopsy samples tend to be well established. Neutrophils may be more conspicuous in early lesions and their short-lived presence in immune responses may have led to an underestimation of their importance. A role of neutrophils was inadvertently suggested when some MS patients, enrolled in experimental protocols of high-dose chemotherapy with peripheral blood stem cell rescue, experienced neurological worsening and new lesion formation while receiving G-CSF. Administration of G-CSF was also associated with severe NMO relapses indicating that mobilization of neutrophils may lead to exacerbation of neuroinflammation in CNS autoimmunity(71-73). Another case of severe exacerbation of RRMS after G-CSF therapy was recently reported(74). After the discovery that G-CSF administration to MS patients was associated with severe MS and NMO relapses, it was shown that G-CSF transcript levels were elevated in MS patient lesion biopsies compared to healthy controls(75). Additionally, neutrophil chemoattractants were elevated in the CSF(17) and serum(76) of some MS patients. Circulating neutrophils from MS patients also show higher levels of activation including increased surface expression of chemokine receptors, increased oxidative burst and degranulation as well as lower levels of apoptosis following stimulation with GM-CSF(77).

While there is emerging evidence that neutrophils may contribute to MS pathology, current disease modifying treatments do not specifically target neutrophils. The most effective treatment in MS is a humanized anti-α4 intergrin antibody, natalizumab, which can block function of α4β1 and α4β7 integrin pairs and restrict peripheral blood mononuclear cell infiltration into tissue, however, neutrophil migration is not affected. Initial clinical trials showed that monthly injections of the antibody could reduce the risk of sustained disability progression by 42 percent in RRMS patients(78). Natalizumab is not effective in all RRMS patients, and SPMS and PPMS patients do not generally respond to natalizumab(79). It has recently been shown that patients that transitioned to SPMS had elevated levels of neutrophil chemoattractant CXCL1 and pro-survival factor G-CSF(80). Current disease modifying therapies were developed based on the assumption that MS is driven primarily by lymphocyte and monocyte/macrophage

populations that require α4 integrin for tissue infiltration. Emerging evidence indicates that these may not be the only cell types and recruitment pathways involved in CNS infiltration and MS pathogenesis.

1d.iv. Role of Neutrophils in cEAE

Although the former discussion focused on the prominence of neutrophils in infiltrates of mice with aEAE, neutrophils also play a role in cEAE. There is evidence in the literature that neutrophils may play a role in early lesion formation during cEAE. Administration of $CXCR2^{+}$ Neutrophils to CXCR2 KO Balb/c mice following immunization restored EAE susceptibility (81). Administration of anti-Ly6G antibody prior to the onset of EAE in C57BL/6 WT mice blocked disease initiation but had no effect when given after onset of disease (82). Additionally, active immunization of G-CSFR KO C57BL/6 mice or administration of anti-CXCR2 antibody to WT mice decreased incidence of cEAE following active immunization(83). While neutrophils do appear to play a role in the induction of cEAE they are not sufficient to induce fulminant cEAE phenotype in C57BL/6 mice. Monocytes have been identified as the most common cells in SC lesions at the peak of cEAE(84). In addition it has been shown by us and other labs that loss off the monocyte chemokine receptor, CCR2, inhibits monocyte infiltration and attenuates cEAE severity(85-87). In conclusion, while neutrophils may contribute to the initial infiltration of cells into the SC they are not sufficient to induce fulminant cEAE.

1d.v. IFNγ promotes monocyte and T cell-dominant SC infiltration in cEAE

IFNγ is the dominant CD4 T cell derived cytokine in cEAE of C57BL/6 mice and may contribute to the predominance of monocytes and T cells at the peak of disease. In the CNS during cEAE, encephalitogenic CD4 T cells are predominantly IFNγ producing. This is true even when the donor cells are IL-23 polarized Th17 cells, since they transition into "ex-TH17" cells by the time they accumulate in the CNS(88). While induction of EAE in WT mice is associated with high levels expression of IFNγ-inducible chemokines CXCL9 and CXCL10 in the CNS, those chemokines are largely absent in IFNγRKO mice(89). CXCL9 and CXCL10 bind the CXCR3 receptor that is expressed by both activated T cells and monocytes(90), potentially driving their recruitment to the CNS . Additionally, it has been shown that IFNγ promotes the

production of CCL2, an agonist for the CCR2 receptor on monocytes, by CNS tissue resident cells(91). IFNγ is also critical for the upregulation of VCAM-1 on the CNS endothelium(92). VCAM-1 is the predominant binding partner of integrin α4β1. α4β1 which is expressed at high levels by monocytes and T cells(93) and is thought to mediate infiltration of encephalitogenic T cells into CNS tissue(94). Circulating neutrophils are not predominant expressors of CXCR3 or CCR2 (95) and do not rely on α4 integrin for adhesion to endothelial cells in humans or mice (96-98). The majority of the IFNγ-responsive pathways upregulated in the CNS during cEAE promote monocyte and T cell, but not neutrophil, infiltration.

1d.vi. IFNγ suppresses neutrophil infiltration in multiple models of inflammation

In addition to actively promoting monocyte and T cell infiltration, there is evidence in multiple disease models that IFNγ also suppresses neutrophil infiltration. Studies examining myelopoiesis during inflammation in mice showed that IFNγ produced by activated CD4 T cells in the bone marrow promotes monopoiesis while suppressing granulopoiesis (99). In a pulmonary tuberculosis model, the loss of IFNγR expression in mice increased neutrophil infiltration into the lung(100). Additionally, it was shown that non-hematopoietic cell production of IFNγ inhibited Th17 cell derived IL-17 production and neutrophil infiltration into the lung(101). Il-17 is known to promote neutrophil recruitment to areas of inflammation(15, 16). The evidence that IFNγ inhibits neutrophil infiltration extends to the EAE model. Actively immunized IFNγKO mice on the Balb/c background have enhanced neutrophil infiltration in both the SC and BS compared with their WT counterpaorts. Loss of IFNγ signaling and increased IL17 signaling results in a heightened incidence of aEAE in multiple model systems(36, 40). (15, 16). The observation that increased pro-neutrophil recruiting cytokine IL-17 and decreased neutrophil suppressing cytokine IFNγ signaling promote aEAE may indicate that, downstream of Th1/Th17 differentiation, neutrophil recruitment to the brain is critical for aEAE pathogenesis. This question has not been explored in the literature.

1e. Distinct migratory pathways to the CNS in aEAE and cEAE

1e.i. Patterns of CNS infiltration in cEAE and aEAE

Neuropathological examination of cEAE and aEAE lesions show distinct patterns of infiltration. In the SC of WT hosts with cEAE, focal infiltration forms around venules and penetrates into white matter tissue. In the IFNγRKO hosts with aEAE, infiltrates accumulate in the meninges surrounding the lateral recess of the 4th ventricle of the BS and appear to extend directly into the BS parenchyma. The difference in lesion pattern may indicate that immune cells migrate to the CNS through distinct pathways to mediate disease.

1e.ii. Blood Brain Barrier (BBB)

The BBB is a highly restrictive barrier between the blood and CNS parenchyma, comprised of multiple layers which can restrict access of toxic compounds, pathogens and circulating immune cells(102). The endothelial cell barrier is strengthened by a combination of transmembrane and cytosolic accessory proteins that comprise the tight junctional complex. The tight junction complex is bound to the endothelial cell actin cytoskeleton and forms an intercellular seal(103) Beyond the endothelium, a parenchymal basement membrane and astrocyte endfeet restrict immune cell migration from the perivascular space into CNS parenchyma(104). While its base function is to restrict entry into CNS tissue, inflammatory conditions can alter BBB function to promote immune cell infiltration.

It has been shown in multiple models of CNS inflammation that factors critical for T cell and monocyte/macrophage infiltration are upregulated at the BBB in response to cytokines or other factors. In the EAE model upregulation of VCAM-1 on the BBB is critical for T cell infiltration into CNS tissue(94). BBB CNS endothelium is positive for CCL2 at pre-clinical and onset timepoints of cEAE(105). BBB endothelium and astrocyte expression of CCL2 was found to be critical for fulminant cEAE pathogenesis (106). While this is a major pathway of immune cell infiltration that fits well with immune cell subsets and histological patterns seen in cEAE there are alternative pathways of infiltration into the CNS.

1e.iii. Blood CSF Barrier (BCSFB)

The BCSFB is the major restrictive barrier between the blood and CSF. CSF circulates throughout the ventricles and within the subarachnoid space over the brain and SC. It is a major protective buffer both to mechanical and chemical stresses and a source of growth factors to CNS tissue. CSF is produced and circulated by the choroid plexus epithelium, which is situated in the ventricles of the brain(107). The BCSFB is comprised of fenestrated endothelium and

choroid plexus epithelium located within the ventricles. The endothelium within the ventricles, unlike that in the BBB, does not play a major barrier function. The choroid plexus is the major barrier to chemical and cellular migration into CSF from the bloodstream mediated by functionally related, although separate, subsets of transmembrane and cytoplasmic proteins that comprise the tight junctional matrix(108).

The BCSFB also differs from the BBB in its role in immune cell surveillance during health and disease. While the CNS is often referred to as an "immune-privileged" site, there is constitutive immune monitoring at the level of the meninges to protect against infection and latent viral reactivation. The choroid plexus expression of P-selectin is thought to be a major pathway for memory CD4 and CD8 T cells to enter during surveillance(109) (110). The choroid plexus also plays a role in immune infiltration during CNS injury. In stroke models, the choroid plexus facilitates neutrophil infiltration via production of neutrophil chemoattractants CXCL1 and CXCL2(111), which bind the CXCR2 receptor that is critical for neutrophil infiltration into inflamed tissue(112). The choroid plexus may be a major gateway that has, as of yet, been unexplored in aEAE.

1f. Summary of rationale and specific aims

MS is a complex CNS autoimmune demyelinating disease. The mechanisms mediating damage in different areas of the CNS are not fully understood. T cells and antigen presenting cells, such as monocytes and macrophages, have been implicated as the primary drivers of destructive neuroinflammation based on GWAS and neuropathological studies. However, therapies that target monocyte, macrophage and T cell infiltration into CNS tissue are only effective in certain RRMS patients and not those with progressive forms of disease, indicating that other cell types and migratory pathways contribute to MS pathology.

Neutrophils are receiving increasing attention in MS pathogenesis. While they are not plentiful in most of the established MS lesions found in autopsy and biopsy specimens, they may play a transient and early role in lesion formation. Administration of neutrophil-mobilizing drugs has been associated with severe relapses in MS patients. Neutrophils isolated from peripheral blood of MS patients show a more activated phenotype compared to healthy controls. Additionally, neutrophil chemoattractants and survival factors were elevated in serum from patients with SPMS compared to RRMS. We previously reported that, in a longitudinal study of

untreated RRMS patients, plasma levels of the neutrophil attracting chemokine, CXCL5, rose in concert with new lesion formation(83).

The role of neutrophil in EAE is not completely understood. While they contribute to the initial infiltration in the SC in some models of cEAE, they appear to be dispensable for disease progression after clinical onset. In the C57BL/6 adoptive transfer cEAE model, CNS inflammation is dominated by IFNγ signaling, which induces factors that promote CD4 T cell and monocyte infiltration into spinal cord tissue. In the absence of either IFNγ production by encephalitogenic T cells or IFNγ responsiveness by host cells, mice predominantly develop aEAE. In the C57BL/6 IFNγKO T cell transfer model, it was shown that loss of IL-17 signaling in the host converted a normally aEAE phenotype to cEAE. Decreases in IFNγ signaling and/or increases in IL-17 signaling have been shown to promote aEAE in other mouse backgrounds as well. A high IL-17 to IFNγ ratio in inflamed tissues is conducive to neutrophil infiltration in other models of inflammation.

In addition to variability in the types of cells recruited to the CNS, differences in lesion location may also be predicated on the pathways by which the inflammatory cells infiltrate the CNS. As mentioned above, intraparenchymal perivascular infiltrates are characteristic of cEAE. Endothelial cells and astroctyes at the blood brain barrier upregulate factors in response to IFNγ that facilitate monocyte and T cell infiltration into the CNS. These factors have been shown to be critical in cEAE induction. The pathway utilized by immune cells to infiltrate the brainstem during aEAE is less well characterized. Of note, the vestibulochoclear nucleus (VCO), which is one of the most heavily infiltrated and damaged areas in aEAE, is adjacent to the lateral recess of the 4th ventricle. The choroid plexus in the lateral recess of the 4th ventricle was shown to upregulate neutrophil chemoattractants in other models of CNS inflammation. Based on these observations, we propose the following model of EAE pathogenesis: **In WT hosts, IFNγ producing myelin-specific CD4 T cells induce VCAM-1 and CCL2 expression in the endothelial cells and astrocytes of the BBB, promoting the recruitment of monocytes and additional CD4 T cells to the CNS where they primarily cause damage in the SC. In IFNγR KO recipients, BBB-dependent migratory pathways are not activated. Instead, myelin-specific CD4 T cells migrate through the choroid plexus of the 4th ventricle and produce inflammatory factors, such as IL-6, TNFα, and IL-17, that activate the choroid plexus**

epithelium and nearby microglia to produce neutrophil attracting chemokines, such as CXCL1 and CXCL2. This leads to the recruitment of neutrophils that initiate an inflammatory cascade leading to demyelination and axonopathy in the VCO and other regions in the posterior fossa. In WT mice IFNγ suppresses the production of the neutrophil attracting chemokines, thereby preventing brainstem infiltration by inflammatory cells.

My thesis project builds upon this model by addressing the following aims:

Aim 1: Chapter 2: Demonstrate that CXCR2-dependent accumulation of neutrophils is critical for aEAE pathogenesis. This finding identifies a specific cell type and recruitment pathway critical for aEAE pathogenesis. In addition, we find that aEAE does not require IL-17 signaling in the absence of loss of IFNγ signaling.

Aim 2: Chapter 3: Demonstrate that IFNγ suppresses neutrophil infiltration into the BS by suppressing monocyte, microglial and neutrophil production of CXCL2. In this study we identify that CXCL1 and CXCL2 are produced by distinct cell subsets. CXCL1 is produced by choroid plexus epithelium and astrocytes in EAE while CXCL2 is produced by $CD45^{+}CD11b^{+}$ cells in the BS parenchyma during aEAE. We also find that CXCL2 transcript production increases with aEAE disease severity and IFNγ suppresses production of CXCL2 by all $CD45^{+}CD11b^{+}$ cell subsets and a positive feedback loop of CXCL2 production in neutrophils.

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CHAPTER 2 - SITE-SPECIFIC CHEMOKINE EXPRESSION REGULATES CNS INFLAMMATION AND DETERMINES CLINICAL PHENOTYPE IN AUTOIMMUNE ENCEPHALOMYELITI2a. Abstract

The adoptive transfer of myelin-reactive T cells into wildtype (WT)[2] hosts results in spinal cord inflammation and ascending paralysis, referred to as conventional experimental autoutoimmune encephalitis (EAE)[3], as opposed to brainstem inflammation and ataxia, which characterize disease in IFNγRKO hosts (atypical EAE). Here we show that atypical EAE correlates with preferential upregulation of CXCL2 in the brainstem, and is driven by CXCR2 dependent recruitment of neutrophils. In contrast, conventional EAE is associated with upregulation of CCL2 in the spinal cord, and is driven by recruitment of monocytes via a partially CCR2-dependent pathway. This study illustrates how regional differences in chemokine expression within a target organ shape the spatial pattern and composition of autoimmune infiltrates, leading to disparate clinical outcomes.

2b. Introduction

A defining feature of multiple sclerosis (MS) is the spatial dissemination of inflammatory demyelinating lesions within the CNS (1). In some patients, lesion burden is concentrated in the spinal cord with little involvement of the cerebrum (as in the opticospinal form of disease that is more common in Asia, or in a significant cohort of individuals with primary progressive MS) (2-4). In others, lesion burden is skewed supratentorially, with little to no involvement of the spinal cord (as in a significant cohort of individuals with relapsing remitting MS in the Western Hemisphere) (5, 6). The factors that regulate leukocyte trafficking to, and accumulation in, particular regions of the CNS are poorly understood.

EAE, widely used as an animal model of MS, classically manifests as an ascending paralysis that correlates with inflammatory demyelination of the lumbosacral spinal cord (7). In

certain instances, an atypical form of EAE has been observed in which afflicted mice exhibit signs of imbalance/ vestibular dysfunction that correlate with lesion formation in the brainstem and/ or cerebellar white matter (8-13). This clinical phenotype occurs most consistently and prominently under circumstances where IFNγ bioactivity is suppressed. Hence, IFNγ- and IFNγ receptor (IFNγR)-deficient mice are significantly more likely to develop atypical EAE than their WT counterparts following active immunization (10). Adoptive transfer experiments have demonstrated that either deficient IFNγ production by encephalitogenic donor T cells or impaired IFNγ signaling into host cells is sufficient for the development of atypical EAE (12-14).

In some experimental paradigms, a high incidence of atypical disease has been observed following the transfer of encephalitogenic T cell lines that contain a high ratio of Th17 to Th1 cells. In contrast, conventional disease is mediated by T cell lines that express a low Th17:Th1 ratio (11). A major function of IL-17, the signature Th17 cytokine, is to induce neutrophil mobilizing/activating factors, such as G-CSF, and chemokines that target granulocytes, such as CXCL1, CXCL2 and CXCL5 (15). Conversely, IFNγ skews myeloid cell differentiation in the bone marrow to favor monocytes over granulocytes during immune activation (16). Therefore, it is not surprising that atypical disease tends to be characterized by neutrophil-rich white matter infiltrates, while monocytes are more prevalent in the infiltrates of mice with conventional disease (10, 13, 14). However, a distinctive requirement for neutrophil mobilizing/ activating factors in atypical EAE versus monocyte mobilizing factors in conventional EAE has yet to be directly demonstrated.

In the current paper we compared atypical and conventional EAE, induced in IFNγRKO and WT hosts, respectively, by transfer of the same population of myelin oligodendrocyte (MOG)[6] peptide-primed, IL-12 polarized $CD4^+$ T cells. We found that atypical EAE correlates with preferential upregulation of CXCL2 in the brainstem of IFNγRKO hosts, and is driven by CXCR2-dependent recruitment of neutrophils to the white matter tracts surrounding the vestibulocochlear nucleus (VCO)[7]. In WT mice, that have an intact IFNγ signaling pathway, brainstem CXCL2 expression is suppressed and spinal cord CCL2 is upregulated. Consequently, the autoimmune assault is redirected to the spinal cord and manifests as a monocyte-predominant infiltrate that is, in part, CCR2 dependent.

