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IMPLICATING CXCL10 and CXCL13 SIGNALING IN T CELL TRAFFICKING TO THE CNS DURING POLYOMAVIRUS INFECTION

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ABSTRACT

Polyomaviruses (PyV) are normally asymptomatic members of the virome of most vertebrate species (including humans). However, some can cause debilitating diseases in immune compromised individuals. The human JCPyV is the causative agent of several high-mortality CNS diseases, of which progressive multifocal leukoencephalopathy has the highest incidence. To control virus infection, CD8 T cells infiltrate the brain, and their cytolytic function along with virus infection causes demyelination and damage to the white matter. The mechanism of CD8 T cell trafficking to the CNS during PyV is unclear, and chemokine/receptor expression varies between tissues and viruses. The overall goal of this work was to identify the chemokines expressed in the brain following PyV infection and their function in recruiting CD8 T cells to the brain using MuPyV. When mice are inoculated with MuPyV intracranially, they develop a PyV-induced encephalitis complete with T cell infiltration and demyelination of the white matter tracts. Using this model, I found CXCL10 and CXCL13 to be expressed as early as 4 d.p.i and peaking at 7 d.p.i. CXCL10 appeared to be expressed by the choroid plexus and subventricular astrocytes, and CXCL13 was expressed by the ependymal lining of the ventricles and the subventricular astrocytes or microglia. A large portion of the CD8 T cells within the brain expressed either CXCR3 or CXCR5, with CXCR5 being favored, and CD8 T cells can be seen surrounding the ventricles and chemokine-expressing cells following MuPyV infection. Both CXCL10 and CXCL13 can be induced by type I IFN signaling, thus implicating this pathway as an important step in the recruitment of CD8 T cells to the site of infection in the brain. Loss of STAT1, a key transcription factor in IFN signaling cascade, has been shown to result in increased hydrocephalus following MuPyV infection. Using global STAT1 knockout mice, I showed that STAT1 is required for full expression of both CXCL10 and CXCL13, suggesting that IFN signaling is required for chemokine expression as well as CD8 T cell recruitment to the brain.

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Chapter 1

Introduction

1.1 An Overview of Polyomaviruses

Polyomaviruses are non-enveloped viruses with double-stranded DNA genomes that can infect a variety of hosts. Of the currently identified Polyomaviruses, there are 14 different viruses that infect humans[1]. Based on seropositivity studies, it is estimated that most humans become infected with these viruses during childhood, which persist as lifelong asymptomatic infections. Infection with multiple human polyomavirus (HPyV) is likely as infection with one HPyV does not prevent subsequent infection by others. Each subject from the seropositivity study was positive for at least 4 of the HPyVs, and on average, were seropositive for about 9 HPyVs [1]. There was also no difference in seropositivity between sexes. In general, the HPyVs cause asymptomatic infections, however, in immunocompromised patients, several have been associated with severe disease. For example, the deadly disease Progressive Multifocal Leukoencephalopathy (PML) is associated with Human Polyomavirus 2 (HPyV2 or JCPyV).

1.2 JCPyV Can Lead to PML

Progressive multifocal leukoencephalopathy (PML) is a rare, but aggressive, brain disease that results in inflammation and progressive damage to the white matter. It was first described in 1958 but wasn't correlated with a virus until 1965 [2,3]. PML is caused by JC Polyomavirus (JCPyV), a 5.1 kilobase (kb) DNA virus that results in an asymptomatic kidney infection in healthy individuals but can cause several diseases of the central nervous system

(CNS) in those who are immunocompromised. Within its genome, JCPyV encodes early and late mRNAs that translate 5 individual proteins [4]. When JCPyV enters the cell, the early transcripts, encoding the T antigens, are expressed, activating host cell pathways to replicate viral DNA. For example, the virus-encoded large tumor (LT) antigen protein can sequester Rb as well as bind p53 to promote progression into S phase of the cell cycle. The late transcripts are expressed after virus replication and encode the capsid proteins: VP1, VP2, and VP3, as well as the agnoprotein[5]. Expression of these late genes indicates productive infection within the cell. The genome also includes a cis-regulatory non-coding control region (NCCR) that drives DNA replication and transcription of the viral genome within the renal, tubular, and urothelial cells in the kidney [6,7]. The mechanism behind the tropism change of JCPyV from the kidney to the CNS is unclear, but when in the brain, astrocytes are the main cell type infected by the virus. Other cell types such as: cerebellar granule neurons, cortical astrocytes, and cortical neurons have been described as having productive infection as well [8–11]. JCPyV isolates from the brain and CSF of PML patients usually vary from the wild type virus due to mutations, deletions, or rearrangements in the NCCR and/or point mutations in VP1[12–16].

With only 230 cases reported before 1984, PML was quite rare, but its prevalence in the population increased with the emergence of the HIV/AIDS epidemic, affecting 5% of AIDS patients and becoming more commonly known as an AIDS-defining illness [17,18]. Co-incidence with AIDS patients began to subside with the use of HAART therapies[19], however, PML has recently become an increasingly common life-threatening complication of immunomodulatory therapies [18,20]. The humanized α4 integrin monoclonal antibody, natalizumab, is the best characterized of these PML-associated therapies. It is used to treat multiple sclerosis (MS) patients by binding to α4 integrin-expressing leukocytes and blocking entry into the CNS [21]. PML was first described in individuals being treated with natalizumab in patients with MS and Crohn's Disease [22–24]. It is estimated that 2 out of every 1,000 patients undergoing natalizumab therapy for

more than 12 months will develop PML. This number increases to 11 out of 1000 if the patient was already on immunosuppressive medications before starting natalizumab and is JCPyV seropositive [25]. While natalizumab is the best characterized drug, the list of immune modulatory drugs associated with PML is continuously growing, including drugs like rituximab and efalizumab [26]. There are currently no antiviral agents targeting JCPyV and no effective treatments for PML. Treatment by halting immune modulatory drugs can result in immune reconstitution inflammatory syndrome (IRIS), which is difficult to distinguish from progression of PML as it causes severe inflammatory and demyelinating lesions with increased infiltration of macrophages and CD8 T cells [27]. This increased infiltration of immune cells could involve dysregulation of chemokine expression disrupts recruitment of macrophages and T cells to the site of neuroinflammation. Clearly, a delicately balanced immune response is crucial to effectively combat PML, making it imperative that we gain an understanding of PML pathogenesis and the immune response to JCPyV.

1.3 MuPyV as a Model to Understand PML Pathogenesis

Studying JCPyV pathogenesis is difficult due to the species-specificity of polyomaviruses and a lack of in vivo models. Our group has developed a MuPyV-CNS model to overcome this hurdle. MuPyV was first identified in the 1950's with a high prevalence in wild mice [28–31]. Both MuPyV and JCPyV cause asymptomatic infections in the host reservoir and have a ~5-kb covalently closed dsDNA genome containing early and late genes that encode T antigens and the capsid proteins, respectively [32–34]. These DNA genomes and organization of the nonstructural and structural protein encoding genes of JCPyV and MuPyV are illustrated in Figure 1. Both viruses induce similar immune responses, including the production of VP1-specific IgG antibodies, which can be measured in the serum long after initial infection [35]. The T cell response is also

crucial for controlling both virus replication and spread [36]. During altered immune status, these viruses can cause disease of the CNS. Mice infected intracerebrally (i.c.) with MuPyV recapitulate the early events of JCPyV pathogenesis and develop several key histopathological features of JCPyV-PML in the context of immunocompetence [37,38,39(p8)]. As other mouse models cannot fully recapitulate human disease, this MuPyV mouse model of CNS disease cannot reproduce all aspects of JCPyV and PML pathogenesis. However, this MuPyV model will further enable us to investigate JCPyV pathogenesis and host immune mechanisms involved in controlling persistent viral encephalitis.

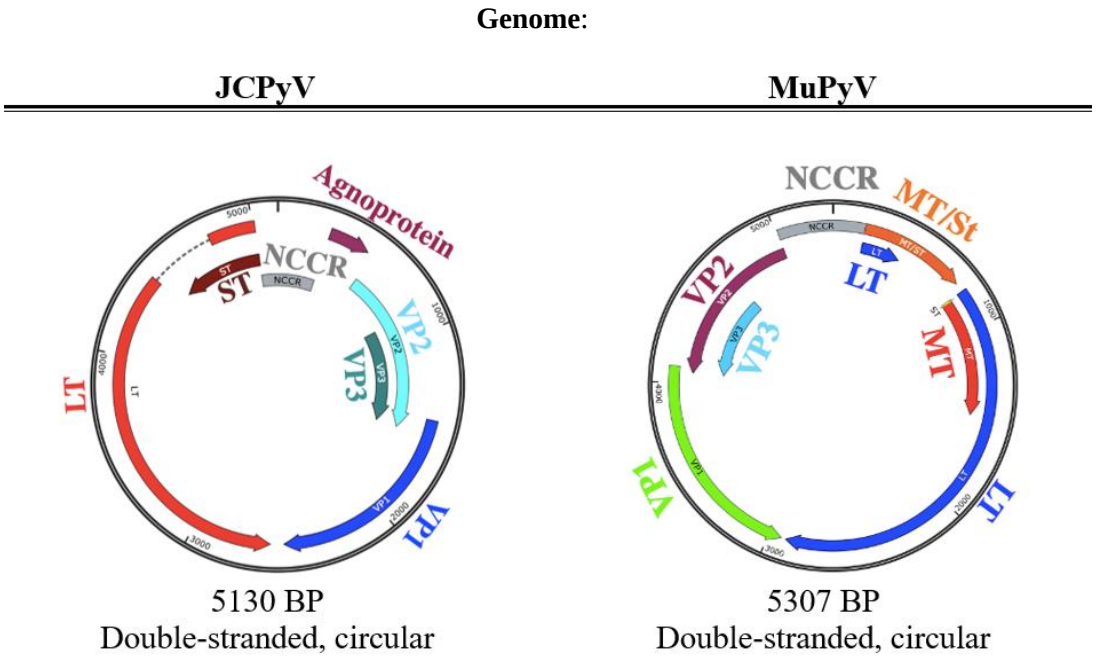

Figure 1-1: JCPyV vs MuPyV Genome. Representations of the circular genomes of both JCPyV and MuPyV for comparison. Early genes including LT (and additional middle T (MT) for MuPyV) and small T (ST) are shown. Both genomes contain an NCCR for viral origin replication and late genes include capsid genes VP1, VP2, and VP3. JCPyV encodes an additional nonstructural agnoprotein.

1.4 CD8 T Cells Control PyV in the Brain

Once activated via their TCR and co-stimulatory receptors, CD8 T cells produce cytokines, such as IFN-γ, TNF-α, and IL-2, and/or can induce apoptosis of the infected cell by releasing cytotoxic granules or expressing FasL [40]. Currently, it is unclear which of these effector activities CD8 T cells use for controlling JCPyV infection due to limitations in human studies. However, inferences can be made based on studies of PML patients. In a study using HLA-A*0201-VP1p100 or -VP1p36 tetramers and ^{51}Cr release assays with overlapping peptide pools of the VP1 protein to identify cytotoxic JCPyV-specific CD8 T cells, or IFN-γ-production after stimulation with VP1 peptide pools, it was found that PML patients with a higher number of JCPyV-specific CD8 T cells in their blood are more likely to survive the disease [41,42]. In another study observing lesions in the brains of PML patients, CD8 T cells were localized surrounding these lesions [43]. Some studies show CD8 T cells in the brain differing from peripheral blood CD8 T cells phenotypically and functionally, with brain CD8 T cell subsets that are CD103−CD69+ and CD103+CD69+ T cells having a phenotypic and transcription factor profile consistent with resident memory T cells [44]. The CD103 expression on T cells in the brain correlates with reduced expression of differentiation markers, increased expression of tissue-homing chemokine receptors, intermediate and low expression of the transcription factors T-box expressed in T-cells (T-bet) and Eomesodermin (Eomes), increased expression of PD-1 and CTLA-4, and low expression of cytolytic enzymes with preserved polyfunctionality upon activation [44]. It is likely that JCPyV-specific brain CD8 T cells are also capable of cytotoxicity and IFN-γ-production, and IFN-γ has been shown to inhibit JCPyV replication in glial cells [45]. This supports the idea that CD8 T cells in the brain control JCPyV using IFN-γ production.

This idea of CD8 T cells controlling PyV infection in the brain is also supported by studies of mice infected i.c. with MuPyV. Immunohistochemistry and in situ hybridization using

T antigen- and VP1-specific probes have identified VP1+ ependymal cells lining the ventricles and CD8 T cells infiltrating the periventricular regions [46]. CD8 T cells infiltrate the brain at 6-8 d.p.i., and studies with DbLT359, an MHC-I tetramer made from four MHC molecules, associated with an LT peptide and bound to a fluorochrome, have found that over 60% of these CD8 T cells are virus specific [47]. Furthermore, depleting CD8 T cells prior to infection, showed a significant decrease in brain-infiltrating CD8 T cells and an increase in viral load in both the brain and spinal cord [39,46]. Most of the brain CD8 T cells have the capacity to produce IFN-γ, but only 5-10% of the brain CD8 T cells are granzyme B+ at any given time throughout infection [48]. These data support IFN-γ as a means by which CD8 T cells control PyV in the brain.

1.5 Chemokines in the CNS

Chemokines are chemotactic cytokines that function by directing cells to a particular tissue or location via gradients. Migratory cells, such as leukocytes, use these gradients, as well as fine-tuned expression of the corresponding receptors, to navigate around the body. Once in the tissue, chemokines can direct interactions between cells, such as antigen-presenting cells and specific T cells. Within the large family of chemokines, some are characterized as "inflammatory", being induced by the inflamed tissue environment, and others are considered "homeostatic", being constitutively expressed within lymphoid sites, such as the spleen and lymph nodes [49]. Of the organs, the brain was once thought to be an immune privileged site. We now know that to maintain tissue homeostasis and control infections in the brain, the immune system continuously surveys the CNS [50]. The ventricular cerebrospinal fluid (CSF) and choroid plexus (CP) are important sites for this immune surveillance, suggested by the increased intensity of immune responses proximal to the ventricles [51]. During infection or inflammation, microglia and perivascular macrophages function to protect the CNS. Microglia become activated, resulting

in an innate immune response and the secretion of inflammatory cytokines as well as antigen presentation, leading to activation of the adaptive immune response [52]. Many studies have found microglia and other CNS resident cells, including astrocytes, oligodendrocytes, neurons, neuroepithelium, CP, ependyma, and meninges capable of secreting chemokines to recruit T cells and other immune cells to confer protection during viral infection [53(p),54–57]. Chemokines and their receptors are critical for immune cell recruitment to and from tissues or lymphoid sites and have been found to function in neurodevelopment, modulation of synaptic transmissions, cellular interactions, and activation of homeostatic signaling pathways [52]. Within the CNS, these chemokines can serve as immunologic checkpoints at barriers to prevent or promote circulating leukocyte infiltration into the brain parenchyma and ventricular/subarachnoid CSF spaces based on the environmental context. Many of the chemokines are expressed by the vasculature of the BBB, such as CXCL12, CXCL19, CXCL20, and CXCL21[54,56], and some are expressed by the CP epithelium[51,54,55], suggesting both the BBB and the BCSFB serve as sites of leukocyte infiltration during inflammation. Involvement of chemokine receptors CCR7, CXCR3, CXCR4, and others in recruiting dendritic cells (DCs) and naïve T cells deep into the brain parenchyma during inflammation has been described [58]. It was also found that CCL20 and its receptor, CCR6, are at least partially responsible for trafficking Th17 cells across the CP during both surveillance and neuroinflammation[54].

1.5.1 Chemokines in the Context of Pathogens

The infiltration of T cells into any tissue and their antiviral functions are required for viral clearance and host survival. However, a consequence of this infiltration and accumulation of leukocytes is potentially fatal neuropathology, commonly seen during persistent infections. For example, lymphocytic choriomeningitis virus (LCMV)-associated meningoencephalitis is caused

by an accumulation of virus-specific cytotoxic T cells that release cytotoxic factors and cause damage to brain-resident cells [59,60]. Therefore, a balance of expression and infiltration is required.