2c. Materials and Methods

2c.i Mice

8- to 14 week old CD45.1 congenic and WT C57BL/6 mice were obtained from NCI Fredrick or Jackson Laboratory. IFNγR knock-out (KO) (B6.129S7-Ifngr1tm1Agt/J)8 and IFNγKO (B6.129S7-Ifngtm1Ts/J) mice were obtained from Jackson Laboratory. Breeding pairs of IL-17RKO originally obtained from from J. Kolls (LSU) and CCR2KO mice from B. Moore (University of Michigan) were bred in our facility. Mice were housed in microisolator cages under specific pathogen-free conditions. All animal protocols were approved by the University Committee on Use and Care of Animals.

2c.ii. Antibodies and Reagents

Rat antibody to myelin basic protein (MBP)[9] (clone 12) was from Millipore. Mouse antibody to unphosphorylated filament –H (SMI)[10] was from Covance. AlexaFluor594 goat-anti-mouse IgG and AlexaFluor488 goat-anti-rat IgG were from Life Technologies. The following antibodies were obtained from ebiosciences: FITC-anti-MHCII (M5/114.15.12), FITC-anti-B220 (RA3-6132), PE-anti-CD45 (Ly5), PE-anti-CD8α (53-6.7), PE-anti-CD4 (Gk1.5), PE-anti-GM-CSF (MP1-22E9), PECy7-anti-CD11b (M1/70), and PECy7-anti-CD4 (RM4-5); PerCpCy5.5-anti-Ly6C (HK1.4), PerCPCy5.5-anti-CD3ε (145-2c11) and PerCPCy5.5-anti-IL17A (1787). The following antibodies were obtained from BD Biosciences: allophycocyanin-anti-CD45.2 (104), FITC-anti-CD44 (IM7), allophycocyanin cy7-anti-Ly6G (IA8), allophycocyanin cy7-anti-CD45.1 (A20), and allophycocyanin cy7-anti-IFNγ (XMG1.2). Rabbit polyclonal antiserum against the amino-terminal ligand binding domain on CXCR2 (MGEFKVDKFNIEDFFSG) was generated by Biosynthesis Inc. as previously described (17, 18). Recombinant mouse (rm)[11] IFNγ and rmIL-12 were from R&D Systems.

2c.iii. Induction and scoring of EAE

Donor mice were immunized subcutaneously with 100μg of peptide MOG_{35-55} (MEVGWYRSP-FSRVVHLYRNGK, Biosynthesis) in CFA (Difco) across four sites over the flanks. Inguinal, axial and brachial lymph nodes were harvested 14 days post-immunization, pooled,

homogenized and passed through a 70μm strainer (BD Falcon). Cells were cultured with MOG_{35-55} (50μg/mL) in the presence of rmIL-12 (6ng/mL), rmIFNγ (2ng/mL) and anti-IL-4 mAb (hybridoma 11B11; 10μg/mL). After 96 h, CD4 T cells were isolated by column separation with CD4 (L3T4) magnetic microbeads, according to manufacturer's instructions (Miltenyi). $5x10^6$ CD4 T cells (85-99% pure) were transferred i.p. into naïve hosts. Adoptive transfer recipients were monitored on a daily basis by an examiner who was blinded to experimental groups. Mice were scored for severity of conventional and atypical signs of EAE using established scales (14, 19). Specifically, mice with conventional EAE were scored as follows: 0, no abnormality; 1, flaccid tail; 2, waddling gait/ difficulty righting from supine position; 3, overt hindlimb weakness; 4, hindlimb paralysis; 5, forelimb and hindlimb paralysis/moribund. Mice with atypical EAE were scored as follows: 0, no abnormality; 1, slight listing/difficulty righting; 2, obvious imbalance but able to ambulate; 3, severely impaired balance/ambulation; and 4, incapacitated due to inability to maintain upright posture/spinning.

2c.iv.Histology

After intracardiac perfusion of mice with 1xPBS and 4% paraformaldehyde (PFA)[12], spinal cords and brainstems were removed. The CNS tissues were then fixed in 4% PFA and cut into 50μm sections on a vibratome (Leica VT1200). Free-floating immunofluorescent staining was performed with primary antibodies against MBP_{82-87} (1:500) and SMI32 (1:1000). Goat anti-mouse IgG AlexaFluor 594 (1:400) and goat anti-rat IgG AlexaFluor 488 (1:1000) were used as secondary antibodies. Sections were incubated with DAPI (100ng/mL) prior to washing and mounting on slides (Prolong Gold anti-fade reagent, Life Technologies). Fluorescent images were acquired with Nikon Eclipse Ti, CoolSnapEZ camera and NIS Elements: Basic Research v3.10. Confocal images were acquired using a Nikon A-1 Confocal microscope (Nikon Plan Fluor 10x/0.30 or Nikon PlanApoVC 60x/1.40 oil) with diode-based laser system and NIS Elements software. 3D reconstruction images were generated with Bitplane software (Imaris) using confocal Z-stack images of equal thickness from each group. Appropriate processing, including image overlays and black level and brightness adjustments, were performed in Adobe Photoshop CS5.1 and applied equally to all samples and controls.

2c.v. CNS Inflammatory Cell Isolation

CNS tissue was harvested and separated into four compartments: the spinal cord, brainstem, cerebellum and cerebrum. Each tissue was homogenized in 1mL PBS containing a protease inhibitor cocktail (Roche) and centrifuged at 800xg for 10 minutes. Supernatants were stored at -80°C. Tissue pellets were digested with collagenase A (1mg/mL) and DNAse I (1mg/mL) in HBSS containing calcium and magnesium. Inflammatory cells were isolated over a 27/63% percoll gradient and counted with a Cellometer AutoT4 automated cell counter (Nexelcom).

2c.vi. Flow Cytometry

For surface staining, cells were suspended in PBS with 2%FCS containing Fc Block (50ng/mL) prior to incubation with fluorochrome-conjugated antibodies. For intracellular staining, cells were stimulated with PMA (50 ng/ml) and Ionomycin (2 μg/mL) and incubated with Brefeldin A (5 μg/mL) for 6-10 hours. Cells were then fixed in 4% PFA, permeabilized with 0.5% saponin and incubated with fluorochrome-conjugated anti-cytokine antibodies. The stained cells were analyzed with a FACS Canto II flow cytometer using FACSDiva software (v6.1.3, Becton Dickenson). Data was analyzed using FlowJo software (v9.3.2, Treestar).

2c.vii. Multiplex Cytokine Analysis

Cytokine levels in homogenate supernatants were measured via a luminex multiplex bead based assay (Millipore). Data was collected on a Bio-Plex 200 system using the manufacturer's protocols. The data shown indicates levels that fell within the linear portion of a corresponding standard curve. Bradford assays (Thermo Scientific) were performed on tissue homogenates in parallel for normalization to total protein.

2c.viii. Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Two-way ANOVA with Sidak's multiple comparisons test was used to compare disease scores over time. Leukocyte cell numbers and percentages, as well as chemokine and growth factor levels, were compared using the unpaired student's t-test. A P value of $<.05$ (*) was considered significant. $P<0.01$ is denoted as (**), $P<0.001$ as (***).

2d. Results

2d.i. IFNγR deficiency in host cells increases the incidence of atypical EAE

Following injection with IL-12 polarized MOG_{35-55}-specific $CD4^+$ T cells, 44 of 47 WT hosts (94%) and 38 of 48 IFNγRKO hosts (79%) developed clinical EAE. The vast majority of afflicted WT mice had pure conventional disease (31 of 44, or 70%), characterized by an ascending paraparesis with no vestibular signs throughout the clinical course (Table 1, Figure2-6). Of the remainder, 4 (9%) had pure atypical disease (vestibular signs with no evidence of limb weakness) and 9 (21%) exhibited a mixed phenotype, marked by early signs of atypical disease (i.e. a head tilt) that invariably evolved into ascending paraparesis within 1-2 days. None of the WT mice that presented with conventional disease subsequently developed atypical signs. Conversely, the majority of IFNγRKO hosts underwent a pure atypical course (24 of 38, or 62%), while 4 (11%) exhibited a pure conventional, and 10 (26%) a mixed, disease phenotype.

Table 2-1: Conventional and Atypical disease incidence in WT and IFNγRKO recipients of 5x106 Th1-polarized MOG35-55-reactive CD4 T cells

	EAE Incidence	Disease Phenotype	Incidence
WT	44/47 (93%)	Pure Conventional	31/44 (71%)
		Pure Atypical	4/44 (9%)
		Mixed	9/44 (20%)
IFNγRKO	38/48 (79%)	Pure Conventional	4/38 (10%)
		Pure Atypical	24/38 (63%)
		Mixed	10/38 (27%)

2d.ii. Inflammatory demyelination is prominent in the spinal cord of mice with conventional EAE and in the brainstem of mice with atypical EAE

CNS tissues were collected from representative adoptive transfer recipients at peak disease and sections were examined by immunofluorescent staining. We consistently observed inflammatory infiltration, demyelination and axonopathy of white matter tracts in the spinal cords of WT mice with conventional EAE but not amongst IFNγRKO mice with atypical EAE (Figure 2-1, A and B,

left panels). In contrast, IFNγRKO mice with atypical EAE displayed pathological changes in white matter surrounding the VCO of the brainstem (Figure 2-1A and 1B, right panels). Inflammation of the vestibulocochlear nerve root has also been highlighted in other models of atypical EAE (9, 12, 20). Consistent with these observations, significantly more CNS cells were isolated from the spinal cords of WT hosts at the peak of conventional EAE than from the spinal cords of IFNγRKO hosts at the peak of atypical EAE, while the reverse was true with respect to the numbers of inflammatory cells isolated from the brainstem (Figure 2-2A). There were no significant differences between the two cohorts in the number of inflammatory cells isolated from either the cerebral hemispheres or the cerebella.

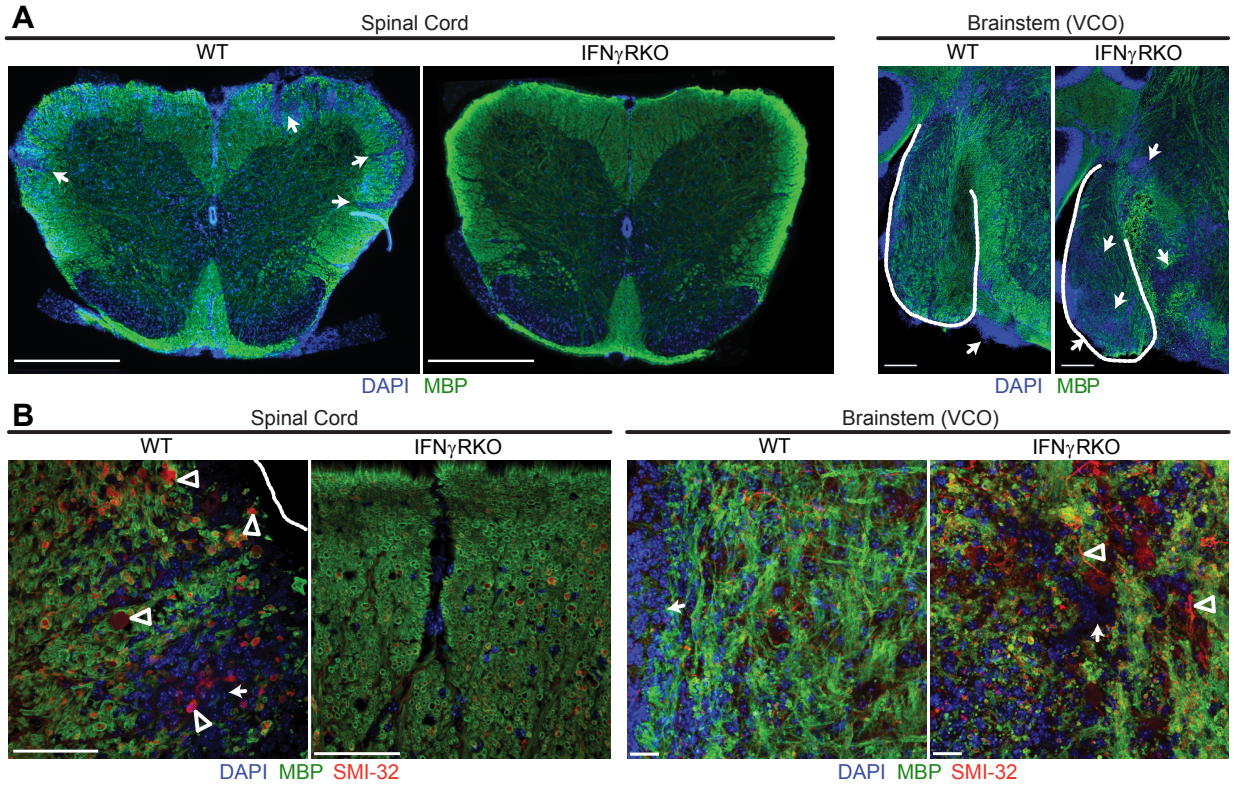

Figure 2-1: The distribution of inflammatory demyelination in the CNS of mice with atypical and conventional EAE. (A) Representative spinal cord (left) and brainstem (right) sections from WT mice with conventional EAE and IFNγRKO mice with atypical EAE. Sections were stained for MBP (green) and DAPI (blue) to define foci of inflammatory demyelination (arrows). Scale bars are 500 μm in the spinal cord images and 100 μm in the brainstem images. (B) Confocal images of spinal cord and brainstem sections stained for MBP (green), SMI-32 (red), and DAPI (blue). Demyelinated axons are SMI-32 positive, MBP negative (arrowheads). Scale bars, 30 μm.

2d.iii. The severity of atypical EAE correlates with the number of brainstem neutrophils, while the severity of conventional EAE correlates with the number of spinal cord monocytes and donor T cells

We performed a detailed analysis of the cellular composition of leukocytes infiltrating different CNS compartments of IFNγRKO mice that exclusively exhibited signs of atypical EAE (hereafter referred to as "pure atypical" disease) or of WT mice that exclusively exhibited signs of conventional EAE (hereafter referred to as "pure conventional" disease). Neutrophils comprised a higher percent of total live cells in all CNS compartments of mice with pure atypical EAE when compared to mice with pure conventional EAE (Figure 2-2B, left panel). The absolute number of neutrophils was significantly increased in the brainstem, cerebrum and cerebellum of mice with pure atypical disease (Figure 2-2B, right panel). In contrast, the percentages and absolute numbers of monocytes/ macrophages and donor T cells were significantly higher in the spinal cords of mice with conventional EAE (Figure 2-2C, 2D). The severity of pure atypical disease directly correlated with the number of neutrophils, but not monocytes/ macrophages, infiltrating the brainstem, while the severity of pure conventional EAE directly correlated with the number of monocytes/ macrophages and donor T cells, but not neutrophils, infiltrating the spinal cord (Figure 2-2F, 2G, and data not shown). Collectively, the above data suggest that pure atypical EAE is driven by accumulation of neutrophils in the brainstem, and pure conventional EAE is driven by accumulation of monocytes/ macrophages and $CD4^+$ T cells in the spinal cord.

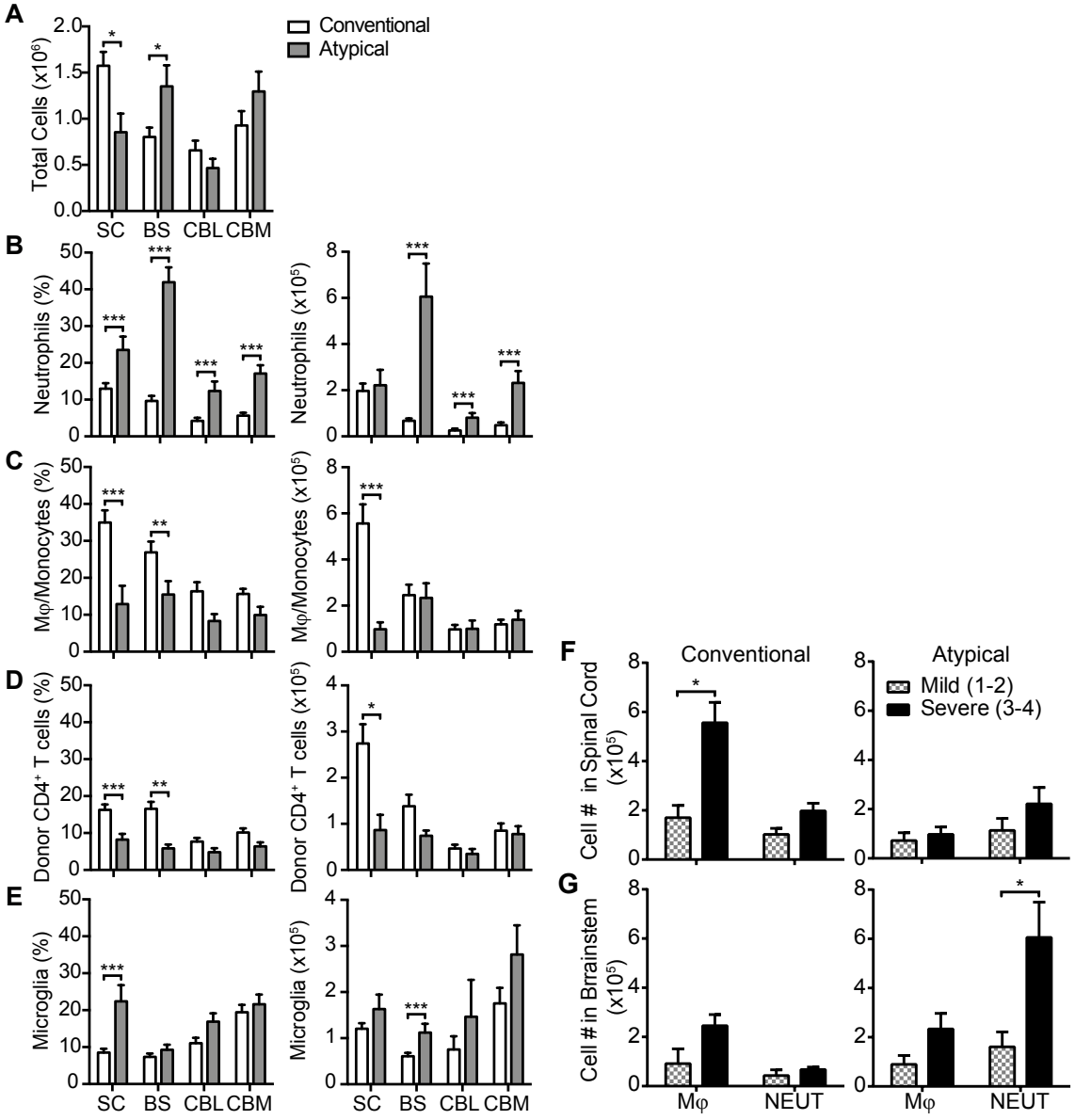

Figure 2-2. Neutrophils are prominent in the brainstem of IFNγRO mice with atypical EAE while monocytes and donor T cells are prominent in the spinal cord of WT mice with conventional EAE. (A) The average number of total cells isolated from the spinal cord (SC), brainstem (BS), cerebellum (CBL), or cerebrum (CBM) of WT mice with pure conventional and IFNγRO mice with pure atypical EAE. All animals had moderate to severe disease (clinical scores of 3-4) at the time of euthanasia. (B-E) Flow cytometry was performed to enumerate the percent and number of infiltrating neutrophils ($CD11b^+CD45^+Ly6G^+$) (B), monocytes/ macrophages ($CD11b^+CD45^{hi}Ly6G^-$) (C), donor $CD4^+$ T cells ($CD3^+CD4^+CD45.1^+$)(D), and microglia ($CD11b^+CD45^{int}Ly6G^-$) (E). (F and G) The absolute number of monocytes (MONO) and neutrophils (NEUT) per spinal cord (F) and brainstem (G) were compared between mice with mild (clinical scores 1-2) or severe (clinical scores 3-4) EAE. Data were pooled from at least 3 experiments with a total of 27 WT and 20 IFNγRKO mice. Flow cytometry gating scheme is illustrated in Fig.2-7. *P<.05, **P<.01,***P<.001