The chemokine expression profile and localization within the brain, as well as other tissues, during healthy and inflamed conditions is elaborate and incompletely understood. Expression differs between tissue type as well as the causes of neuroinflammation (i.e., virus infection). For the wide variety of neurotropic viruses, infection results in a signature chemokine expression pattern, with varying levels of chemokines upregulated and their cellular sources, but most converge at inflammatory cell infiltration. Studies investigating these differing patterns of expression between viruses show induction or upregulation of several chemokines, including CCL2, CCL3, CCL5, CXCL10, and CXCL12 [61–64]. Cellular sources of these chemokines can vary between virus infections. During LCMV, mouse hepatitis virus (JHMV), herpes simplex virus type 1 (HSV-1), and human immunodeficiency virus (HIV) infection, astrocytes and microglia are the source of the chemokines [57,61,65,66]. During West Nile virus (WNV) and HIV infection, neurons mainly produce chemokines [67(p10),68]. During simian immunodeficiency virus-induced encephalitis, endothelial cells are the main source of chemokines [69]. These variations even occur between strains of the same virus. For example, CXCL10 and CXCR3 are critical for T cell infiltration during LCMV (Taub) infection [70,71] but are dispensable for T cell infiltration and neuroinflammation during LCMV (Armstrong) infection [72]. Understanding the chemokine expression profiles and chemotactic mechanisms during one viral infection does not confer understanding of the mechanisms to another virus. The chemokines induced or upregulated during polyomavirus infection and their functions are unknown and represents the focus of my studies.

1.5.2 CXCL10 and CXCL13 Mediated Trafficking to the CNS

CXCL10, also known as IP-10, was initially identified in human U937 cells (a histiocytic lymphoma cell line), human placenta, and spleen as an IFN-γ inducible chemokine [73]. It has a small molecular weight of about 10 kDa and shares its receptor, CXCR3, with CXCL9 and CXCL11. It has been functionally described as an "inflammatory" chemokine and is transcriptionally regulated by signals induced by IFN-γ, IFNα/β, TNF-α, and LPS [74–77]. CXCL10 has been established as an important chemoattractant for recruitment of monocytes, macrophages, and activated T cells and NK cells to different tissues [78–80]. CXCL10 is produced by many cell types, including endothelial cells [81], fibroblasts [82], keratinocytes [83], mesenchymal cells [84], neutrophils [85], monocytes [86], dendritic cells [87], hepatocytes [88], and astrocytes [89]. Previous studies with neurotropic viruses have found CXCL10 to be involved in attracting activated, CXCR3-expressing T cells to the CNS during HSV-1, JHMV, and WNV infection [57,67,88,90,91]. Genetically silencing CXCL10 or CXCR3 results in a reduction of CD8 T cell infiltration into the CNS after infection with LCMV [70(p10),71]. CXCL10-expressing astrocytes and CXCR3-expressing T cells are also seen surrounding the demyelinating lesions in mouse hepatitis virus (MHV) infected spinal cords [57,88,92], and neutralization of the chemokine results in reduced T cell infiltration and survival [61,65].

CXCL13, also known as BCA-1, was initially identified as a potent B cell chemoattractant. It has a molecular weight of about 12 kDa and is mostly known for its function in secondary lymphoid organs. CXCL13, produced by the stromal and follicular dendritic cells, governs the migration and compartmentalization of CXCR5+ B cells and follicular helper T cells (T_{FH}) to the correct location to properly form follicles and germinal centers [93–96]. These germinal centers, located in the lymph nodes, spleen, and Peyer's patches, are where B cells mature and acquire higher affinity B cell receptors. CXCL13 has additionally been found in nonlymphoid

organs, recruiting cells to form ectopic lymphoid structures that resemble the GC in secondary lymphoid organs in the lung [97], the salivary glands of patients with Sjögren's disease [98], and gastric mucosa following *Helicobacter pylori* infection [99–101]. CXCL13 has also been found in the CNS during inflammatory diseases. For example, increased CXCL13 in the CSF and CNS occurs in patients with primary CNS lymphoma [102], Lyme Neuroborreliosis [103(p13),104(p13)], MS [105], and in the experimental autoimmune encephalomyelitis (EAE) mouse model [106]. The CXCL13/CXCR5 chemokine axis has also been found to recruit CXCR5+ CD4 T cells to the CSF during neuroinflammation [107]. The source of CXCL13 in the CNS is unclear as some studies identify CXCL13-expressing microglia while others identify CXCL13-expressing astrocytes or neurons [108–111]. Preliminary data from the Lukacher group has shown CXCR5 and CXCR3 to be expressed by MuPyV-specific T cells within the ventricular-subventricular zone (V-SVZ), the predominant site of MuPyV infection in the brain (Unpublished results). Previous work in the field and within the Lukacher lab suggest a role for the CXCR3:CXCL10 and/or CXCR5:CXCL13 axis in the control of persistent viral CNS infection via chemotaxis of CD8 T cells to the correct location in the brain during MuPyV, and potentially JCPyV, infection. However, the mechanisms of T cell trafficking and localization in the brain are unknown.

1.6 IFN Signaling and Induction of Chemokine Expression

Expression of both CXCL10 and CXCL13 is induced by Interferon (IFN) signaling. IFN signaling induces a range of antiviral activity by regulating both innate and adaptive immunity to create a host cell-intrinsic antiviral state [112–115]. There are 3 types of IFNs: type I, type II, and type III. The type I IFN family includes IFN-α, IFN-β, IFN-ω, IFN-ε, and IFN-κ, which all signal through the IFN α/β receptor (IFNAR) complex [116]. Cell types capable of both producing and responding to type I IFN include almost all brain resident neurons and glial cells [117]. The type II

IFN family consists only of IFN-γ, which signals through the IFN-γ receptor (IFN-γR) and is produced by T cells, NK cells, and NKT cells [118,119]. The type III IFN family includes IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4, and is produced only by cells of an epithelial or endothelial lineage, and signals through the heterodimeric IFNLR (IFNLR1 and IL10RB) [120,121]. Although each family consists of different individual cytokines and receptors, all members signal through Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathways, specifically signaling through STAT1. During the IFN signaling cascade, heterodimers of activated STAT1/STAT2 or STAT1 homodimers move to the nucleus to bind promoters of IFN-related genes that contain IFN-sensitive response elements and gamma-activated sequences [119]. These IFN-stimulated genes (ISGs) induce an antiviral state and potent defenses against viral infections. Differential distribution of the IFN receptors as well as JAK/STATs to different cell types results in tissue-specific immune programs and responses to varying pathogens. STAT1 is also used in cytokine pathways of the common γ chain and β chain receptor families [122].

STAT1 signaling has been found to be important for controlling acute and persistent MuPyV infection of the brain. Loss of STAT1 in mice results in hydrocephalus, suggesting that STAT1 signaling may engage antiviral defense mechanisms in the ependymal cells lining the ventricles [39]. Depletion of CD8 T cells in STAT1-deficient mice results in higher viral loads as well as early mortality, indicating that STAT1 and CD8 T cells work in concert to control PyV infection and potential injury in the brain [39]. CXCL10 is induced by type I IFNs and IFN-γ. CXCL13 is induced by type I IFNs, suggesting that IFN-I signaling, and specifically STAT1 signaling, could be important for recruitment of CXCR3+ or CXCR5+ CD8 T cells and other immune cells to the CNS and to the site of infection within the brain during PyV infection.

1.7 Overarching Hypothesis and Specific Aims

Each neurotropic virus induces unique chemokine patterns that differ in the chemokines being expressed and their cellular sources. This prevents the application of findings from one virus infection model to another. Thus, there are gaps in knowledge in the effects of polyomavirus infection on brain resident cells and the immune cell recruitment mechanisms employed by the CNS to confer protection and aid in virus control. In this thesis, I attempted to fill these gaps and further the understanding of T cell trafficking and infiltration during polyomavirus infection of the CNS, thus aiding in the potential development of therapies designed to induce more T cell recruitment for viral control or block recruitment to prevent immunopathology. The central hypothesis of this thesis is that MuPyV infection of the ependyma and other brain resident cells results in the release of type I IFNs that initiate the IFN signaling cascade and induce expression of ISGs, including the chemokines CXCL10 and CXCL13. CXCL10 and/or CXCL13 form gradients that guide CXCR3+ and CXCR5+ CD8 T cells to the brain and to the site of infection within the brain. The CD8 T cells then initiate their cytotoxic and effector functions to control MuPyV CNS infection. This hypothesis was tested via the following specific aims:

Aim 1: Define expression conditions of CXCL10 and CXCL13 and their corresponding receptors during MuPyV infection

Hypothesis: CXCL10 and CXCL13 are induced within ependymal cells lining the ventricles after MuPyV infection.

Specific questions asked regarding this aim were:

1. When are CXCL10 and CXCL13 expressed in the brain?
2. Which cell types express CXCL10 and/or CXCL13?

3. Do T cells in the brain express CXCR3 and/or CXCR5?
4. Are T cells co-localized with chemokine-expressing brain resident cells?