2d.iv. $CD4^+$ donor T cells traffic to the brainstem and upregulate CD25 and CD69 to a similar extent in WT and IFNγRKO hosts during the preclinical phase

EAE lesion formation is initiated several days prior to clinical onset (21, 22). The development of CNS infiltrates is contingent upon the passage of myelin epitope-specific effector T cells across the blood-brain-barrier (BBB)(13) or blood-cerebrospinal fluid barrier (BCSFB)(14) and their reactivation by local antigen presenting cells (23). We questioned whether the distribution of EAE infiltrates in atypical versus conventional EAE reflects differences in trafficking patterns or reactivation of encephalitogenic T cells in the CNS during the preclinical phase. To address that possibility, we adoptively transferred MOG_{35-55}-primed, IL-12 polarized $CD4^+$ T cells bearing a CD45.1 congenic marker into $CD45.2^+$ WT and IFNγRKO hosts. Representative mice in each group were euthanized at 1-2 days prior to expected clinical onset and at peak EAE, to analyze CNS infiltrating inflammatory cells by flow cytometry. There was a trend toward a higher number of $CD45.1^+CD4^+$ donor T cells in the brainstem, as well as the spinal cord, of WT compared with IFNγRKO hosts at the preclinical time point (Figure 2-3A, 3B). A higher percent of $CD45^{hi}CD11b^+$ cells expressed MHC Class II in WT versus IFNγRKO CNS infiltrates (Figure 2-3C), demonstrating the presence of immunocompetent APCs in the brainstem of WT hosts. There were no significant differences between the groups with respect to the number of $CD11b^+CD11c^+$ cells in either the brain or spinal cord (data not shown). The percent of $CD25^+CD69^+$ cells (indicative of recently activated cells) within the $CD44^+$donor T cell population was also comparable between groups (Figure 2-3D). Furthermore, we measured similar quantities of IL-2 protein in CNS tissue homogenates from WT and IFNγRKO hosts at clinical onset (data not shown). $CD45.1^+CD4^+$ donor T cells in the brainstem and spinal cord of WT hosts continued to outnumber those cells in IFNγRKO hosts at peak EAE (Figure 2-3A, 3B). Collectively, these results led us to conclude that the low incidence of atypical EAE in WT mice could not be attributed to impairment in the early migration of encephalitogenic T-cells to the brainstem, or in their reactivation once they had infiltrated the brainstem. However, increased accumulation of donor $CD4^+$ T cells in the spinal cord could be, at least in part, responsible for the enhanced susceptibility of WT hosts to conventional EAE.

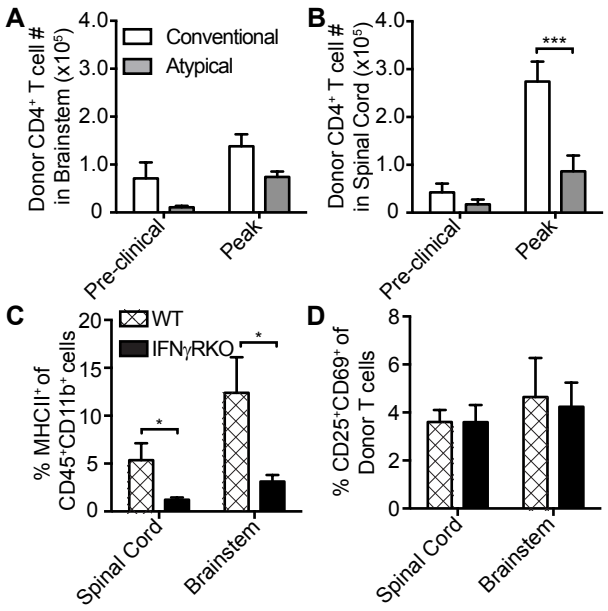

Figure 2-3. Enumeration of activated donor CD4$^+$ T cells and antigen presenting cells in the CNS of WT versus IFNγRKO mice. CNS-infiltrating cells were analyzed by flow cytometric analysis. (A and B) The average numbers of donor CD45.1$^+$ CD4$^+$ donor T cells (CD3$^+$CD4$^+$CD45.1$^+$) the brainstem (A) or spinal cord (B) of WT and IFNγRKO hosts at preclinical and peak stages of EAE. (C and D) Percent of CD45$^+$CD11b$^+$ that are MHCII$^+$ (C) and donor CD44$^+$CD4$^+$ T cells that are CD25$^+$CD69$^+$ (D) in spinal cord and brainstem of WT and IFNγRKO at day 6 post-transfer (preclinical time point). The data are representative of three experiments with 3-6 mice per group. *P<.05,***P<.001

2d.v. Neutrophil attracting chemokines are preferentially upregulated in the brainstem during pure atypical EAE, while monocyte attracting chemokines are preferentially upregulated in the spinal cord during pure conventional EAE

We next focused on events downstream of effector T cell homing and reactivation. The development of clinical signs in EAE coincides with CNS infiltration by a secondary wave of circulating leukocytes, in large part composed of myeloid cells (24). To interrogate the factors that differentially recruit neutrophils to the brainstem of IFNγRKO hosts, and monocytes to the spinal cord of WT hosts, we collected CNS tissue homogenates from representative mice at clinical onset, and measured levels of candidate chemokines. CXCL2, CCL3, and CCL4 were preferentially expressed in brainstem homogenates of IFNγRKO hosts, while CCL2, CCL5, CXCL9 and CXCL10 were expressed at relatively high levels in spinal cord homogenates of WT hosts (Figure 2-4). Surprisingly, the neutrophil attracting chemokine CXCL1 was upregulated in the spinal cord

and brainstem of WT hosts. The paucity of neutrophils in the CNS infiltrates of WT mice could be secondary to IFNγ-mediated suppression of CXCR2 on neutrophils (25).

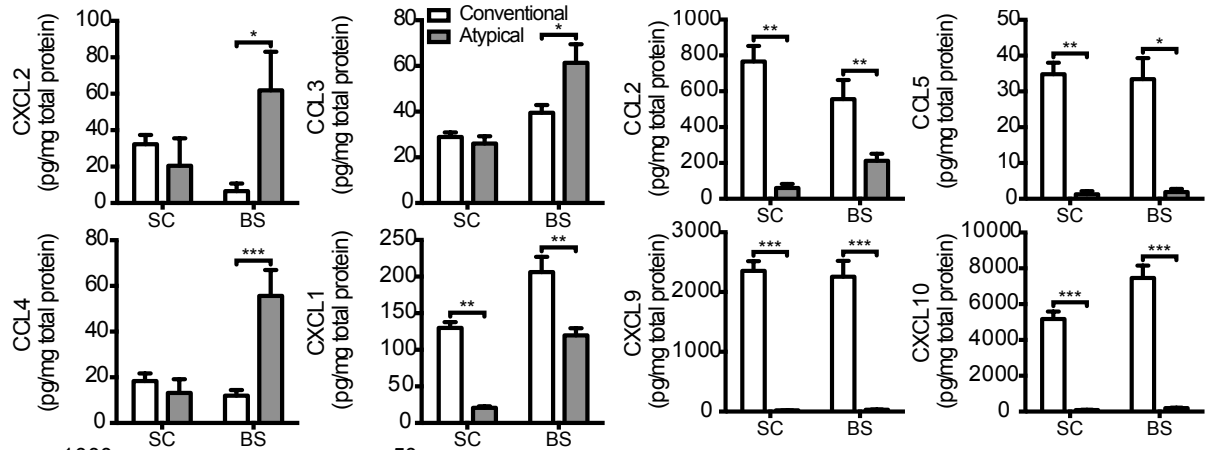

Figure 2-4. Chemokine and growth factor expression in the spinal cord and brainstem of mice with pure conventional or pure atypical EAE. Supernatants of spinal cord and brainstem homogenates obtained at clinical onset were subjected to Luminex based multiplex assays to measure levels of a panel of candidate chemokines. Data are pooled from 2 experiments with a total of 6 WT and 8 IFNγRKO mice per group. *P<.05, **P<.01,***P<.001

2d.vi. IL-12 polarized T cells mediate atypical EAE in IFNγRKO hosts via an IL-17-independent pathway

IL-17 is a potent inducer of CXCL2 (15). In an independent model of EAE induced in the C3H strain, atypical EAE occurs only when Th17 effector cells outnumber Th1 effector cells and is suppressed by IL-17 blockade (11). Although, in our model, donor T cells are polarized with IL-12 and exhibit a classic Th1 profile prior to adoptive transfer (Figure 2-8), we entertained the possibility that they upregulate IL-17 themselves, or induce other cell types to produce IL-17, following transfer into IFNγRKO hosts. Therefore, we measured intracellular cytokine levels in CNS infiltrating leukocytes from symptomatic WT and IFNγRKO hosts. A significant percent of transferred CD45.1$^+$ CD4$^+$ donor T cells expressed IFNγ (30-40%) and/ or GM-CSF (17-22%), irrespective of the tissue they had accumulated in, or of host genotype. Conversely, we detected very few IL-17 expressing donor CD45.1$^+$ CD4$^+$ T cells or host CD45.2$^+$CD4$^+$ T cells in any of the tissues analyzed (< 5%). Consistent with these findings, IFNγ protein was elevated in homogenates of brainstem and spinal cord tissues from WT and IFNγRKO hosts (620-1200 pg/mg), whereas IL-17 was undetectable or expressed at marginal levels (< 5 pg/mg protein). Th2 cytokines (IL-4,

IL-5 and IL-13) were also undetectable or at the borderline of detection. To more definitively assess the functional role of IL-17 in our experimental system, we transferred MOG_{35-55}-primed, IL-12 polarized donor T cells into IL-17RKO, IFNγRKO or IFNγRKO x IL-17RKO mice. The majority of IL-17R single knock-out mice (63%) developed pure conventional EAE without atypical features (Figure 2-5A). IL-17R/IFNγR double knock-out mice exhibited a similar severity and incidence of atypical EAE to IFNγRKO mice (Figure 2-5A, and data not shown). Taken together, these experiments demonstrate that, in our experimental paradigm, atypical EAE is IL-17 independent.

2d.vii. CXCR2 blockade abrogates atypical EAE, while CCR2 deficiency abrogates conventional EAE

Based on the chemokine expression patterns shown in Figure 4, we hypothesized that CXCL2 plays an instrumental role in the recruitment of neutrophils to the brainstem of IFNγRKO hosts. However, CCL3 and CCL4 were also induced in the brainstem of IFNγRKO adoptive transfer recipients (Figure 2-4), potentially providing a default chemokine pathway for leukocyte recruitment should CXCL2 be blocked. Neutrophils are responsive to a broad array of chemoattractants, including formylated peptides and eicosanoids, that could also function in a redundant manner (26). To directly assess the importance of ELR^+ CXC chemokines in our experimental system, we treated WT and IFNγRKO hosts with anti-sera to CXCR2 (the receptor for CXCL1, CXCL2 and CXCL5) or control sera. Preliminary studies showed that anti-CXCR2 treatment selectively inhibited neutrophil infiltration of the CNS (Figure 2-9). CXCR2 blockade abrogated atypical EAE in IFNγRKO hosts, but did not alter the severity of conventional EAE in WT hosts (Figure 2-5B). A small percentage of WT hosts treated with control antibody, but none of the WT hosts treated with anti-CXCR2, exhibited atypical signs (data not shown).

The onset of conventional EAE in WT mice is associated with spinal cord expression of the monocyte-attracting CC chemokine CCL2 (Figure 2-4). In parallel studies, we transferred MOG_{35-55}-primed, IL-12 polarized WT or IFNγKO $CD4^+$ T cells into syngeneic WT or CCR2KO hosts. The severity of conventional EAE was significantly attenuated in CCR2KO compared with WT mice that received WT Th1 cells (Figure 2-5C). Conversely, the low incidence of mice that

developed atypical features, and the severity of atypical signs, did not differ significantly between the groups. Interestingly, CCR2KO hosts almost exclusively developed atypical EAE following transfer of IFNγKO Th1 donor cells, while WT hosts exhibited a mixture of conventional and atypical features (Figure 2-5D).

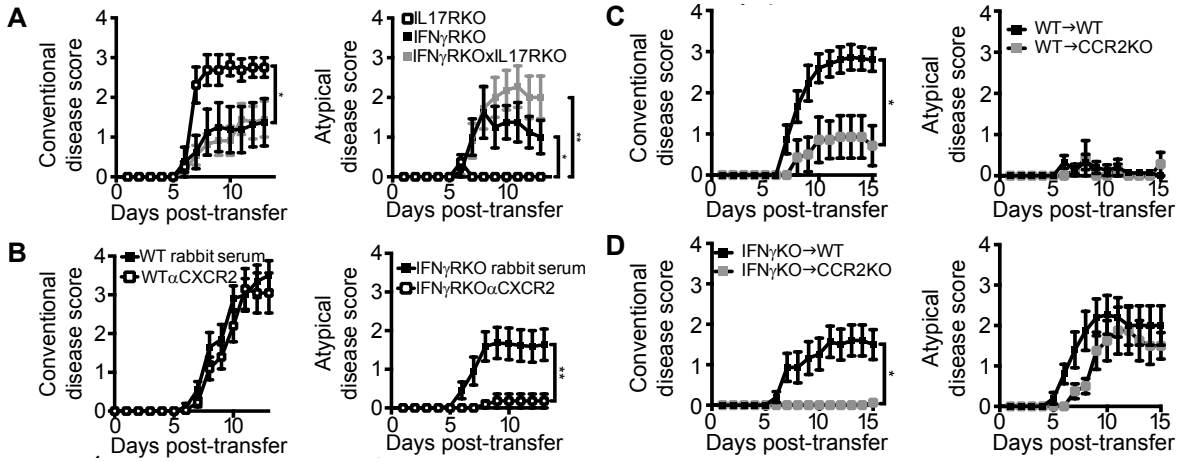

Figure 2-5. The pathological role of signature cytokines and chemokines in IFNγRKO and WT adoptive transfer recipients. (A) MOG_{35-55} primed, IL-12 polarized $CD4^+$ T cells were transferred into IL-17RKO, IFNγRKO or IFNγRKOxIL-17RKO mice. Data are pooled from two independent experiments with a total of 8 IL-17RKO, 8 IFNγRKO and 11 IL-17RKOxIFNγRKO mice. (B) WT and IFNγRKO mice were injected i.p. with ✔CXCR2 antisera or control rabbit serum on days 0, 2, 4, 6, and 8 following the adoptive transfer of MOG-primed, Th1-polarized $CD4^+$ T cells. Conventional and atypical disease scores were averaged over 4 experiments with a total of 20-23 mice per group. (C, D) WT and CCR2KO mice were injected with MOG_{35-55}-primed, Th1-polarized $CD4^+$ WT or IFNγKO T cells and rated for signs of conventional and atypical EAE. WT T cell transfer data are pooled from two representative experiments with 15 WT and 7 CCR2KO recipients. IFNγKO T cell transfer data are from two representative experiments with 15 WT and 9 CCR2KO recipients. The experiment was repeated 4 times with similar results. *P<.05, **P<.01

2e. Discussion

Atypical EAE in IFNγRKO and conventional EAE in WT mice display diametrically different patterns in the spatial distribution of lesions across the neuroaxis. Our data indicate that this is the consequence of distinct CNS region-specific chemokine production following the initial infiltration and reactivation of encephalitogenic T cells. In IFNγRKO mice, CXCL2 is preferentially upregulated in the brainstem. Its cellular source remains to be identified. Astrocytes and choroid epithelial cells are capable of producing ELR^+ CXC chemokines (27-29), which is consistent with the recent observation that IFNγ suppresses atypical EAE via modulation of a non-

hematopoietic, radioresistant cell type (10). If brainstem astrocytes are the source of CXCL2, then they might comprise a distinct subset not found in the spinal cord. More definitive conclusions await experiments with IFNγR conditional knockout mice. The factors that induce CXCL2 in the brainstem of IFNγRKO hosts are also unknown. IL-17 was a logical candidate, since it stimulates the production of neutrophil attracting chemokines in a wide variety of cells (15) and has been implicated in alternative models of atypical EAE (11, 14). However, our data demonstrate that atypical EAE induced by the transfer of MOG_{35-55}-primed, Th1 polarized WT cells into IFNγRKO hosts is IL-17 independent (Figure 2-5). Other candidates include IL-1, TNF ✔ and GM-CSF, although the levels of those molecules were similar in brainstem and spinal cord homogenates from mice with atypical EAE, and were lower than their respective levels in homogenates from mice with conventional EAE (data not shown).

In WT hosts, CCL2 is upregulated in both the spinal cord and brainstem, but only facilitates parenchymal infiltration of the spinal cord. Donor T cells and monocytes appear to initially cross the BCSFB at the level of the fourth ventricle in WT hosts, but fail to penetrate deep into the brainstem distal to the choroid plexus (Figs 1 and 2). This disparity may be the consequence of paradoxical effects of IFNγ-modulated molecules that regulate leukocyte trafficking when expressed in different locations. For example, CXCL12 facilitates neuroinflammation when expressed on the luminal aspect of the cerebrovasculature, but curtails the migration of leukocytes beyond the perivascular space when translocated to the abluminal side (30). IFNγ and IL-17 have opposing effects on the internalization of CXCL12 by brain microvessel endothelial cells. We are currently investigating the effects of IFNγ, produced by encephalitogenic T cells, on the expression of adhesion molecules and chemokines by choroid plexus epithelial cells, glia and cerebrovascular/ meningovascular endothelial cells in vivo, and the repercussions for brainstem and spinal cord inflammation.

Lesions are widely distributed throughout the neuroaxis in the majority of MS patients. However, across the spectrum of human demyelinating disease, there are subpopulations in which inflammatory demyelination is consistently focused in a particular region of the CNS, resulting in distinct clinical phenotypes (2, 3, 5, 6). Analogous to atypical and conventional EAE, the pattern of inflammation in human disease tends to associate with particular CNS chemokine profiles and leukocyte subsets. Although EAE does not perfectly simulate MS, the current data illustrates, in principle, how site-specific interactions between infiltrating immune

cells and CNS resident cells determine clinical and histopathological phenotypes of autoimmune demyelinating disease. Ultimately, the current line of research might lead to the identification of clinical and radiological features that correlate with CNS immune profiles, thereby facilitating clinical management.

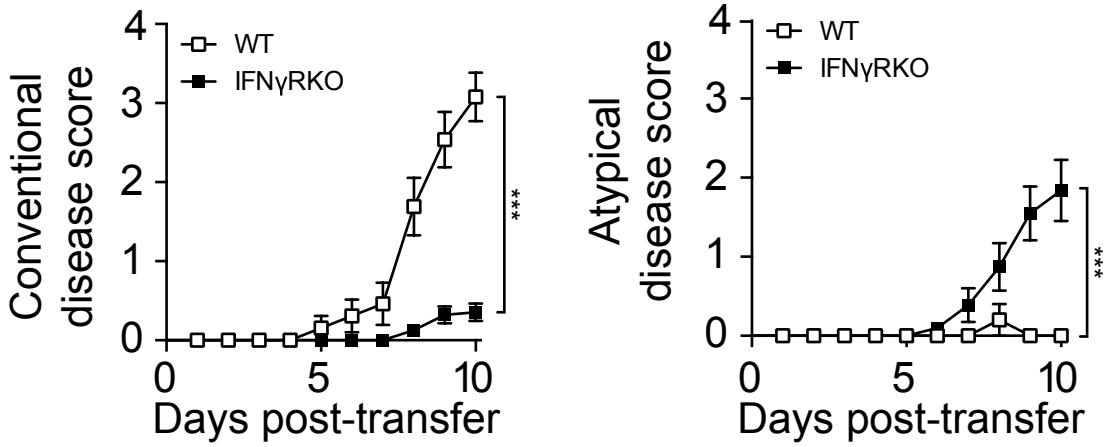

Figure 2-6. Conventional and atypical disease scores for WT and IFNγRKO recipients of Th1-polarized MOG-primed CD4 T cells. WT n=14, IFNγRKO n=29.

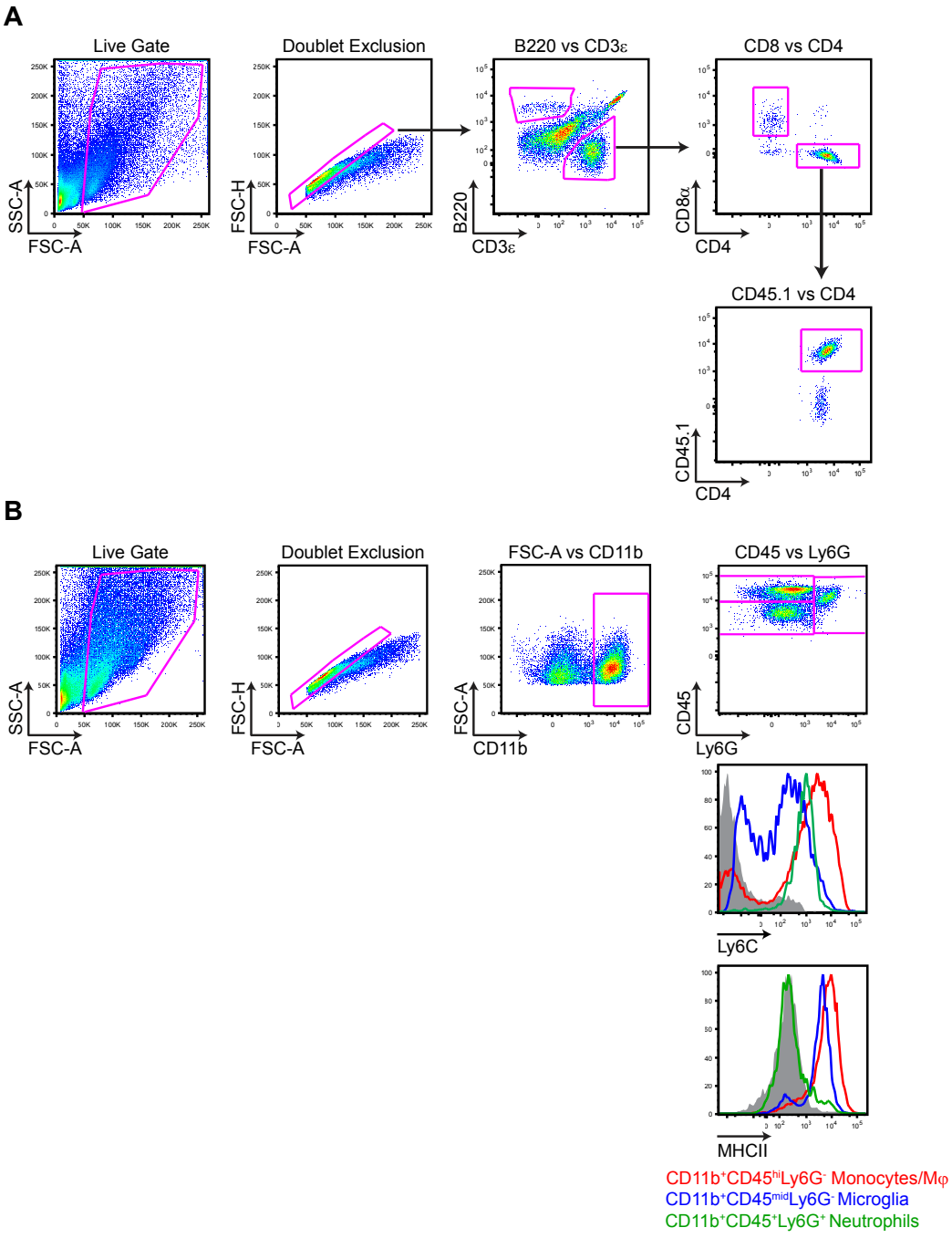

Figure 2-7. Gating schemes for identifying myeloid and lymphoid cell subsets in CNS tissue. (a) Flow cytometry gating scheme for identifying donor $CD4^+$ T cell populations as $B220^-CD3\varepsilon^+CD4^+CD45.1^+$ cells. (b) Myeloid cells ($CD11b^+$) were separated into microglia ($CD45^{int}Ly6G^-$), monocytes ($CD45^{hi}Ly6G^-$), and neutrophils ($CD45^+Ly6G^+$). MHCII and Ly6C expression were used to confirm phenotypes of microglia, monocytes and neutrophils.

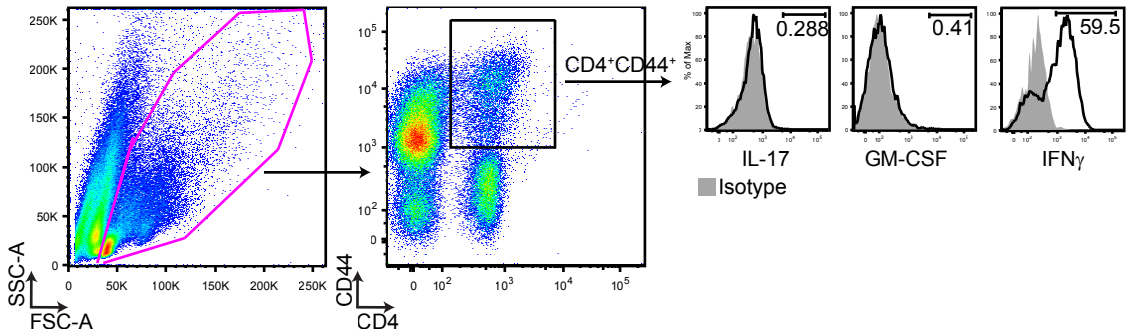

Figure 2-8. Cytokine production by Th1-polarized T cells following *in vitro* stimulation. MOG_{35-55}-primed, IL-12 polarized CD4 T cells were stimulated with PMA and ionomycin prior to intracellular staining and flow cytometric analysis. Histograms are gated on $CD4^+CD44^+$ T cells.

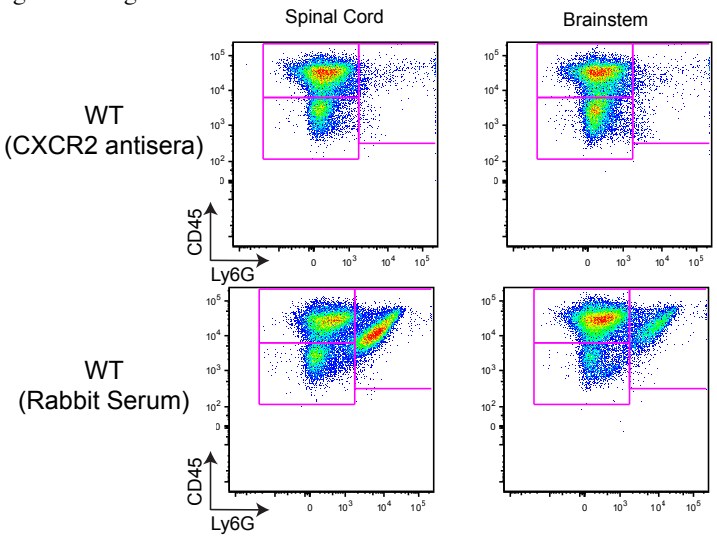

Figure 2-9. Administration of CXCR2 antisera inhibits neutrophil accumulation in the spinal cord and brainstem in WT mice with EAE. Flow cytometric analysis of the spinal cord and brainstem isolated from score-matched mice d9 post-CD4 T cell transfer to examine neutrophil infiltration.

2f. Acknowledgements

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CHAPTER 3 - IFNγ MEDIATED REGULATION OF MYELOID DERIVED CXCL2 DETERMINES LESION LOCALIZATION DURING EAE

3s. Abstract

The spatial distribution of lesions in the central nervous system (CNS) can vary widely among patients with multiple sclerosis (MS), or mice with experimental autoimmune encephalomyelitis (EAE). Cytokines produced by infiltrating leukocytes may control the sites where demyelinating lesions form. Hence, IFNγ producing encephalitogenic Th1 cells induce conventional EAE (cEAE) in syngeneic wildtype hosts, characterized by ascending paralysis and monocyte predominant spinal cord inflammation. Conversely, adoptive transfer of the same Th1 effector cells into IFNγ receptor (IFNγR) deficient mice results in atypical EAE (aEAE) characterized by gait imbalance and neutrophil-predominant brainstem inflammation. CXCL2, a neutrophil attracting chemokine, is upregulated in the brainstem during aEAE; blockade of its cognate receptor, CXCR2, abrogates disease. Here we demonstrate that myeloid cells are the primary CNS source of CXCL2 during aEAE. We present evidence for an IFNγ regulated CXCR2/ CXCL2 autocrine feedback loop in neutrophils that drives their progressive accumulation in brainstem white matter. In mice with cEAE, IFNγ directly suppresses CXCR2 expression by neutrophils, and CXCL2 expression by CNS infiltrating myeloid cells. These data reveal a novel mechanism by which IFNγ and CXCL2 interact to direct regional recruitment of inflammatory cells in the CNS, resulting in distinct clinical phenotypes.

3b. Introduction

In multiple Sclerosis (MS), a multifocal inflammatory demyelinating disease of the central nervous system (CNS), the distribution of lesions can vary widely between patients, resulting in distinct clinical phenotypes. In some patients lesion burden is dispersed across CNS compartments, while in others it is skewed towards the spinal cord or cerebral white matter. (1, 2) Little is known about the factors that determine which CNS regions are targeted in a given individual. Several models of experimental autoimmune encephalomyelitis (EAE) also demonstrate heterogeneity in the distribution of lesions. A common theme that has emerged is that IFNγ production by encephalitogenic T cells has a defining effect on lesion localization. Hence, adoptive transfer of Th1 polarized, encephalitogenic T cells derived from C57BL/6 wildtype (WT) mice into IFNγ receptor deficient (IFNγRKO) hosts, or transfer of IFNγKO T cells into WT hosts, results in a high incidence of atypical EAE (aEAE) characterized by imbalance and brainstem or cerebellar inflammation(3, 4). We previously demonstrated that induction of aEAE in C57BL/6 IFNγRKO mice is dependent on CXCR2 mediated neutrophil infiltration of the pons, at the level of the vestibular cochlear nucleus(5). Conversely, transfer of WT T cells into WT hosts induces cEAE. with an ascending paralysis and infiltration of the thoracolumbar spinal cord. IFNγ promotes spinal cord inflammation by inducing the local expression of chemokines, such as CCL2 and CXCL10(6), and adhesion molecules, such as VCAM-1(7), which mediate the passage of T cells and monocytes across the BBB(8). Hence, cEAE is dependent on CCR2 and α4β1 integrin, the receptors for CCL2 and VCAM-1, respectively.

In some EAE models the induction of cEAE versus aEAE correlates with the ratio of Th1 over Th17 cells within the transferred donor T cell population(9). IL-17 is a strong inducer of G-CSF and ELR+ CXC chemokines, factors that drive neutrophil mobilization and recruitment to sites of inflammation(10). IFNγ is known to suppress the differentiation of Th17 cells and production of IL-17. However, IL-17 may or may not play a critical role in aEAE pathogenesis depending on mouse strain and mode of disease induction. For example, IL-17 signaling is required for the induction of aEAE by IL-23 polarized T cells in C3H/Feb mice(9), but it is dispensible for the induction of aEAE by IL-12 polarized T cells in C57BL/6 IFNγKO mice(5). In the former model, astrocytes were identified as the major source of CXCL2 transcripts in the

brain(11). In the current study we investigate the source of CXCL2 in the brainstem of C57BL/6 IFNγKO mice with aEAE, the factors that promote its production, and the mechanism by which IFNγ regulates neutrophil infiltration and clinical aEAE.

3c. Methods

3c.i. Mice.

8- to 14 week old CD45.1 congenic and WT C57BL/6 mice were obtained from NCI Fredrick. IFNγRKO mice were originally obtained from Jackson Laboratory and bred in the University of Michigan animal facilities. Mice were housed in microisolator cages under specific pathogen-free conditions. All animal protocols were approved by the University Committee on Use and Care of Animals.

3c.ii. Antibodies and Reagents.

For flow cytometry the following antibodies were obtained from ebiosciences: PECy7-anti-CD11b (M1/70), eFluor450-anti-CD45 (30-F11) and PerCpCy5.5-anti-Ly6C (HK1.4). Allophycocyanin cy7-anti-Ly6G (IA8) was from BD biosciences. For immunofluorescent histology Rabbit anti-GFAP (Gibco), rat anti-mouse CD45 (IBL-5/15, Millipore), goat anti-mouse CXCL2, and anti-mouse CXCL1 (R&D Systems), rat anti-mouse Ly6G (IA8, BD Biosciences) and Ham anti-Mouse CD3ε were used as primary antibodies. Secondary antibodies AlexaFluor594 donkey-anti-goat IgG, AlexaFluor488 goat-anti-rat IgG, goat anti-hamster and AlexaFluor647 goat-anti-rabbit IgG were obtained from life technologies. For *in vitro* cultures, recombinant mouse (rm) IFNγ, rmIL-12, rmCXCL2, rmIL-1β and rmCXCL1 were from R&D Systems.

3c.iii. Induction and scoring of EAE.

Donor mice were immunized subcutaneously with 100μg MOG_{35-55} (MEVGWYRSP-FSRVVHLYRNGK, Biosynthesis) in CFA (Difco) across four sites over the flanks. Inguinal, axial and brachial lymph nodes were harvested 14 days post-immunization, pooled,

homogenized and passed through a 70μm strainer (BD Falcon). Cells were cultured with MOG_{35-55} (50μg/mL) in the presence of rmIL-12 (6ng/mL) and rmIFNγ (2ng/mL). At 96 h, CD4 T cells were isolated by column separation with CD4 (L3T4) magnetic microbeads, according to manufacturer's instructions (Miltenyi). $5x10^6$ CD4 T cells (80-99% pure) were transferred i.p. into naïve hosts. Adoptive transfer recipients were monitored on a daily basis by an examiner who was blinded to experimental groups. Mice were scored for severity of conventional and atypical signs of EAE using established scales(4),(20).

3c.iv. BMT.

Bone marrow was isolated from femur and tibia of IFNγRKO or tdTomato mice and mixed at 1:1 ratio. Following 2 6.5 Gy doses spaced out over 3 hours from an orthovoltage source to eliminate the recipient hematopoetic compartment. $5x10^6$ cells from bone marrow mixture were injected into irradiated mice via the tail vein. Adoptive transfer experiments were performed at least 6 weeks post-transplant.

3c.v. Histology.