Aim 2: Determine the impact of STAT1 signaling on CXCL10 and CXCL13 expression in brain resident cells

Hypothesis: Loss of STAT1 signaling will result in a significant decrease in both CXCL10 and CXCL13 expression in brain-resident cells.

Specific questions asked regarding this aim were:

1. Is STAT1 signaling required for CXCL10 and CXCL13 mRNA expression in the periventricular zone?
2. Is STAT1 signaling required for CXCL10 and CXCL13 protein expression in the brain?
3. Does loss of STAT1 alter localization of CXCL10 and CXCL13 expression and cellular sources of these chemokines in the brain?

Chapter 2

2.0 Materials and Methods

Study Design

The primary objective of this study was to identify the chemokines responsible for guiding CD8 T cells to the site of PyV infection within the brain. Due to our lab's previous findings of CXCR3 and CXCR5 expression on CD8 T cells within the brain post infection [123], I hypothesized that the corresponding chemokines, CXCL10 and CXCL13, may be released from brain resident cells to guide CXCR3+ and CXCR5+ CD8 T cells to the infected ependyma. To determine which chemokines were expressed in the brain before and after PyV infection, I carved out the periventricular zone from mice at days 4, 7, and 15 post MuPyV infection and used the isolated mRNA for RTqPCR with primers specific for various chemokine mRNAs, including *Cxcl10* and *Cxcl13*. CXCL10 and CXCL13 protein expression and localization in the brain was examined by immunofluorescence microscopy to identify CXCL10+ and CXCL13+ brain resident cells. Corresponding chemokine receptor (CXCR3 and CXCR5) expression on CD8 T cells in the brain was determined by flow cytometry staining, and CD8 T cell localization in the brain was observed with immunofluorescence microscopy. To understand the importance of STAT1 signaling in chemokine expression and CD8 T cell infiltration, STAT1 KO mice were used to characterize potential differences in CXCL10 and CXCL13 expression and CD8 T cell localization after MuPyV infection using RTqPCR and immunofluorescence microscopy.

Mice:

C57BL/6NCr (stock #OIC55) and B6-ly5.1/Cr (stock #564) mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). *Stat1*$^{-/-}$ mice were obtained from Dr. Christopher Norbury (Penn State College of Medicine) with an approved Materials Transfer Agreement from Rutgers New Jersey Medical School. All knockout mice are on a C57BL6 background. Dcdc2Cre mice (C57BL/6J-Tg (Dcdc2a-cre)NIDA056Htz/Mmjax, MMRRC stock # #043796-JAX) and *Stat1*$^{fl/fl}$ mice (B6;129S-Stat1^{tm1Mam}/Mmjax, MMRRC stock #032054-JAX) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in accordance with the guidelines of the Institutional Animal Care and Use Committees and the Department of Comparative Medicine at the Pennsylvania State University College of Medicine. The Pennsylvania State University College of Medicine Animal Resource Program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The Pennsylvania State University College of Medicine has an Animal Welfare Assurance on file with the National Institutes of Health's Office of Laboratory Animal Welfare; the Assurance Number is A3045-01.

MuPyV Infection:

As previously described, 7-12 week-old mice were anesthetized with ketamine and i.c. inoculated by injecting the right frontal lobe with 6 x 10^5 PFU of MuPyV strain A2 in 30 µL of DMEM [47].

Quantification of MuPyV Genomes and LT-Ag mRNA:

TaqMan real-time PCR was performed in an ABI StepOnePlus (Applied Biosciences) with 10 ng of template DNA. DNA was purified from the brain by homogenization in nuclear lysis solution (Promega, Madison, WI) followed by incubation with protein precipitation solution (Promega, Madison, WI) to obtain DNA in the supernatant. The supernatant was then incubated with isopropanol to precipitate the DNA. The precipitated DNA was washed in ethanol and dissolved in molecular grade water. Primers and amplification parameters are previously described [124].

Lymphocyte Cell Isolation:

For isolation of lymphocytes, mice were euthanized, and brains and spleens were removed. Brains and spleens were minced and digested with Collagenase I (40mg/100ml) for 20 minutes at 37° C. Brain homogenates were subjected to a two-step percoll gradient (44%/66%) to remove myelin and cell debris. Cells were collected from the 44%/66% interface and washed with 5% FBS DMEM before flow cytometry staining. Mononuclear cells were isolated from spleens as described previously [125].

Flow Cytometry:

Spleen and brain cells were exposed to Fixable Viability Dye (eBioscience, San Diego, CA) prior to staining for CD8 tetramers, surface molecules, and/or intracellular molecules. Allophycocyanin-conjugated DbLT359-638 tetramers (NIH Tetramer Core Facility, Atlanta, GA) and antibodies to the following molecules were used: CD8α (53-6.7), CXCR5-biotin (2G8), Streptavidin, and CD45 (30-F11) purchased from BD Biosciences (San Diego, CA); CD4 (RM4-

5) and CXCR3 (CXCR3-173) purchased from BioLegend (San Diego, CA). To identify cells in the brain vasculature, mice were injected i.v. with 30 ug of CD45 (30-F11) 3 minutes prior to being euthanized [126]. Samples were acquired on an LSR II or LSR Fortessa (BD Biosciences, San Diego, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Immunofluorescent Microscopy:

For frozen sections, brains were removed and hemisected sagittally or coronally, frozen in optimal cutting temperature compound (OCT), and cut in 10 µm sagittal sections using a TN-2085 TANNER Scientific Cryostat. Prior to immunofluorescent antibody labeling, sections were fixed to the slides with ice cold acetone for 10 seconds, then permeabilized with 0.3% Triton-X for 20 min, and finally blocked with 10% Donkey Serum in 5% BSA for 1 hour. Primary antibodies and dilutions used were: 1:1000 goat anti-GFAP (Abcam, ab53554), 1:900 rabbit anti-GFAP (Dako, Z0334), 1:250 monoclonal rat anti-vimentin (R&D, MAB2105), 1:500 rabbit anti-Iba1 (Wako, SAJ2266), 1:100 polyclonal rabbit anti-CXCL13 (Invitrogen, PA5-28827), and 1:150 goat anti-CXCL10 (R&D, BAF466). Secondary antibodies were diluted 1:500 in 5% BSA and include bovine anti-goat AF488 (Jackson ImmunoResearch, West Grove, PA), donkey anti-rabbit AF647 (Jackson ImmunoResearch, West Grove, PA), donkey anti-rabbit AF488 (Jackson ImmunoResearch, West Grove, PA), and donkey anti-rat AF555 (Abcam, Cambridge, United Kingdom, ab150154). Donkey anti-goat AF647 (Jackson ImmunoResearch, West Grove, PA) was diluted 1:250. ProLongTM Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) was used to mount the coverslips. Images were acquired using a Leica DM4000 B LED microscope (Leica Biosystems, Wetzlar, Germany).

RNA Isolation and qPCR to Measure Chemokine Expression:

Brains were extracted from mice before resecting the periventricular zone. Brains were cut into coronal sections from the olfactory bulb to the cerebellum. Under a dissecting microscope, the area surrounding the lateral and fourth ventricles from each section was carved out using a scalpel and fine point tweezers. RNA was harvested from the periventricular zones of each individual brain using Trizol reagent (ThermoFisher) and isolated by phenol: chloroform extraction followed by isopropanol precipitation. cDNA was prepared with 1–2 ug of RNA using random hexamers and Revertaid RT (ThermoFisher). cDNA was used in qPCR reactions using the following primers from IDT (Coralville, Iowa): 40x GAPDH (Primetime qPCR, NM_002046(1)), CXCL9 (FWD = 5'-TGCACGATGCTCCTGCA-3' REV= 5'-AGGTCTTTGAGGGATTTGTAGTGG-3'), CXCL0 (FWD= 5'-GACGGTCCGCTGCAACTG-3' and REV 5'-GCTTCCCTATGGCCCTCATT-3'), CXCL11 (FWD= 5′-AAGCTCGCCTCATAATGCAG-3′ REV= 5′-CACAGTCAGACGTTCCCA-3′), CXCL12 (FWD= 5′-CCAGAGCCAACGTCAAGCAT-3′ REV= 5′-CAGCCGTGCAACAATCTGAA-3′), and CCL2 (FWD= 5'-AGCAGGTGTCCCAAAGAA-3' and REV=, 5'-TATGTCTGGACCCATTCCTT-3'). The thermocycler conditions were 10 min at 95°C followed by 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30s. Transcript levels were calculated relative to the housekeeping gene GAPDH using the $2^{-(\Delta\Delta Ct)}$ formula, where CT is determined as the threshold cycle at which the fluorescent signal becomes significantly higher than that of the background.