After perfusion with 1xPBS and 4% PFA CNS tissue was post-fixed in 4% PFA for 96h, decalcified in 0.5M EDTA for 96h and transferred into 30% sucrose for at least 48h prior to embedding in OCT and storage at -80°C. 10μm spinal cord and brain sections were were cut in cryostat at -20°C. For staining, in a humidified chamber, sections were incubated 1x PBS to remove OCT, then blocking solution (1x PBS 7.4pH, 10% Normal Donkey Serum, 0.5% Triton-X100) for 1 hour prior to addition of primary antibodies overnight at 4°C. For secondary antibody staining, sections were incubated with AlexaFluor594 donkey-anti-Goat IgG, washed, then incubated with AlexaFluor488 goat-anti-rat IgG and AlexaFluor647 goat-anti-rabbit IgG. Subsequently, sections were incubated with DAPI (100ng/mL) prior to washing and mounting on slides (Anitfade Reagent, Southern Biotech). Confocal images were acquired using a Nikon A-1 Confocal microscope (Nikon PlanApoVC 20x, 40x or 60x/1.40 oil) with diode-based laser system and NIS Elements software. Appropriate processing including image overlays, black level and brightness adjustments were performed in Adobe Photoshop CC2014 and applied equally to all samples and controls.

3c.vi. CNS Mononuclear Cell Isolation.

CNS tissue was harvested and separated into the spinal cord and brainstem. Each tissue was homogenized in 1mL PBS containing a protease inhibitor cocktail (Roche) and centrifuged at 800xg for 10 minutes. Supernatant from homogenization was frozen down for subsequent chemokine analysis, and tissue pellets were further homogenized in 27% percoll and spun at 500xg for 20 minutes with slow brake. Cell pellets were counted on a Cellometer AutoT4 automated cell counter with trypan blue exclusion used to assess viability (Nexelcom).

3c.vii. Flow Cytometry.

For surface staining, cells were resuspended in PBS+2% FCS containing Fc Block (50ng/mL) and Fixable Viability Dye efluor 506 (ebioscience) prior to incubation with fluorochrome-conjugated antibodies. For intracellular staining, cells were incubated with Brefeldin A (10 μg/mL) for 4 hours in the presence or absence of stimulation conditions. Cells were labeled with fluorochrome-conjugated cell surface antibodies as described above, fixed in 4% PFA, permeabilized with 0.5% saponin and incubated with fluorochrome-conjugated anti-cytokine or chemokine antibodies. Stained cells were run on a FACS Canto II flow cytometer (v6.1.3, Becton Dickenson) or sorted on the FACS Aria II using FACS Diva software. Data was analyzed using FlowJo software (v10.3.2, Treestar).

3c.viii. BM and Neutrophil Isolation and Stimulation

8-14 week old mice were sacrificed, femur and tibia were flushed and passed through cell strainer (70μm) with repeated washes. For monocyte stimulation, whole BM was plated in complete media (RPMI with 10% FCS, L-Glut (2mM, Gibco), Pen/Strep (1:100, Gibco), Soduim Pyruvate (12.5μM, Gibco) and 2-mercaptoethanol (55μm, Gibco)) in the presence of Brefeldin A (10μg/mL) in stimulation conditions containing LPS (1μg/mL) in the presence or absence of IFNγ (2ng/mL) for 4 hours, then isolated for intracellular flow cytometric analysis of CXCL2. Neutrophils were purified by positive selection from bone marrow cell suspension using anti-Ly6G microbead kit (Miltenyi). Purified neutrophils were plated complete media in the presence or

absence of CXCL2 (20ng/mL), CXCL1 (20ng/mL), G-CSF (25ng/mL), IFNγ (2ng/mL) or IL-1β (10ng/mL) and isolated following 1h in culture to examine CXCL2 mRNA expression.

3c.ix. RNA isolation

Cells from FACS or *in vitro* cell culture were spun down and resuspended in 1mL Trizol (Life Technologies). For RNA extraction, 200μL of chloroform was added to samples and mixed prior to centrifugation at 18000xg. Chloroform layer was moved to fresh tube with 500μL cold isopropanol, mixed and incubated for 15 minutes prior to purifying the RNA out using the RNeasy MiniKit (Qiagen) with on column DNase digestion per manufacturer's instructions.

3c.x. RT- and q-PCR

RT-PCR was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) per manufacturer's instructions. For q-PCR, TaqMan Universal Master Mix and primer/probe sets for CXCL2, CXCR2 and GAPDH were purchased from Applied Biosystems and run on a MyIQ system using iQ5 software (BioRad) as described in manufacturer's instructions.

3c.xi. Statistics

Statistical analyses were performed using GraphPad Prism software. Comparisons of cell number, transcript levels in transfers into WT and IFNγRKO mice were done using an unpaired Student *t* test. Comparisons of transcript levels in transfers into mixed bone marrow chimeras were or *in vitro* experiments were done using a paired Student *t* test. A p value < 0.05 (*) was considered significant, with $p < 0.01$ (**) and $p < 0.001$ (***).

3d. Results

3d.i. T cells and neutrophils infiltrate the meninges of mice with cEAE as well as aEAE, but only migrate into the BS Parenchyma in mice with aEAE

It has been suggested that the choroid plexus (located in the ventricles of the brain) is the initial portal of entry of encephalitogenic T cells into the CNS during cEAE(12), despite the fact that clinical deficits of those mice localize to the spinal cord. We questioned whether inflammatory cells traverse the choroid plexus and migrate into the meninges as early steps in the pathogenesis of both cEAE and aEAE, but only penetrate deeper into the brainstem parenchyma in mice with aEAE(9). Indeed, immunofluorescent histological studies revealed the presence of T cells ($CD3\varepsilon^+$ (green)) and neutrophils ($Ly6G^+$(Red)) in the meninges, adjacent to the lateral recess of the 4^{th} ventricle, at the onset of cEAE in WT recipients of MOG-specific Th1 cells (Figure 3-1 left panels). However, inflammatory cells were not present in the underlying parenchymal white matter. In contrast, a dense neutrophil-rich infiltrate extended from the meninges into the BS parenchyma of IFNγRKO at the onset of aEAE (Figure 3-1 right panels). T cells were also detected, clustering around blood vessels, in the BS parenchymal tissue of the IFNγRKO hosts. This suggests that in cEAE, as opposed to aEAE, inflammatory cells are actively confined to the meninges, and/ or do not receive, or respond to, sufficient chemotactic stimuli to migrate into the adjacent white matter.

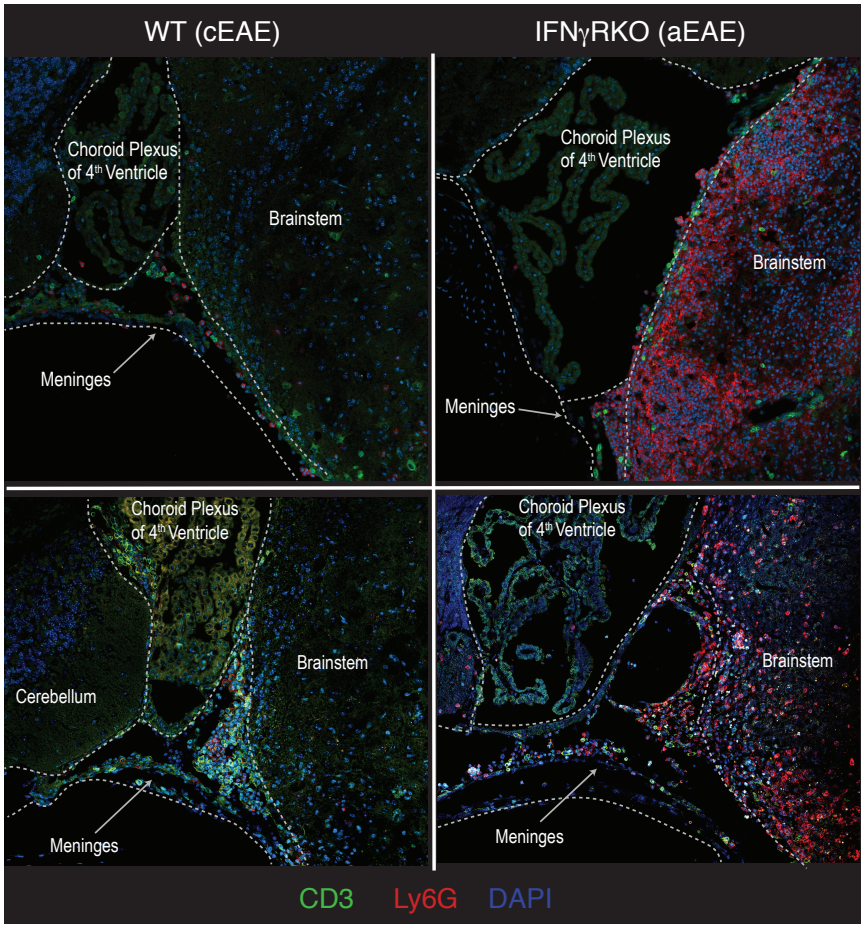

Figure 3-1: Neutrophils migrate into BS parenchyma at the onset of aEAE. Immunofluorescent histology for CD3ε (green), Ly6G (red) and DAPI (blue) in WT mice at the onset of cEAE (left panels) and IFNγRKO mice at the onset of aEAE (right panels).

3d.ii. IFNγ suppresses expression of CXCR2 by CNS-infiltrating neutrophils

We previously demonstrated that the neutrophil attracting chemokine CXCL2 is upregulated in the brainstem of IFNγRKO, but not WT mice, following the adoptive transfer of encephalitogenic WT Th1 cells. Furthermore, blockade of CXCR2, the cognate receptor for CXCL2, abrogates brainstem inflammation and clinical aEAE in IFNγRKO hosts, but has no impact on cEAE in WT hosts. These observations led us to question whether IFNγ regulates CXCR2 expression by WT neutrophils as they infiltrate the CNS. CXCR2 is rapidly internalized following ligation by its cognate chemokines. Therefore, cell surface CXCR2 levels are not always a reliable indicator of CXCR2 production. Therefore, we measured CXCR2 transcripts in neutrophils harvested from mice with aEAE or cEAE by qRT-PCR. CXCR2 transcript levels

were significantly higher in neutrophils isolated from the brainstems and spinal cords of IFNγRKO hosts at aEAE onset compared with analogous neutrophils isolated from WT hosts at cEAE onset. There was no difference in CXCR2 expression by bone marrow neutrophils isolated from IFNγRKO versus WT hosts at the same time point (Figure 3-2A).

We constructed mixed bone marrow chimeric mice in order to directly compare CXCR2 mRNA expression in IFNγ responsive versus unresponsive myeloid cells within the same CNS microenvironment during EAE. Lethally irradiated CD45.1 congenic mice were reconstituted with a mixture of bone marrow cells from WT and IFNγRKO donors. WT donor cells were distinguished by expression of a tdTomato reporter. The mixed BM chimeras were subsequently injected with MOG-specific Th1 cells to induce EAE, and CNS infiltrating cells, and bone marrow cells were harvested at clinical onset. Neutrophils derived from each donor pool were FACS sorted and subjected to RNA extraction and qRT-PCR analysis. CXCR2 mRNA levels were reproducibly higher in IFNγRKO neutrophils compared to paired WT neutrophils isolated from the CNS of the same mouse. In contrast, there were no differences in CXCL2 mRNA expression comparing WT versus IFNγRKO myeloid cells isolated from the bone marrow (Figure 3-2B). This suggests that IFNγ directly inhibits CXCR2 production by neutrophils in the CNS.

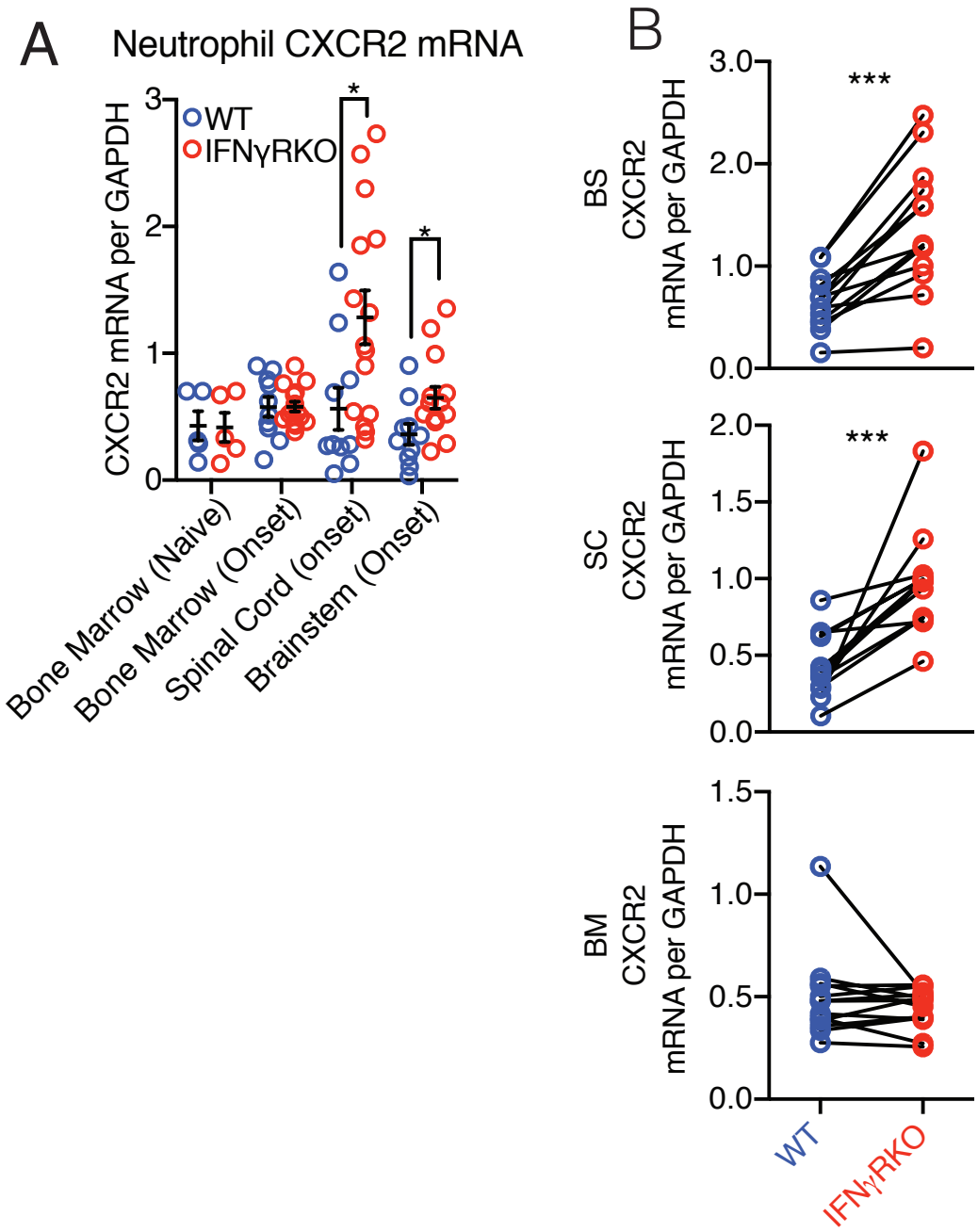

Figure 3-2: IFNγ signaling directly regulates CXCR2 expression on neutrophils. (A) $CD45^+CD11b^+Ly6G^+$ cells were FACS sorted from naïve BM as well as BM, SC and BS of mice at the onset of cEAE in WT and aEAE in IFNγRKO mice to examine CXCR2 transcript levels relative to GAPDH. WT n=10 IFNγRKO n=15 from two experiments. (B) $CD45^+CD11b^+Ly6G^+$ cells were sorted into WT and IFNγRKO subsets from mixed BM chimeras at the onset of EAE out of the BM, SC, and BS and examined for CXCR2 transcript expression relative to GAPDH. n=13 from two experiments.

3d.iii.Myeloid cells are the primary source of CXCL2 in the BS of mice with aEAE

The primary cellular source(s) of CXCL2 in the brainstem of C57BL/6 IFNγRKO injected with MOG-specific Th1 cells has not previously been identified. Using in situ hybridization, we detected CXCL2 transcripts within intraparenchymal brainstem infiltrates, but not in adjacent uninflamed white matter or in the meninges, at the peak of aEAE (Figure 3-3A and data not shown). Immunohistochemical studies indicated that the CXCL2 producers were exclusively $CD45^+$, indicative of hematopoietic cells. The majority of the $CD45^+$ cells that stained positively for CXCL2 had multilobulated nuclei, consistent with neutrophils. Although we were able to visualize $GFAP_+$ astrocytes at the border of the infiltrates, none were CXCL2 positive (3-3B). The above data were corroborated by qRT-PCR analysis of cell subsets FACS sorted from the brainstem at the onset of aEAE. Hence, CXCL2 transcript levels were highest in purified $CD45^{hi}CD11b^+Ly6G^+$ neutrophils, followed by $CD45^{hi}CD11b^+Ly6G^-$ monocytes/ macrophages, and $CD45^{int}CD11b^+Ly6G^-$ microglia (Figure 3-4A). CXCL2 mRNA expression in brainstem-infiltrating neutrophils, macrophages and microglia correlated with aEAE severity (Figure 3-4B). CXCL2 mRNA was barely detectable above background levels in CNS-infiltrating cells that were $CD45^+CD11b^-$, a cell surface phenotype consistent with lymphocytes. Similarly, intracellular staining and flow cytometric analysis demonstrated that neutrophils were the predominant producers of CXCL2 protein during aEAE (in terms of frequency of cells within the CXCL2+ population), followed by microglia and macrophages/ monocytes (Figure 3-4C). We did not detect CXCL2 protein in lymphoid cells or non-hematopoeitic CD45- cells.