Brain Resident Cell Sorting and RTqPCR:

Mice were perfused with 10mL of 1x heparin in saline, and brains were isolated before being placed on ice. Brains were minced with a scalpel in petri dishes before being digested in a mix of Collagenase I (1mg/mL), DNAse I: 40ug/mL, and pre-warmed 1X HBSS for 15 minutes at 37˚C. Brains were mashed through a 100uM filter in a 50mL conical tube and centrifuged for 7 minutes at 421xg. The pellet was resuspended in 10mL of 37% Percoll and centrifuged without brakes for 10 minutes at 500xg. The cell pellet was washed and distributed in a 96 well plate for staining. Cells were stained for 30 minutes at 4˚C using the following antibodies diluted in FACS buffer: Oligodendrocytes- 1:20 Anti-O4 (Militenyi Biotec, 130-095-891), 1:200 Astrocytes- Anti-GLAST (Miltenyi Biotec, ACSA-1), 1:200 CD45 (BioLegend, 30-F11), and 1:200 CD11b (BD Biosciences, M1/70). Samples were washed with FACS buffer before being fixed in 2% PFA and FACS sorted using the BD Aria Sorter. Cells were sorted into oligodendrocytes, astrocytes, microglia, and monocytes, and RNA was isolated from each group to be used in RTqPCR with primers amplifying CXCL13 (primers and thermocycler conditions described previously).

Statistical Analysis:

p values were determined by the Mixed-effects model, Tukey's multiple comparisons test, Sidak's multiple comparisons test, One-way Anova, Holm-Sidak's multiple comparisons test, and unpaired Student's T test using GraphPad Prism software (La Jolla, CA). All p values $\leq$ 0.05 were deemed significant.

Chapter 3

Results

3.1 CXCL10 and CXCL13 are Responsible for Guiding CXCR3+ and CXCR5+ CD8 T cells to the Site of MuPyV Infection

3.1.1 CXCL10 and CXCL13 Expression Increases During MuPyV Infection

During virus infection, the chemokine expression profile varies between tissues as well as viral strains. Therefore, one cannot generalize chemokine expression profiles in the brain with different viruses. It is unclear which chemokines are expressed in the brain following PyV infection. In attempt to to identify these chemokines, I infected WT mice i.c. with MuPyV, and their brains were dissected at days 4, 7, and 15 post infection. Because MuPyV primarily infects the ependymal lining of the ventricles, I wanted to examine expression in the cells within this area, so I dissected the periventricular zone of the lateral and fourth ventricles for RNA isolation. Extracted RNA from brains at different timepoints was used in RTqPCR with primers specific for the chemokines CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, and CCL2. Chemokine expression was compared using the fold change based on expression in mock infected mouse brains. Among the chemokines tested, CXCL10 and CXCL13 were the most highly expressed following infection with expression peaking at 7 d.p.i. (Figure 3-1a). This suggests that CXCL10 and CXCL13 may be induced by infection and may be the chemokines responsible for recruiting CD8 T cells to the site of MuPyV infection.

To determine if virus infection directly affects chemokine expression, virus levels in each periventricular zone sample were compared to the chemokine expression fold change. MuPyV levels were measured via the number of LT mRNA transcripts from the mouse brains at days 4, 7, and 15 post infection. There is a minor positive correlation between CXCL10 and CXCL13 and

virus levels with R^2 values of 0.6164 and 0.4791 respectively (Figure 3-1b). This suggests that MuPyV infection may be directly or indirectly causing chemokine expression by activating the IFN signaling cascade to induce CXCL10 and CXCL13 expression by brain cells. As MuPyV levels in the brain increase, more IFN signaling occurs which then induces more expression of each chemokine. However, it is unclear which cells in the brain are expressing CXCL10 and CXCL13 and whether these cells are infiltrating or brain resident.

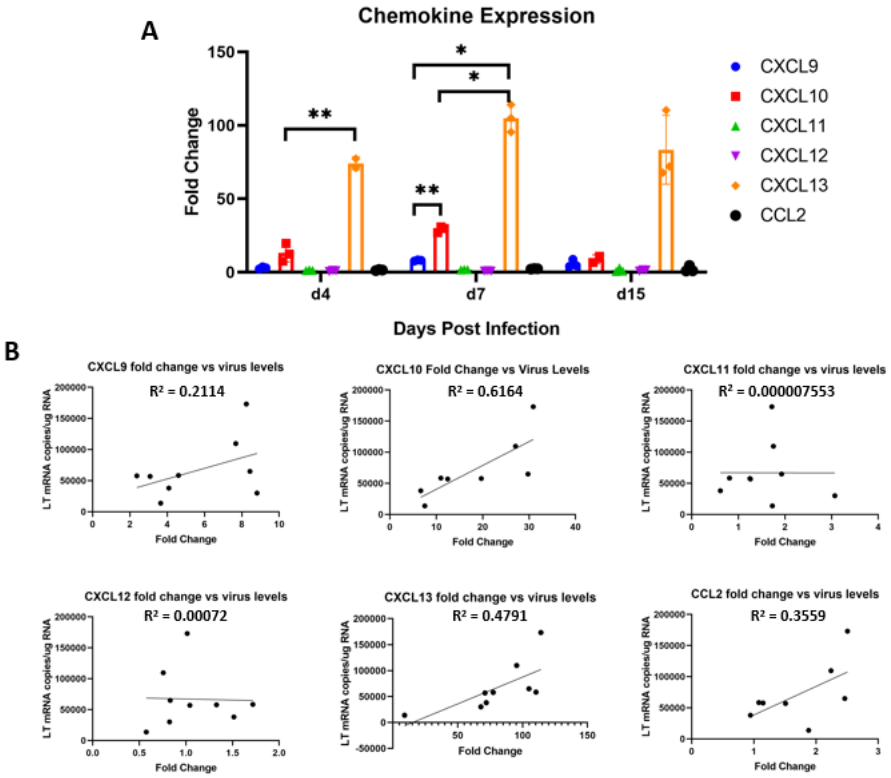

Figure 3-1: CXCL10 and CXCL13 Expression Peaks at 7 d.p.i and Increases with Virus Levels. (A) The periventricular zones of mouse brains were isolated, and RNA was extracted and used in RTqPCR to measure chemokine expression on days 4, 7, and 15 post infection. The $2^{-(\Delta\Delta CT)}$ formula was used to compare the fold changes between the chemokines CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, and CCL2 at each timepoint. **(B)** Correlation between the fold change of each chemokine and the virus levels (based on LT mRNA copies/ug of RNA) of the periventricular zones. **Statistics: (A)** Mixed-effects model with the Geisser-Greenhouse correction and Tukey's multiple comparisons test. Data is not significant unless noted. Not shown: At d7 CXCL9 vs CXCL11, CXCL9 vs CXCL12, CXCL9 vs CCL2, CXCL10 vs CXCL12, CXCL10 vs CXCL12, CXCL10 vs CCL2, CXCL11 vs CXCL12, CXCL12 vs CCL2 =

. CXCL11 vs CXCL13, CXCL11 vs CCL2, CXCL13 vs CCL2 = *. **(B) R^2 values for each correlation are depicted in the corresponding graph. Sy.x values: CXCL9 = 45517, CXCL10 = 32830, CXCL11 = 51256, CXCL12 = 51237, CXCL13 = 36994, and CCL2 = 42542. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$, ****$p \leq 0.001$. Data are from 1 experiment with 3 infected and 3 sham infected mice.