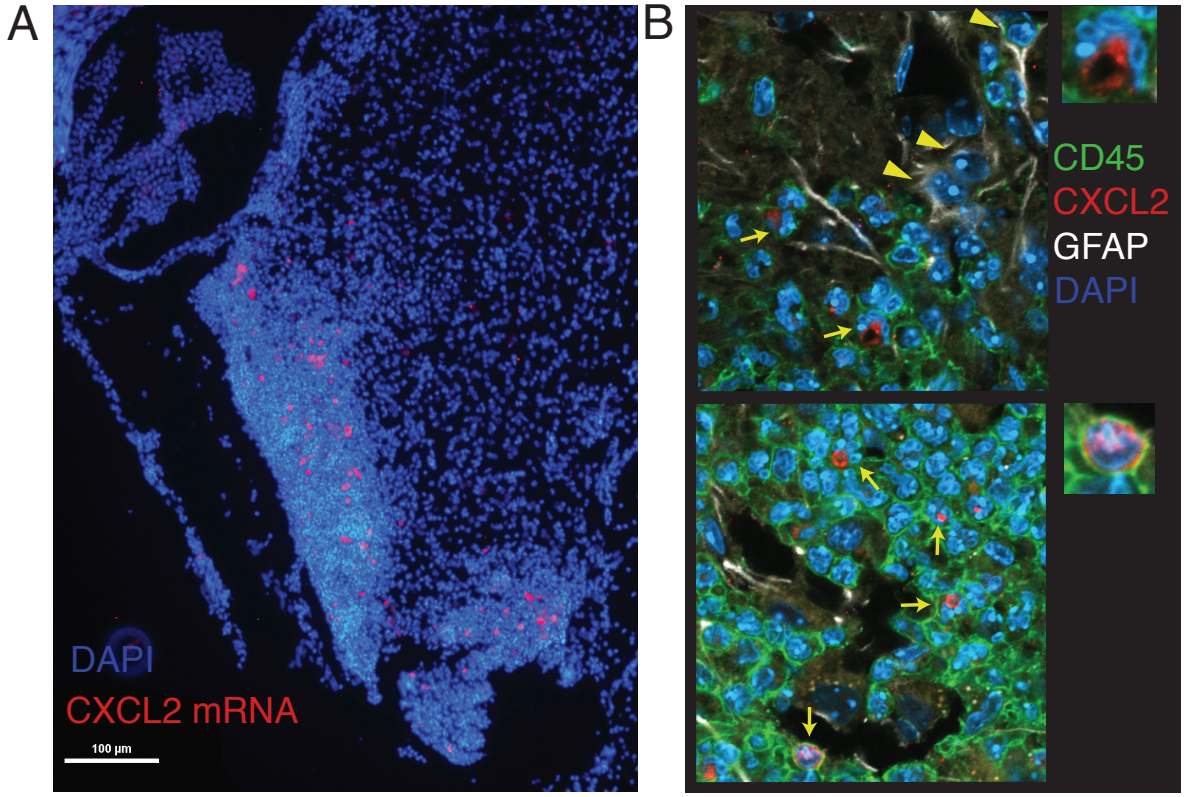

Figure 3-3: CXCL2 is made by CD45+ cells in the BS parenchyma. CXCL1 is made by choroid plexus epithelium and astrocytes. (A) *in situ* hybridization of the BS at the onset of aEAE for CXCL2 mRNA (red) combined with DAPI (Blue) counter stain. (B) Immunofluorescent histology of BS infiltrates in aEAE staining for CD45 (green), GFAP (white), DAPI (blue) and CXCL2 (red).

3d.iv. IFNγ directly suppresses CXCL2 expression by neutrophils

While the data in (Figure 3-2A) demonstrates that IFNγ could prevent brainstem inflammation and the development of aEAE via inhibition of CXCR2 expression on neutrophils, we questioned whether IFNγ also regulates CXCL2 production. Indeed, CXCL2 mRNA levels were reproducibly higher in IFNγRKO neutrophils and monocytes isolated from the brainstems of mixed bone marrow chimeric mice when compared with their paired WT counterparts (Figure 3-4D). In contrast, there was no difference in CXCL2 mRNA expression comparing WT versus IFNγRKO myeloid cells isolated from the bone marrow of the chimeric mice (Figure 3-4D). This suggests that, similar to its effects on CXCR2 expression by neutrophils, IFNγ directly inhibits CXCL2 production by myeloid cells within the CNS.

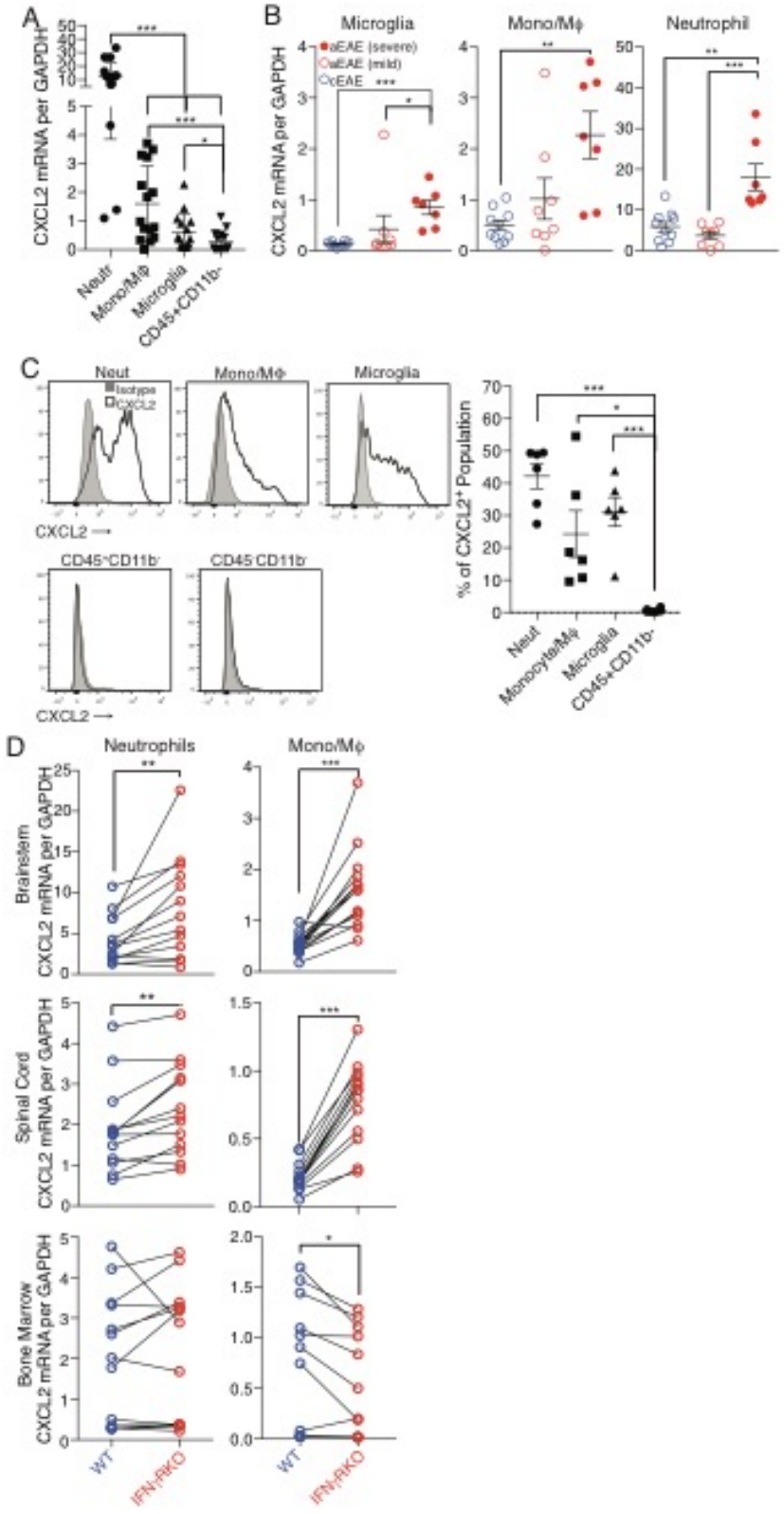

Figure 3-4: CD45^{+}CD11b^{+} cells are the primary source of CXCL2 in BS during aEAE. (A) neutrophils (CD45^{+}CD11b^{+}Ly6G^{+}), monocyte/macrophage (CD45^{hi}CD11b^{+}Ly6G^{--}), microglia (CD45^{lo}CD11b^{+}Ly6G^{-}) and non-myeloid immune cells (CD45^{+}CD11b^{-}) were FAC sorted out of the BS of mice at the onset of aEAE and examine

for CXCL2 mRNA levels relative to GAPDH. IFNγRKO n=15 from two experiments. (B) Examination of cell number and CXCL2 mRNA expression per GAPDH of microglia, monocyte/macrophages and neutrophils at the onset of cEAE and aEAE. aEAE analysis was separated into mild onset (score=1) and severe onset (score≥2). WT n=10 IFNγRKO n=15 from two experiments (C) BS infiltrates at the onset of aEAE were plated for 4 hours in the presence of brefeldin A and examined by flow cytometry to identify cellular subsets that were positive for CXCL2. n=6 from two experiments. (D) CXCL2 transcript per GAPDH was measured in neutrophils sorted out of mixed BM chimeras. n=13 from two experiments.

3d.v. CXCL1 is expressed by non-hematopoietic CNS resident cells during EAE

CXCL1 and CXCL2 are both ligands for CXCR2. In a previous study we made the paradoxical observation that, while CXCL2 is preferentially upregulated in the brainstem of IFNγRKO mice with aEAE, CXCL1 expression is elevated in the brainstem and spinal cord of WT hosts compared to IFNγKO hosts(5). It was unclear why CXCL1, produced in the CNS of WT hosts, did not compensate for low CXCL2 levels to stimulate local neutrophil infiltration. One possibility is that CXCL1 and CXCL2 are produced by different cell types at distinct locations within the brainstem. Indeed, immunohistochemical analyses showed that choroid plexus epithelial cells are a major source of CXCL1 during EAE in IFNγRKO mice (Figure 3-5B). This finding was corroborated by qRT-PCR, which revealed an upregulation of CXCL1 transcripts in the choroid plexus epithelium during aEAE (Figure 3-5A). We also detected CXCL1 in astrocytes and cerebrovascular endothelial cells in the spinal cords of WT hosts with cEAE (Figure 3-5B). We did not detect CXCL1 in infiltrating inflammatory cells, irrespective of the CNS compartment or genotype of the host.

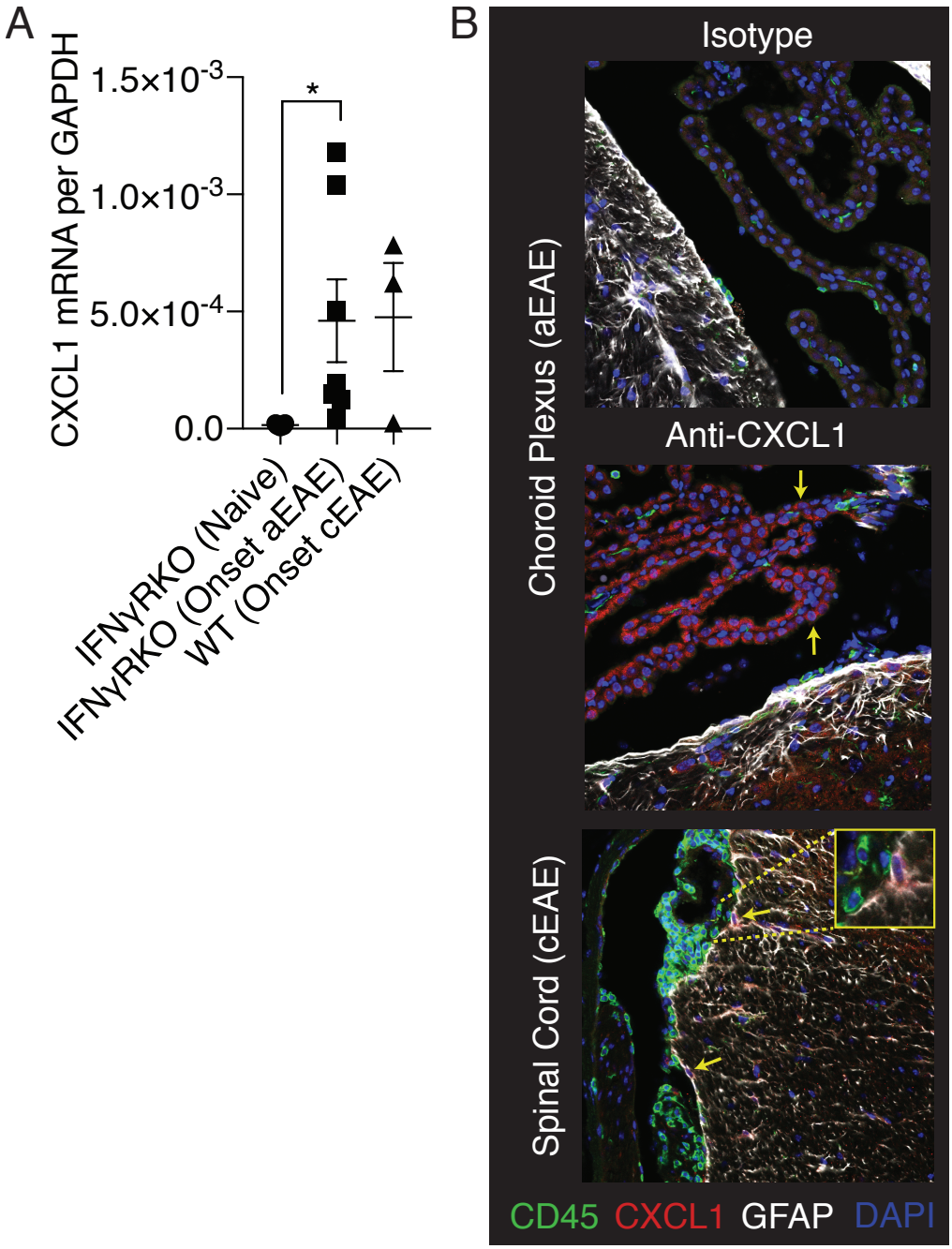

Figure 3-5: CXCL1 transcript and protein is expressed by CNS-resident cells during EAE(A) qRT-PCR analysis of choroid plexus isolated from onset of cEAE, aEAE and IFNγRKO naïve mice (B) Immunofluorescent histology stain of choroid plexus and BS parenchyma in aEAE (600x) and SC in cEAE (400x) for CD45 (green), GFAP (white), DAPI (blue) and CXCL1 (red).

3d.vi. IFNγ regulates a CXCL2 autocrine feedback loop in neutrophils

IL-17 is a potent inducer of ELR+ CXC chemokines and has been shown to play a critical role in initiating neutrophil migration to the brainstem white matter in some models of aEAE. However,

in our model system aEAE is IL-17 independent. This raises the question of which factors drive CXCL2 production in the brainstem of IFNγRKO hosts. To address that issue, we assessed the ability of soluble factors that are upregulated in the brainstem during aEAE to stimulate CXCL2 expression by bone marrow neutrophils *in vitro*. We found that CXCL2 mRNA was upregulated in neutrophils cultured with recombinant G-CSF, as well as with CXCL2 itself. Induction of CXCL2 mRNA expression by CXCL2 signaling was enhanced by the addition of IL-1b. IFNγ suppressed CXCL2 mRNA levels in neutrophils stimulated with either CXCL2 alone or combined with IL-1b, but had no effect on CXCL2 mRNA levels in neutrophils stimulated with G-CSF (Figure 3-6A and B). Ligation of TLRs on monocytes, macrophages and microglia is known to induce CXCL2 expression (13, 14). To examine whether CXCL2 expression in non-granulocytic myeloid cells could regulated by IFNγ, we cultured bone marrow derived monocytes with LPS in the presence or absence of IFNγ. LPS stimulation induced CXCL2 production in BM monocytes which was inhibited by IFNγ (Figure 3-6C).

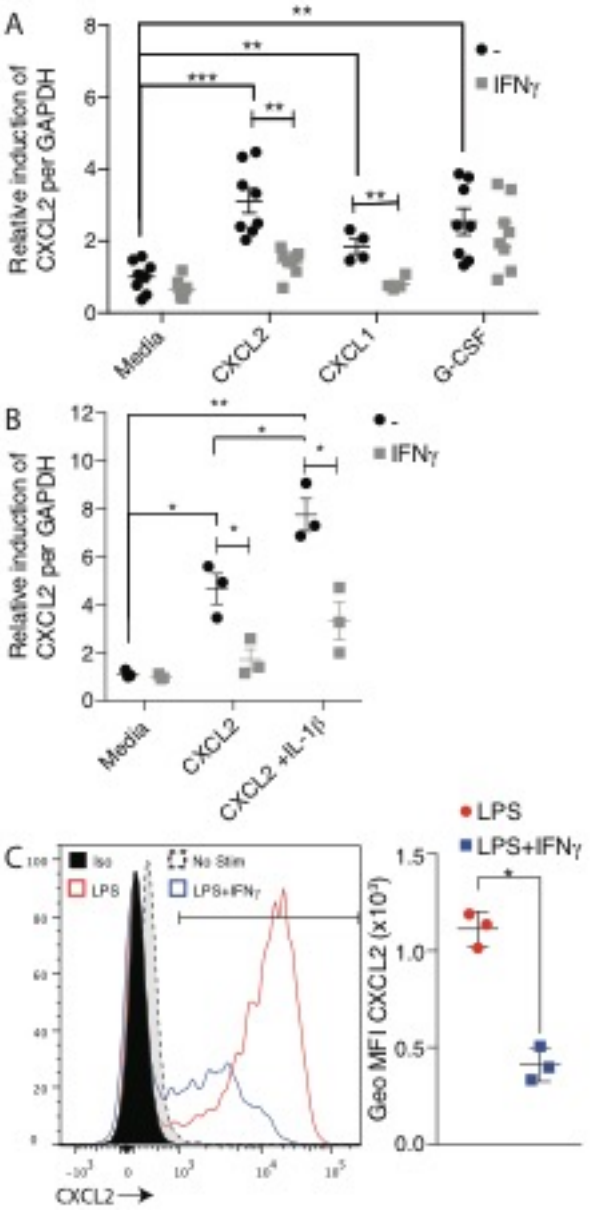

Figure 3-6: IFNγ directly suppresses distinct pathways of neutrophil and monocyte CXCL2 expression (A-B) Neutrophils isolated from the BM were incubated in conditions for 1h to stimulate CXCL2 expression in the presence and absence of IFNγ. n=8 from 3 experiments in figure A, n=3 in representative experiment shown in figure B of two replicates. (C) BM monocytes were stimulated in the presence of Brefeldin A with LPS in the presence and absence of IFNγ for 4h. CXCL2 protein expression was analyzed by flow cytometry. N=3 in representative experiment of 3 replicates.