3.1.2. CXCL10 and CXCL13 are Expressed by Choroid Plexus, Ependyma, and Subventricular Astrocytes

Once I identified CXCL10 and CXCL13 as chemokines expressed by cells in the brain following MuPyV infection, I wanted to elucidate which cells are expressing these chemokines. To visualize these cells, WT mice were infected i.c. with MuPyV, and sacrificed at 7 d.p.i. Brains were dissected and frozen in OCT before being cut into coronal sections. Sections were stained with fluorescent antibodies targeting the markers GFAP (astrocytes), Iba1 (microglia and monocytes), vimentin (ependyma), and CXCL10 or CXCL13. Using immunofluorescent microscopy, stained sections were imaged, and colocalization of each chemokine was observed with astrocytes, microglia, and ependyma. CXCL10 colocalized with the choroid plexus inside the ventricles as well as the subventricular zone (SVZ) astrocytes, indicated by the GFAP co-staining (Figure 3-2a). This corroborates previous studies that detected astrocytes expressing CXCL10 in the brain [88]. Immunofluorescence microscopy is not sensitive enough to visualize the chemokine gradients outside of the cells, but the staining observed was localized within the cytoplasm of the cells, potentially being prepared to be released into the lumen. It also seems that expression is limited to certain astrocytes surrounding the ventricles as not every SVZ astrocyte expressed CXCL10. The CXCL10 expression by the choroid plexus suggests the CXCR3+ CD8 T cells may be infiltrating the brain via the choroid plexus epithelium and the blood-CSF barrier to reach the infected ependyma. CXCL13 appeared to be co-localized with the ependymal lining of the ventricles, indicated by vimentin co-staining, and the SVZ astrocytes, indicated by the GFAP co-staining (Figure 3-2b). However, as with CXCL10, not all astrocytes surrounding the

ventricles co-stained with CXCL13. This could be due to select astrocytes being infected by MuPyV or exhibiting active IFN signaling to induce CXCL10 or CXCL13 expression.

To further elucidate which cell types are expressing chemokines in the brain, mice were i.c. infected with MuPyV, and their brains were dissected at 15 d.p.i. Dr. Heather Ren, a previous member of the Lukacher lab, prepared whole brains for FACS cell sorting, separating the brain resident cells based on staining of astrocytes via anti-GLAST, oligodendrocytes via anti-O4, monocytes and microglia via CD11b and CD45. Sorted cells were used in RNA isolation and RTqPCR, using primers to amplify CXCL13. Based on levels of mRNA, microglia express the most CXCL13 in the brain post infection (Figure 3-2c). This does not align with the previous immunofluorescence data showing astrocytes expressing CXCL13 near the ventricles. This could be due to changes in CXCL13 expression over time following infection. Chemokine expression could shift from astrocytes and ependyma to the microglia as infection persists.

I next confirmed the localization of CXCL13 expression in the area surrounding the ventricles as observed via immunofluorescence microscopy. Mice were i.c. infected with MuPyV, and at 7 d.p.i. their brains were dissected with the periventricular zone carved out and separated from the other parts of the brain, including the cortex, hippocampus, and cerebellum. I isolated RNA from these brain samples which was used for RTqPCR with primers to amplify CXCL13. I found that compared to the combined other areas of the brain, the periventricular zone expressed a significant amount of CXCL13 (Figure 3-2d). This confirms that a majority of the CXCL13 expression in the brain following MuPyV infection surrounds the infected ependyma lining the ventricles and the periventricular zone/SVZ.

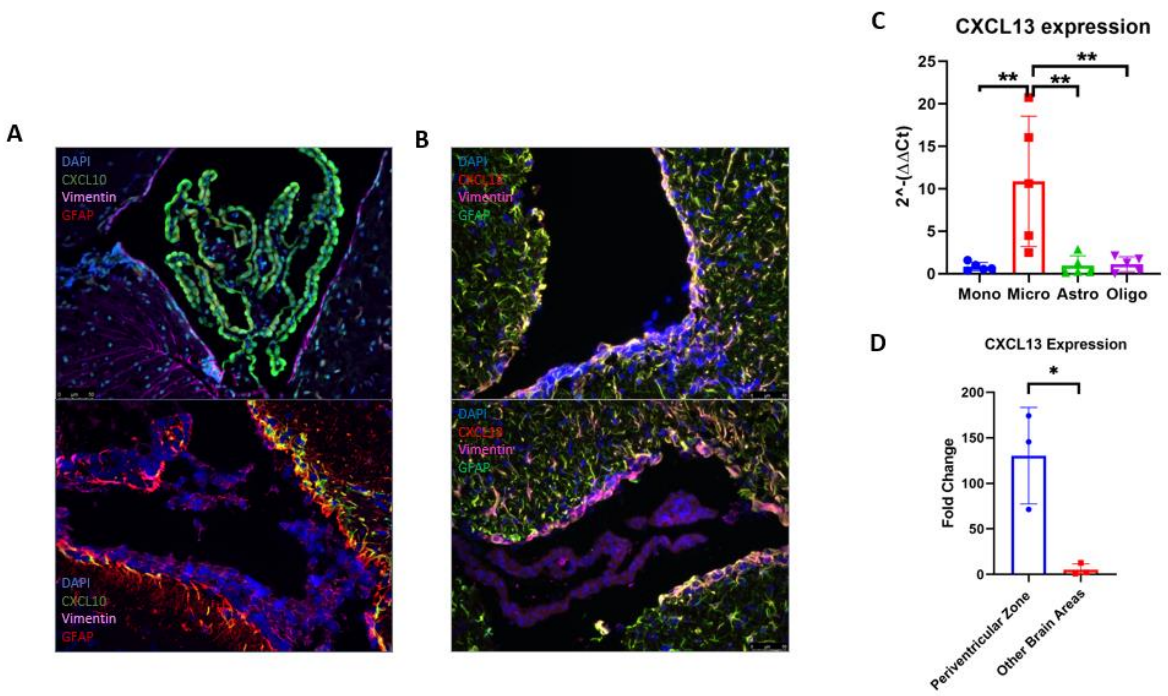

Figure 3-2: Cellular Sources of CXCL10 and CXCL13 and Their Localization. (A) Immunofluorescence (IF) images of the CXCL10-expressing choroid plexus and subventricular astrocytes (GFAP) inside and surrounding the ventricles after MuPyV infection at 5 d.p.i. Yellow indicates overlap of GFAP and CXCL10. **(B)** IF images of CXCL13-expressing ependyma (Vimentin) and subventricular astrocytes (GFAP) surrounding the ventricles after MuPyV infection (5 d.p.i). Yellow indicates overlap of CXCL13 and GFAP. White indicates overlap of CXCL13 and Vimentin. **(C)** CXCL13 expression levels using RTqPCR of RNA isolated from sorted glia cells of infected mouse brains, including mono(cytes), micro(glia), oligo(dendrocytes), and astro(cytes) at 8 d.p.i. **(D)** CXCL13 expression in the resected periventricular zone vs other areas of the brain combined, including the cortex, hippocampus, and cerebellum at 7 d.p.i. **Statistics: (C)** one-way Anova and Tukey's multiple comparisons test. **(D)** Unpaired T test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$, ****$p \leq 0.001$. Data are from 1 experiment with 5 or 3 infected and sham infected mice.

3.1.3. CD8 T Cells Express CXCR3 or CXCR5 and Localize to the Periventricular Zone

Previous work in the Lukacher lab showed expression of the CXCL10 and CXCL13 corresponding chemokine receptors, CXCR3 and CXCR5 respectively, on the CD8 T cells within the brain after MuPyV infection [123]. To confirm this expression, mice were infected i.c. with MuPyV and sacrificed at days 8 and 15 post infection. Brains were dissected out and lymphocytes

were extracted from whole brains before being stained using immunofluorescent markers. Using flow cytometry, I found that more MuPyV-specific CD8 T cells infiltrating the brain expressed CXCR5 versus CXCR3, with about 1% of cells expressing CXCR3 and about 80% of cells expressing CXCR5 (Figure 3-3a, b). This suggests that the CXCR5:CXCL13 axis is favored in recruiting CD8 T cells to the brain and the site of infection within the brain. However, not all MuPyV-specific CD8 T cells, or total CD8 T cells, in the brain express one or both of these chemokine receptors. This result suggests that another chemokine and receptor pair may be responsible for recruiting the remaining CD8 T cells to the brain after PyV infection.

I then wanted examined whether the infiltrating CD8 T cells were localizing around the chemokine-expressing cells in the brain. To do this, I infected mice i.c. with MuPyV and extracted their brains at 15 d.p.i., which were frozen in OCT and sectioned for immunofluorescence microscopy. Sections were stained for CXCL10, CXCL13, and CD8α to observe potential colocalization. I found that the CD8 T cells infiltrating the brain localized within the choroid plexus, where CXCL10 is expressed, as well as around the ependyma and the periventricular zone where CXCL13 and both chemokines are expressed (Figure 3-3c, d). This result further suggests the function of the CXCL10:CXCR3 and CXCL13:CXCR5 signaling axes to be involved in recruiting CD8 T cells to the brain and to the periventricular zone, so the T cells can exert their anti-viral functions.