3e. Discussion

Based on our data we propose the following model by which an IFNγ/CXCL2 axis regulates the regional localization of inflammatory infiltrates during EAE. CXCL1 is released by choroid plexus epithelial cells in the early stages of both cEAE and aEAE, and promotes neutrophil

migration into the meninges. In IFNγRKO hosts the neutrophils are then stimulated to migrate further into the brainstem by a CXCL2 concentration gradient, possibly initiated by microglia and then amplified by neutrophils and monocytes as they accumulate in the brainstem white matter. CXCL2 production by neutrophils escalates during the progression of aEAE, driven by an autocrine positive feedback loop. In WT hosts, IFNγ, produced by myelin-specific T cells upon reactivation by APCs in the brainstem parenchyma, suppresses CXCL2 production by local myeloid cells. Consequently, production of CXCL1 by choroid plexus endothelium is not counterbalanced by a CXCL2 gradient from cells originating in the white matter parenchyma, effectively trapping neutrophils in the meningeal space.

Our model is supported by the presence of similar regulatory mechanisms in other settings. Hence, in a model of type III hypersensitivity, it was shown that neutrophils respond to CXCL1 and CXCL2 by upregulating CXCL2 transcript(15) and have the highest level of CXCL2 transcript of all cells within inflamed tissue. Additionally IFNγ signaling is known to regulate neutrophil expression of CXCR2(16), which may directly suppress neutrophil response to and production of CXCL2.

Although CXCL2 is critical in both models of aEAE, *Simmons et al*. show that IL-17 and IFNγ induce CXCL2 transcript expression in the BS and SC, respectively. In their model CXCL2 transcript levels are highest in compartments where neutrophil numbers are greatest. If neutrophils are the predominant source of CXCL2 then the tissue compartment most heavily infiltrated by neutrophils would also have the highest levels of CXCL2 transcript. A LysM-Cre $SOCS3^{fl/fl}$ model of active immunization aEAE, also dependent upon neutrophil infiltration of the BS and cerebellum, found that neutrophils from SOCS3 deficient animals produced higher levels of CXCL2 in the BS and cerebellum during aEAE compared to WT controls(17, 18). While they found that other chemokines such as CXCL10 and CCL2 were also increased in their SOCS3 deficient neutrophils, this is not entirely unsurprising as IFNγ signaling remains intact in this model.

This CXCL2 dependent neutrophil recruitment represents a novel pathway of immune cell recruitment in CNS autoimmunity and indicates that multiple mechanisms exist by which immune cells can infiltrate the CNS and regulate lesion localization. Subsets of patients with distinct lesion localization patterns also had distinct cytokine and chemokine expression patterns in their serum and CSF(19). This damage to different areas of the CNS generates distinct clinical

deficits that may be indicative of recruitment pathways activated in individual patients, and what disease modifying therapies may be effective in their treatment. Analysis of chemokine levels in patient serum and CSF to lesion localization and patient response to disease modifying therapies could help to tailor treatment to individual patients.

3f. Acknowledgements

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CHAPTER 4-DISCUSSION AND FUTURE DIRECTIONS

Previous work had outlined the importance of IFNγ signaling in regulating lesion localization in EAE. In adoptive transfer models, loss of IFNγ production by transferred myelin-specific T cells or IFNγ response in recipient mice altered disease focus from the spinal cord to the brainstem. In this work, we sought to identify critical points within the disease process where IFNγ altered the focus of CNS inflammation and damage. In particular, we focused on how the host response to IFNγ, following the transfer of a Th1-polarized myelin-specific T cell population, would alter each stage of disease in order to identify a potential divergence in the immune response that could elucidate what drives these distinct disease phenotypes.

4a. The pattern of initial Encephalitogenic T cell infiltration is unaltered in the absence of IFNγ signaling

We and others found that aEAE in IFNγRKO adoptive transfer recipients (1,2) correlates with preferential infiltration of the brainstem, compared to WT mice with predominant cEAE due to preferential inflammation of the spinal cord (Table 2-1 and Figure 2-1). As the first stage of EAE is the recruitment of myelin-specific T cells, we questioned whether the infiltration patterns of encephalitogenic T cells at preclinical time points informed the localization of subsequent immune cell infiltration in WT and IFNγRKO mice. We found that there was not preferential accumulation of transferred T cells in the spinal cord of WT mice or the brainstem of IFNγRKO mice prior to disease onset (Figure 2-3A and B). Nor were there differences in the frequency of activated myelin specific T cells, as measured by $CD25^{+}CD69^{+}$ frequency, between genotypes or CNS compartments (Figure 2-3D). There were slightly more myelin-specific T cells in the brain vs the spinal cord of both WT and IFNγRKO recipients at preclinical time points (unpublished

data). Our laboratory has previously detected transferred T cells in the choroid plexus by immunofluorescent histology 4-5 days following adoptive transfer (2-3 days prior to standard disease onset) (unpublished data). This led us to hypothesize that encephalitogenic T cells initially infiltrate the CNS via the choroid plexus in both WT and IFNγRKO mice. IFNγ production by donor T cells doesn't occur until they are reactivated in the presence of cognate antigen presented by a CNS-resident antigen presenting cell. Therefore, we would not expect there to be a divergence in the evolution of cEAE and aEAE until after the until migration of T cells to the CNS. The choroid plexus has been identified as a key entry point for CD4 and CD8 T cells into the CNS during immune surveillance (3,4). A common portal of initial entry of T cells into the CNS at the inception of cEAE and aEAE would also explain the predominance of myelin-specific T cells in the brain compartments prior to disease onset, since the choroid plexus is situated within the ventricles of the brain. Investigators have identified the choroid plexus and BCSFB as key early entry points in other models of EAE(5,6).

4b. α4 integrin is required for initial infiltration of Th1-polarized encephalitogenic T cells into the CNS prior to EAE onset

While the pattern of infiltration of myelin-specific CD4 T cells does not differ in WT and IFNγRKO mice, this is not the case as disease progresses. At the peak of EAE we observed significant increases in transferred CD4 T cell accumulation in the SC of WT mice compared to IFNγRKO mice (Figure 2-2D, Figure 2-3B). We sought to identify a mechanism by which IFNγ promoted encephalitogenic T cell accumulation in the spinal cord. It is known that interactions between α4β1 integrin on T cells and VCAM-1 on cerebrovascular endothelium are critical for T cell infiltration into the spinal cord during cEAE pathogenesis(7). In a toxoplasma model of neuroinflammation, IFNγ signaling was critical for upregulating VCAM-1 expression in the CNS endothelium and mediating T cell infiltration to sites of infection(8). In our adoptive transfer model, we found that VCAM-1 was only upregulated in the spinal cord and brainstem of WT but not IFNγRKO recipients over naïve controls (Figure 4-1A) while other markers of activated endothelium were elevated during both aEAE and cEAE.

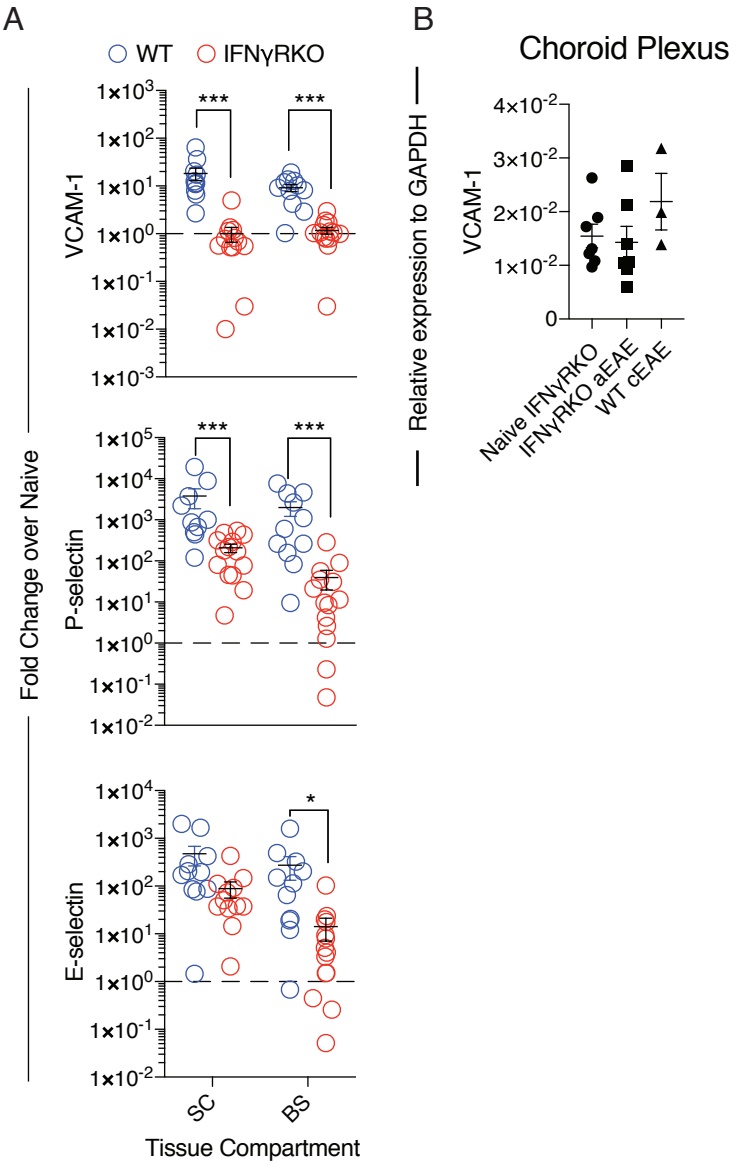

Figure 4-1:V-CAM-1 expression is not upregulated in the CNS of IFNγRKO mice (A) Expression of VCAM-1, P-selectin and E-selectin at the onset of cEAE or aEAE in whole CNS compartments normalized to endothelial marker (Tie2) and housekeeping gene (GAPDH). Shown has fold change over naïve control (dashed line). From two experiments WT: Naïve (n=3), cEAE (n=11) and IFNγRKO: naïve (n=3), aEAE n=14. (B) Expression of VCAM-1 relative to GAPDH in the choroid plexus IFNγRKO: Naïve (n=7), onset aEAE (n=7) and WT: onset cEAE (n=3) Statistical analysis was performed using an unpaired students T-test. * p>0.05, ** p>0.01, ***p>0.001

Blockade of α4 integrin is known to inhibit α4 VCAM-1 interactions that are required for the manifestation of cEAE. We speculated that aEAE in IFNγRKO mice would be independent of α4 integrin expression since VCAM-1 is not upregulated in CNS compartments during aEAE. Surprisingly, inhibition of α4 integrin signaling via administration of blocking antibodies following adoptive transfer completely inhibited aEAE as well as cEAE. T cell interactions with

endothelium in cEAE are thought to be α4β1 integrin mediated, however, α4 is also critical for α4β7-mediated migration of T cell subsets. Administration of anti-α4β7 integrin did not have any effect on cEAE incidence, however, aEAE incidence was partially inhibited (Table 4-1). Based on our findings with integrin blocking antibodies we hypothesized that cEAE required α4β1-VCAM-1 interactions while α4β7-MadCAM-1 interactions were more critical in aEAE due to the absence of VCAM-1 upregulation. However, antibody-mediated blockade of VCAM-1 significantly decreased cEAE and aEAE incidence while anti-MadCAM-1 had no effect on cEAE or aEAE incidence (Table 4-2). Our findings indicate that α4-mediated interactions with VCAM-1 are critical for encephalitogenic Th1 T cell infiltration into the CNS. Other labs have shown that Th17, but not Th1-polarized, encephalitogenic α4 KO T cells can induce EAE following adoptive transfer(9). Th17 cells migrate through the choroid plexus via a CCR6-dependent mechanism to induce EAE(6), indicating that α4-mediated pathways may be uniquely critical for EAE initiation in Th1 polarized cell subsets.

Table 4-1: cEAE incidence in WT and aEAE incidence in IFNγRKO micetreated with α4 integrin blocking antibodies (0.1mg/dose) or α4β7 blocking antibodies (0.5mg/mL) every other day starting on d0 post-T cell transfer Each row represents an individual adoptive trasnfer experiment. Anti-α4 treatment blocked cEAE and aEAE incidence entirely. A Mantel-Haenszel chi-squared with continuity corrections was used to compare incidence of aEAE in control antibody vs anti-α4β7 treatment groups. Anti-α4β7 significantly inhibited aEAE compared to control antibody: Common odds ratio of 3.9653 with $p<0.05$.

α4 blockade

Conventional Incidence in WT Recipients			
Rat IgG		Anti-α4	
# sick	# recip	# sick	# recip
5	5	0	5
4	5	0	5
9	**10**	**0**	**10**
90%		**0%**	

Atypical Incidence in IFNγRKO Recipients			
Rat IgG		Anti-α4	
# sick	# recip	# sick	# recip
4	5	0	6
5	5	0	5
9	**10**	**0**	**11**
90%		**0%**	

α4β7 blockade

WT Conventional Incidence			
Rat IgG2a		Anti-α4β7	
# sick	# recip	# sick	# recip
3	4	4	5
4	4	3	3
7	**8**	**7**	**8**
88%		**88%**	

IFNγRKO Atypical Incidence			
Rat IgG2a		Anti-α4β7	
# sick	# recip	# sick	# recip
3	5	2	4
3	5	2	5
2	6	1	6
5	10	1	10
3	8	0	8
2	6	1	6
18	**40**	**7**	**39**
45%		**18%**	

*

Previous work has shown that VCAM-1 is expressed on the choroid plexus in naïve mice and is upregulated following active immunization(5). However, the administration of CFA and

pertussis toxin alone can upregulate adhesion molecule expression (10) and its expression in adoptive transfer models has not been examined. Our transcript analysis of choroid plexus samples showed VCAM-1 is expressed by the choroid plexus in naïve mice and is not significantly upregulated at the onset of cEAE in WT mice or aEAE in IFNγRO mice (Figure 4-1B). We believe that constitutive expression of VCAM-1 in the choroid plexus during homeostasis is sufficient to promote the initial infiltration of Th1-polarized encephalitogenic T cells via α4β1 or α4β7-dependent mechanisms. In order to directly assess the requirements of VCAM-1 expression on the choroid plexus epithelium during EAE conditional KO (Lymphotrophic papovavirus control region (LPV)-Cre(11)xVCAM-$1^{fl/fl}$) may be useful.

Table 4-2: cEAE incidence in WT and aEAE incidence in IFNγRKO mice treated with anti-VCAM-1 (0.2mg/dose) or anti-MadCAM-1 (0.5mg.dose) blocking antibodies every other day starting on d0 post-T cell transfer Each row represents an individual adoptive transfer experiment. A Mantel-Haenszel chi-squared test with continuity corrections was used to compare incidence of EAE in control antibody vs experimental treatment. VCAM-1 significantly decreased cEAE incidence: common odds ratio of 16 with $p<0.01$, and aEAE incidence: common odds ratio of 10.8 with $p<0.05$. Anti-MadCAM-1 treatment did not significantly decrease EAE incidence in WT or IFNγRKO recipients.

VCAM-1 blockade

WT Conventional Incidence			
Rat IgG2b		Anti-VCAM-1	
# Sick	# injected	# Sick	# injected
4	4	0	4
2	5	1	5
5	5	0	5
5	5	2	5
16	19	3	19
84%		16%	

**

IFNγRKO Atypical Incidence			
Rat IgG2b		Anti-VCAM-1	
# Sick	# Recip	# Sick	# Recip
4	13	0	13
2	11	1	11
3	6	0	6
9	30	1	30
30%		3%	

*

MadCAM-1 blockade

WT Conventional Incidence			
Rat IgG2a		Anti-MadCAM	
# sick	# recip	# sick	# recip
3	4	4	5
4	4	2	3
7	**8**	**6**	**8**
88%		**75%**	

IFNγRKO Atypical Incidence			
Rat IgG2a		Anti-MadCAM	
# sick	# recip	# sick	# recip
3	5	2	4
3	5	2	4
6	**10**	**4**	**8**
60%		**50%**	

If VCAM-1 expression is required for the initial infiltration of Th1-polarized donor T cells via the choroid plexus during both cEAE and aEAE, we must alter our experimental

approach to assess the requirement for VCAM-1 upregulation specifically in the spinal cord endothelium in cEAE progression. We inhibited α4 integrin or VCAM-1 signaling by administering blocking antibodies starting the same day as the adoptive transfer itself. This likely blocked the initial infiltration of T cells via the choroid plexus. To interrogate the importance of VCAM-1 α4 integrin interactions following initial infiltration of T cells we could initiate anti-α4 or anti-VCAM-1 treatments at the clinical onset of cEAE or aEAE. We anticipate that administration at this time point would block the progression of cEAE but not aEAE. Additionally, adoptive transfers into mice with genetic ablation of VCAM-1 expression restricted to endothelium (human von Willebrand factor (hVWF)-Cre(11)xVCAM-$1^{fl/fl)}$) could be used to distinguish the requirement of VCAM-1 expression by the BBB endothelial cells vs choroid plexus epithelial cells.

4c. IFNγ promotes spinal cord inflammation by upregulating chemokines and adhesion molecules required for monocyte infiltration in the spinal cord

We observed significant accumulation of monocytes in the spinal cord at the peak of cEAE but not IFNγRKO mice at the peak of aEAE (Figure2-2C). Previous studies by our lab and others identified Ly6C^{hi} monocytes as critical drivers of cEAE(12,13). Examination of chemokine levels in the spinal cord and brainstem showed that WT mice had significantly higher levels of CCR2-binding chemokines CCL2 and CCL5 as well as CXCR3-binding chemokines CXCL9 and CXCL10 (Figure 2-4). Both receptors are known to be expressed by monocytes(14,15). IFNγ signaling can upregulate CXCL9 and CXCL10 levels in the CNS(16) and induce CCL2 production by microglia and astrocytes(17,18). Other studies from our lab showed that deficiencies in CXCR3 or CXCL10 were dispensable for fulminant cEAE induced by Th1-polarized myelin specific T cell adoptive transfers(19). In my own experiments, CCR2 KO recipients of Th1-polarized myelin specific T cell transfers had a lower incidence of cEAE compared to WT controls (Figure 2-5C). Some mice still had mild cEAE, indicating that CCR2-dependent infiltration was critical for severe disease at peak but not induction of cEAE. Our findings are consistent with those of Fife *et al*. (20). Subsequent studies demonstrated suppressed monocyte and CNS homing in CCR2 deficient mice compared to WT controls following active immunization(13). While our findings contribute to the idea that CCR2 expression on monocytes

is critical for their infiltration in cEAE this has not been proven definitively. In order to assess the absolute requirement of CCR2 signaling in monocyte infiltration of the SC adoptive transfers into mixed BM chimeras containing both $CCR2^{+/+}$ and $CCR2^{-/-}$ BM could be utilized to examine how deficiency in CCR2 affects monocyte recruitment to the SC during EAE.