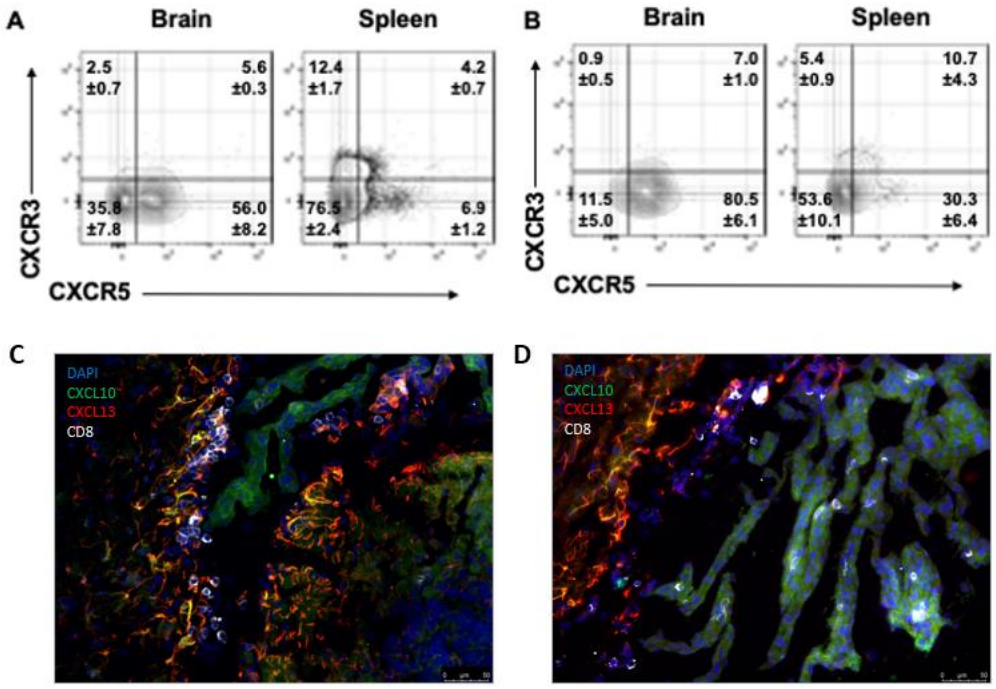

Figure 3-3: CD8 T cells Express CXCR3 or CXCR5 and Localize to the Periventricular Zone During MuPyV Infection. (A) Representative flow plots of total CD8 T cells in the brain and spleen showing expression of CXCR5 and CXCR3. Mean ±SD. **(B)** Representative flow plots of DbLT359+ CD8 T cells in the brain and spleen showing expression of CXCR5 and CXCR3. Mean ±SD. **(C)** IF image showing CD8 T cells localized near the chemokine-expressing ependyma and subventricular astrocytes 14 d.p.i. **(D)** IF image showing CD8 T cells localized in the CXCL10-expressing choroid plexus inside the ventricle 14 d.p.i.

3.2 CXCL10 and CXCL13 Expression in the CNS Requires STAT1 Signaling

It has been previously shown that STAT1 signaling in combination with CD8 T cells is important for protecting the CNS from injury due to virus infection [39]. Loss of STAT1 results in hydrocephalus, which is exacerbated by loss of CD8 T cells. Both CXCL10 and CXCL13 can be induced by IFN signaling, with CXCL10 being induced by IFN-γ and CXCL13 being induced by type I IFNs. The two chemokines are induced by different families of IFN, but all IFN family members signal through the JAK/STAT pathway. This led us to hypothesize that STAT1

signaling is required for CXCL10 and CXCL13 expression by brain resident cells and that loss of STAT1 will disrupt the chemokine gradients responsible for trafficking the virus specific CD8 T cells to the site of infection. Therefore, virus control will be dramatically reduced, and damage from MuPyV infection will cause hydrocephalus over time.

3.2.1 Loss of STAT1 Significantly Reduces CXCL10 and CXCL13 Expression

To investigate the importance of STAT1 signaling in chemokine expression, I extracted brains from WT and STAT1 KO mice at days 4, 7, 15, and 30 post infection. I dissected out the periventricular zones as before and isolated RNA. RTqPCR was performed using primers amplifying *Cxcl10* and *Cxcl13* mRNAs, in WT mice and STAT1 KO mice. I found that loss of STAT1 dramatically reduced expression of CXCL13 in the periventricular zone but CXCL10 expression was only moderately reduced (Figure 3-4a). This suggests that CXCL13 is more directly regulated by STAT1 signaling with a majority of its expression in the brain relying on the IFN cascade. However, there is still some CXCL13 remaining after the loss of STAT1 (Figure 3-4a). This could be due to other pathways being utilized to induce CXCL13 expression in the absence of IFN signaling. This is also likely the case for CXCL10 as its expression decreased by ~50% in the STAT1 KO mice compared to WT mice. Therefore, CXCL10 induction relies more on additional pathways besides JAK/STAT and the IFN signaling cascade.

To further examine changes in chemokine expression and localization within the brain in the absence of STAT1 signaling, I i.c. infected WT and STAT1 KO mice and sacrificed them at days 4 and 7 post infection. Their brains were frozen in OCT before being sectioned for immunofluorescence microscopy. Sections were stained for CXCL10 or CXCL13, GFAP (astrocytes), and vimentin (ependyma). CXCL10 and CXCL13 expression was compared between the WT and STAT1 KO mice. Both CXCL10 and CXCL13 expression appeared to

decrease in the areas surrounding the ventricles at both days 4 and 7 post infection (Figure 3-4b, c). This further suggests that STAT1 signaling is important for expression of CXCL10/CXCL13 in the brain and potentially guiding CD8 T cells to the infected areas. Combined, this data shows that CXCL10 and CXCL13 expression at both the mRNA and protein levels is at least partly regulated by IFN and STAT1 signaling, but the remaining expression suggests that other pathways are involved in chemokine regulation as well.

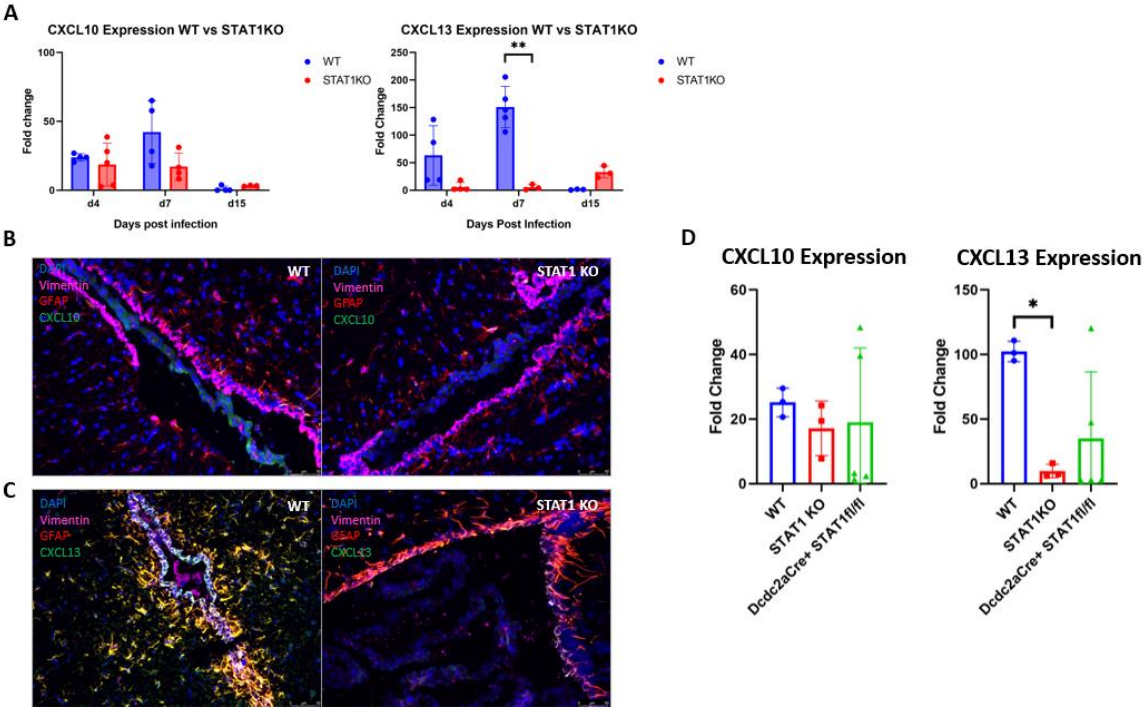

Figure 3-4: CXCL13 Expression Requires STAT1 Signaling. (A) RTqPCR of resected periventricular zones from WT and STAT1 KO mice comparing CXCL10 and CXCL13 expression at 7 d.p.i. **(B)** IF images comparing CXCL10 expression and localization between WT and STAT1 KO mouse brains. **(C)** IF Images comparing CXCL13 expression and localization between WT and STAT1 KO mouse brains. **(D)** CXCL10 and CXCL13 expression via RTqPCR of periventricular zones from WT, STAT1 KO, and Dcdc2aCre+ STAT1$^{fl/fl}$ mice. **Statistics:** Data is not significant unless noted. **(A)** Mixed effects model with the Geisser-Greenhouse correction and Sidak's multiple comparisons test. **(D)** One-way Anova and Holm-Sidak's multiple comparisons test (CXCL10) or Tukey's multiple comparisons test (CXCL13).