Few studies have focused on the potential role for VCAM-1 in monocyte CNS infiltration. Monocytes are known to utilize α4β1 integrin for infiltration into inflamed tissue(14). It is now well established that anti-α4 and anti-VCAM-1 treatment inhibits cEAE; the importance of α4 VCAM-1 interactions in monocyte infiltration into the SC during cEAE could be examined via adoptive transfers into mixed BM chimeras reconstituted with α4 integrin sufficient and deficient hematopoietic stem cells. We would predict that α4 integrin sufficient monocytes would have a competitive advantage over their α4 deficient counterparts for accumulation in the spinal cord.

Taken together our data identify IFNγ as a critical regulator of multiple factors that promote monocyte and myelin-specific T cell infiltration into the SC and cEAE pathogenesis. The direct cellular target(s) of IFNγ are what remains to be identified. Conditional KO of the IFNγR would allow us to target individual CNS resident cell types astrocytes (GFAP-Cre), endothelial cells of the BBB (VWF-cre) and choroid plexus epithelium (LPV-Cre), to determine where responses to IFNγ are critical for promoting spinal cord inflammation in cEAE.

4d. CXCR2 dependent neutrophil accumulation in the brainstem is critical for aEAE pathogenesis

In the absence of IFNγR signaling we observed a distinct pattern of CNS infiltration at the peak of EAE characterized by neutrophil accumulation in the brainstem (Figure 2-2a). This correlated with higher levels of CXCL2 in the brainstem at the onset of aEAE when compared to cEAE (Figure 2-4). Blockade of CXCR2 specifically inhibited aEAE pathogenesis and had no effect on cEAE in our model. In parallel to our own studies, Simmons *et al*. showed that CXCR2 antagonists block clinical disease in an IL-17-dependent model of aEAE(21). Additionally, in a LysM-Cre $SOC3^{fl/fl}$ mouse model of atypical EAE, administration of a Ly6G-blocking antibody decreased the incidence and severity of aEAE(22). Hence infiltration of the brain by neutrophils

in a CXCR2-dependent manner is critical for multiple models of aEAE. We sought to identify the specific factors that promote CXCL2 production in the BS during aEAE and mechanisms by which IFNγ could suppress CXCL2 production and hence neutrophil accumulation in the brainstem and aEAE.

4e. CXCL1 and CXCL2 are produced by distinct cell subsets and are differentially regulated by IFNγ during EAE

Next we sought to identify the cellular sources of CXCR2-binding in adoptive transfer recipients. Immunohistology at the onset of EAE identified CNS-resident cell types such as choroid plexus epithelial cells and astrocytes as $CXCL1^+$, while $CD45^+CD11b^+$ cell subsets were $CXCL2^+$ (Figure 3-5). CXCL2 transcript and protein levels were elevated in $CD45^+CD11b^+$ subsets within the brainstem parenchyma (Figure 3-4). Models of neutrophil recruitment to sites of inflammation differ on the requirement for CXCL1 versus CXCL2 signaling. Blockade of CXCL1 or CXCL2 inhibited neutrophil extravasation into peritoneal fluid following LPS injection(23), while neutrophil infiltration into tumor draining lymph nodes only requires CXCL2(24). The differential pattern of CXCL1 and CXCL2 expression in aEAE may signify distinct roles for CXCL1 and CXCL2 in promoting neutrophil migration out of circulation and migration into CNS parenchyma.

4f. IFNγ suppresses monocyte and microglial production of neutrophil chemoattractant CXCL2

CXCL2 is elevated in the brainstem at the onset of aEAE compared to cEAE. Protein and mRNA analysis of brainstem cell subsets at the onset of aEAE identified myeloid cells as predominant producers of CXCL2. Further examination of purified microglial and monocyte populations from WT and IFNγRKO recipients at the onset of EAE showed that CXCL2 transcript levels were elevated in IFNγRKO microglia and monoctyes (Figure 3-4B). We also found that CXCL2 expression was directly attenuated by IFNγ signaling specifically in the CNS during EAE. IFNγRKO monocyte/macrophage populations isolated from the CNS at the onset of EAE in

WT/IFNγRKO mixed BM chimeric mice had significantly higher levels of CXCL2 transcript compared to WT counterparts (Figure 3-4). In contrast, there was no difference in CXCL2 expression comparing WT and IFNγRKO cells derived from the BM in the mixed BM chimeras. In other model systems, stimulation of TLR4 on monocytes, macrophages and microglia induces CXCL2 expression (25,26). *In vitro* experiments showed that IFNγ inhibits CXCL2 protein production by BM monocytes following LPS stimulation (Figure 3-6C). Taken together, our data shows that IFNγ directly regulates CXCL2 production by monocytes and microglia, however the factors that induce CXCL2 production in each cell type during aEAE is not known.

There are multiple potential stimuli of CXCL2 production from resident microglia and macrophages at the onset of aEAE. Infiltrating T cells produce TNFα and GM-CSF following reactivation. This can induce IL-6 and IL-1β production from CNS support cells and infiltrating immune cells. All of these factors are present in the brainstem at the onset of aEAE (Data not shown) and can contribute to microglial/macrophage activation and induce neutrophil chemoattractant production(27) (28). Co-cultures of CNS microglia/macrophages with MOG-specific T cells and MOG peptide, or in the presence of candidate pro-inflammatory factors can be used to identify whether these stimuli are sufficient to induce CXCL2 production. Additionally, we have identified TLR activation as a potential mechanism for CXCL2 production by monocytes, however the specific stimulus that induces its production in aEAE is not known. Following via TLR engagement by danger associated molecular patterns released following CNS damage microglia and CNS-resident macrophages can be activated (29). While we have shown stimulation of TLR4 promotes CXCL2 production by bone marrow monocytes *in vitro*, we haven't examined potential danger signals that may be present in the brainstem during aEAE and their ability to stimulate CXCL2 by microglial and macrophage populations isolated from the CNS. Analysis of danger signals present in the brainstem at the onset of aEAE particularly those that bind TLR4, such as High mobility group box-1 (HMGB-1) and Heat Shock Protein (HSP-60 and 70), and subsequent stimulation of microglial, macrophage and monocyte cultures may identify a particular stimulus of CXCL2 production in response to early CNS damage in aEAE. Myd88$^{-/-}$ and TLR2$^{-/-}$ hosts are resistant to adoptive transfer cEAE however TLR4$^{-/-}$ hosts are not (30,31) indicating that multiple pathways of DAMP responses may be critical for induction of EAE in different model systems. Inquiry into the TLR and

responses critical for aEAE may allow us to identify distinct DAMP signals that promote cEAE and aEAE.

4g. IFNγ dampens response to and production of CXCL2 by neutrophils in the CNS

We believe the initial production of CXCL2 by microglia and early infiltrating myeloid cells is critical for the initiation of aEAE, however, subsequent activation of a CXCL2 response and production loop in neutrophils is critical for aEAE progression. At the onset of EAE we found that CXCL2 transcript levels were highest in neutrophil populations sorted out of the CNS (Figure 3-4A). Flow cytometric analysis of intracellular CXCL2 identified neutrophils as a major source of CXCL2 in the brainstem during aEAE (Figure 3-3C). CXCL2 transcript expression in neutrophils isolated from the brainstem correlated with scores at aEAE onset (Figure 3-3B). This increased CXCL2 transcript production correlates with increased neutrophil accumulation in the brainstem during aEAE (Figure 4-2). This observation led us to hypothesize that neutrophil accumulation drives increased CXCL2 production in an autocrine/paracrine loop of CXCL2 response by neutrophils stimulating CXCL2 production. We found that CXCL2 production by neutrophils was upregulated by incubation with either CXCL2 or CXCL1 alone. In the presence of IFNγ, CXCR2-dependent upregulation of CXCL2 transcript was significantly inhibited, however G-CSF-dependent upregulation of CXCL2 was not. Indicating that IFNγ may be directly affecting CXCL2 production in response to CXCR2 binding chemokines (Figure 3-6A and B). Analysis of neutrophils isolated from mixed WT/IFNγRKO BM chimeras at the onset of EAE showed that IFNγ directly inhibits neutrophil production of CXCR2 (Figure 3-2) and CXCL2 (Figure 3-4D) in the CNS but not in the periphery.

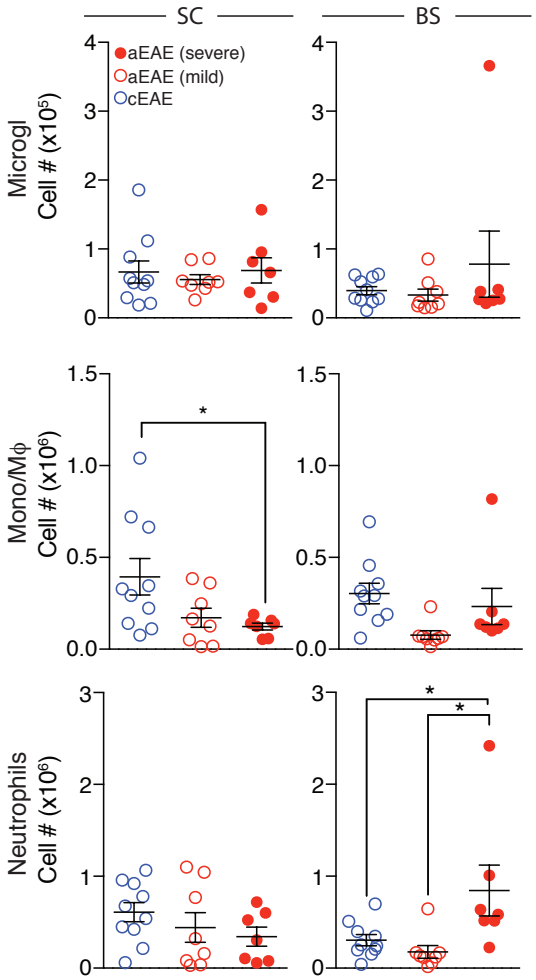

Figure 4-2: Microglia ($CD45^{lo}CD11b^{+}Ly6G^{-}$), monocyte ($CD45^{hi}CD11b^{+}Ly6G^{-}$), and neutrophil ($CD45^{+}CD11b^{+}Ly6G^{+}$) cell numbers from the spinal cord and brainstem of mice at the onset of EAE in WT and IFNγRKO mice. cEAE (n=10), mild onset of aEAE (score=1) (n=8) and severe onset of aEAE (score≥2) (n=7). Statistical analysis was performed using an unpaired students T-test. * p>0.05, ** p>0.01, ***p>0.001

CXCR2 dependent production of CXCL2 by neutrophils has been demonstrated in other models of inflammation(32), but the role of IFNγ in regulating that pathway has not been studied. Our data demonstrate that IFNγ signaling in neutrophils directly suppresses CXCR2-dependent CXCL2 production, however the exact pathway that IFNγ is acting on is unknown. *In vivo* evidence showed that IFNγ can directly downregulate CXCR2 expression. *In vitro* evidence points to an additional rapid mechanism of IFNγ-mediated suppression of CXCL2 signaling, since addition of IFNγ to cultures of neutrophils with CXCL2 is sufficient to suppress

upregulation of CXCL2 (Figure 3-6A and B) after a 1 hour incubation period in response to CXCR2 stimulation without affecting CXCR2 transcript levels (data not shown)

There are multiple pathways downstream of G-protein coupled receptor (GPCR) signaling that IFNγ could be acting on to suppress CXCR2 signaling. One rapid pathway of GPCR downregulation is heterologous desensitization, where activation of secondary messengers downstream of one receptor can lead to the phosphorylation, and subsequent inactivation, of another. Since this process only requires second messenger interactions the effect can often be seen within 30 minutes(33). One major heterologous desensitization pathway works through cAMP-PKA mediated phosphorylation of GPCRs(34). IFNγ can induce cAMP PKA (35) and could inhibit CXCR2-meditated upregulation of CXCL2 transcript through heterologous desensitization in addition to direct suppression of CXCR2 transcript expression.

If IFNγ doesn't suppress CXCL2 mRNA induction via heterologous desensitization, further elucidation of the exact pathway by which CXCR2 signaling induces CXCL2 expression may help to identify the target. Using the inhibitors listed in Figure 4-3 we can interrogate which pathways downstream of CXCR2 ligation are critical for CXCL2 production. If we are able to identify a specific pathway required for CXCL2 production, we can then identify potential mechanisms by which IFNγ signaling could inhibit CXCR2 dependent second messenger signals of CXCL2 production.

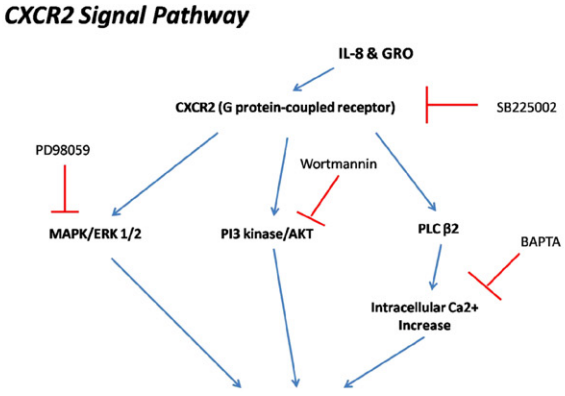

Figure 4-3: CXCR2 signaling pathway with inhibitors of each step in the pathway indicated with red block arrows(36).

IFNγ may also regulate the access of CXCR2-dependent transcription factors to CXCL2 promoter regions via epigenetic modifications. A recent study demonstrated epigenetic suppression of LPS-induced production of IL-6, IL-1β and CXCL1 in BM macrophages

mediated by IFNγ STAT-1 dependent pathways(37). Further examination of transcription factor binding and methylation states of CXCL2 and CXCR2 promoter regions in WT and IFNγRKO neutrophils may identify novel epigenic mechanism of IFNγ mediated regulation of the neutrophil CXCL2 autocrine/paracrine positive feedback loop.

Taking all of our data together on CXCR2-binding chemokines in aEAE, we propose that CXCL1 production by the choroid plexus in response to master inflammatory cytokines such as IL-6 and TNF-α, released following encephalitogenic T cell activation in the CNS promotes the initial recruitment of neutrophils through the BCSFB. However, in the absence of subsequent CXCL2 responses that are suppressed by IFNγ, parenchymal infiltration of the brainstem by neutrophils and subsequent aEAE pathogenesis are inhibited. Antibody blockade of CXCL1 or CXCL2 individually in our Th1-polarized adoptive transfer into IFNγRKO mice would elucidate the absolute requirement of CXCL1 and CXCL2 for aEAE. If both chemokines are important for aEAE pathogenesis, subsequent experiments focusing on conditional deletions of CXCL1 in different CNS resident cell subsets and CXCL2 in different myeloid cell subsets can help us identify what cells are critical sources of these chemokines during aEAE.

4h. Examining mechanisms of neutrophil damage to CNS parenchyma

The CXCL2-CXCR2 axis of neutrophil recruitment is critical for aEAE pathogenesis but the mechanisms by which neutrophil infiltration causes damage to the brainstem are unknown. Activated neutrophils produce matrix metalloproteases (MMPs) which can promote inflammation and digest extracellular matrix proteins to promote breakdown the tight junctional barriers in the CNS(38). In human neutrophils, CXCR2 engagement triggered MMP-9 release from tertiary granules(39). MMP-9, induced downstream of CXCR2 signaling, promotes neutrophil migration into uroepithelium in a bacterial infection model(40). MMP-2 and MMP-9 have been shown to mediate β-dystroglycan digestion around astrocyte endfeet and subsequent immune cell infiltration into the spinal cord in EAE(41), but their role in neutrophil migration into CNS parenchyma has not been studied. Using adoptive transfers of encephalitogenic

IFNγKO CD4 T cells into MMP-9 and/or MMP2 KO mice we can examine whether MMPs play a role in aEAE.

4i. Examining the heterogeneity of CNS autoimmunity through analysis and characterization of immune cell recruitment profiles

This project characterizes two distinct pathways of immune cell infiltration into the CNS which are modulated through distinct chemokine pathways and which recruit variable cell types to the CNS. Direct correlation between lesion localization in rodent models and MS patients is unlikely due to significant differences in CNS physiology, however animal models can still be used to study the diverse pathways by which immune cells infiltrate the CNS. Current MS therapeutics target a restricted panel of immune related molecules with variable results for individual patients. Most of these therapies have been developed with the assumption that the vast majority of MS pathophysiology is driven by infiltration of T cell, B cell and monocyte/macrophage subsets, however expansion in our understanding of the complexity of MS progression has generated new potential targets for disease therapy.

The role of neutrophils in CNS autoimmunity has gained a lot of traction recently, however therapies targeting them are not widely utilized. In addition to clinical studies showing the administration of G-CSF induced relapses in MS patients(42-45), elevated levels of neutrophil chemoattractants have been identified in progressive MS patients(46) as well as those with opticospinal form of MS(47). Neutrophils are also predominant in lesions of NMO patients who also have a predominantly spinal cord and optic-nerve focused disease(48). The increased presence of neutrophils NMO and progressive forms of MS may account for their non-responsiveness to α4 integrin-blocking therapies. The effectiveness of α4 integrin in opticospinal forms of MS has not been examined. While some studies have identified correlates between lesion distribution and cytokine/chemokine levels in patient serum and CSF(47), the detailed analysis of these factors and immune cell subset characterization in MS patients has not been well described. The heterogeneity of migratory pathways utilized in immune cell infiltration into the CNS has been well described in our model and throughout the literature, however detailed analysis of patient blood and CSF for cytokine, chemokine and immune cell subset characterization has not yet been performed. To increase the efficiency of disease modifying therapy selection and better understand how these drugs are working it is imperative that studies

are undertaken to develop profiles of patient immune responses and identify markers of responders and non-responders to specific therapies. This work could inform diagnosis and treatment, and also identify profiles of disease that do not respond to current therapies which informs potential cell and molecular targets to pursue in the future.

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