3.2.2 STAT1 Expression in the Ependyma May Be Important for Chemokine Expression

Previous experiments were conducted with global STAT1 KO mice, but we have seen that chemokine expression is limited to specific cell types within the periventricular zone. To test the importance of STAT1 more specifically in these cell types, such as the ependyma, I wanted to knockout STAT1 selectively in the ependyma. Dcdc2Cre+ mice were crossed to *Stat1*$^{fl/fl}$ mice, generating Dcdc2Cre+ *Stat1*$^{fl/fl}$ mice where STAT1 expression was ablated in only the ependymal cells. These mice along with STAT1 KO and WT mice were subjected to i.c. MuPyV infection before being sacrificed at 4 and 7 d.p.i. The periventricular zone was carved out and used to isolate RNA for RTqPCR analysis. Primers were used to amplify *Cxcl10* and *Cxcl13* mRNAs to measure chemokine expression and compare between the three groups of mice. As seen previously, CXCL10 and CXCL13 expression was dramatically reduced in the STAT1 KO mice; however, the Dcdc2Cre+*Stat1*$^{fl/fl}$ mice exhibited high variability (Figure 3-4d). Based on averages there was a decrease in expression of both CXCL10 and CXCL13 as seen with the STAT1 KO mice, but the variability of the Dcdc2Cre+ *Stat1*$^{fl/fl}$ mice makes it difficult to measure. In later experiments done by Lukacher lab members, it was found that in the male Dcdc2Cre+ *Stat1*$^{fl/fl}$ mice, Cre was not functioning properly, likely due to Cre not being expressed, and therefore, Stat1 was not deleted (data not shown). This sexual dimorphism could explain the variability in chemokine expression seen in the Dcdc2Cre+ *Stat1*$^{fl/fl}$ mice as both male and female mice were used in the experiments. It is unclear how this sex difference emerged, and if this is due to the Cre insertion site in the genome. Future experiments will utilize only female Dcdc2Cre+ mice.

Chapter 4

Discussion

The chemokine expression profile of the brain and other tissues is known to change based on the infection type and strain of virus. The expression profile of chemokines in the brain following PyV infection is unknown. MuPyV CNS pathogenesis results in hydrocephalus and demyelination of the white matter of the brain, which can be due to lack of virus control and an influx of CD8 T cells that attack and kill the infected cells in the brain [39]. Therefore, knowing how the T cells traffic to the brain could offer insight into therapeutic interventions to either promote T cell surveillance of the CNS or mitigate PML-IRIS in immune-reconstituted patients.

In my thesis work, I provide evidence that the chemokines CXCL10 and CXCL13 are expressed by brain resident cells. Specifically, the choroid plexus and SVZ astrocytes express CXCL10, and the ependyma and microglia express CXCL13. Expression of these chemokines is detected as early as 4 d.p.i and peaks at 7 d.p.i. of i.c. inoculated MuPyV. Based on RTqPCR and IF results, the chemokine expression is localized to cells surrounding the ventricles, or the periventricular zone/SVZ, but not all cells in this location express either chemokine. This could be due to chemokine expression being limited to infected cells or only certain cells responding to relevant signals (i.e., IFN). Ideally, I would confirm expression at the protein level by resecting the periventricular zone and making cell lysates for western blot immunoassays to detect CXCL10 and CXCL13 expression. There are some discrepancies in the literature and my data as to which cells express CXCL13. My IF experiments show expression of CXCL13 by the SVZ astrocytes and ependyma, but the FACS sorting and subsequent RTqPCR data show expression by microglia. This could be due to chemokine expression changes over time with expression initiating in astrocytes and later in microglia to continue recruiting more immune cells. There

could also be some regulation differences with microglia only expressing CXCL13 at the mRNA level and not the protein level, which would explain why I don't see microglia expressing CXCL13 using IF. This experiment was also done using whole brain rather than just the periventricular zone. Therefore, it is possible the non-expressing astrocytes from other areas of the brain diluted the CXCL13 expression results, resulting in astrocytes appearing to express the chemokine at a low level. I would want to perform a western blot using lysates from sorted brain resident cells as previously described to confirm which cell types are expressing each chemokine as well as repeat the previous RTqPCR. This should help resolve the discrepancies over which cells express CXCL13. Although these experiments show expression of CXCL10 and CXCL13 in the brain, it is still unclear if the chemokines are in fact being secreted into the CSF to form gradients. To determine this, I would isolate CSF from mice infected i.c. with MuPyV at 7 d.p.i., the peak of CXCL10 and CXCL13 expression, and perform an ELISA using the CSF and mouse serum. If the chemokines are being secreted and move into the blood, I would expect to detect high levels of each chemokine in both the CSF and blood.

The ependymal cell layer lining the ventricles is the main site of MuPyV infection, and there appears to be a correlation between virus levels measured by LT mRNA and fold change of CXCL10 and CXCL13. This suggests that MuPyV infection is likely inducing chemokine expression in the infected and surrounding cells. It is unclear if the virus is directly inducing expression by infection and expression of its own genes or indirectly by activating the innate immune response inside the cells to create an antiviral state and activate downstream chemokine expression. To investigate this further, I would observe whether the cells expressing each chemokine are infected themselves or are simply surrounding infected cells. To do this I would conduct IF staining for MuPyV (using anti-VP1 or anti-LT antibodies) as well as each chemokine. I would observe any co-localization of virus and chemokine staining to see if all infected cells express CXCL10 or CXCL13, and if all CXCL10 or CXCL13-expressing cells are

infected. I could also test this using RNA in situ hybridization to observe co-localization of chemokine and virus mRNAs. RNA *in situ* hybridization, such as RNAScope or RNAView, would allow for more accurate observation of both chemokine expression and identification of infected cells.

The corresponding chemokine receptors, CXCR3 and CXCR5, are expressed on the infiltrating CD8 T cells in the brain, which suggests that CXCL10 and CXCL13 and their receptors indeed have chemotactic function to help recruit T cells to the correct site in the brain. Further supporting this idea is the localization of CD8 T cells in the periventricular area via IF where CXCL10 and CXCL13 are expressed post infection. To confirm that these CD8 T cells localized around the ventricles and the CXCL10 and CXCL13 expressing cells are expressing either chemokine receptor, I would use IF and stain the brain sections for CD8α, CXCR3, and CXCR5. As not all T cells in the brain express either chemokine receptor, this experiment would implicate expression of one or both of these chemokine receptors with T cell migration to the infected areas of the brain. Further testing and experiments need to be done to further confirm this idea. For example, expression of each receptor on the infiltrating CD4 and CD8 T cells could be confirmed by FACS-sorting T cells from the brain at 8 and 15 dpi and isolating RNA to be used in RTqPCR with primers specific for CXCR3 and CXCR5. To investigate the importance of CXCR5 and CXCR3 expression on T cells for proper recruitment, receptor KO mice could be obtained from JAX: *Cxcr5*$^{-/-}$ mice (JAX #006659) and *Cxcr3*$^{-/-}$ mice (JAX #005796). An adoptive transfer system could be used to monitor the movement of receptor KO and WT CD8 T cells into the brain. T cells from [CD90.1+/CD90.2+] WT and [CD90.2+ CXCR5-] CD8 T cells from TCR-I donor mice could be purified and transferred into recipient CD90.1+ WT mice via tail vein injection (d = -1) at a 1:1 ratio. The next day (d = 0) recipient mice would be infected i.c. with MuPyV.LT206 at 1x10^6 PFU. On day 8 p.i., lymphocytes would be isolated from the brains and spleens of the recipient mice. Using flow cytometry, I would compare the ratio of CXCR5 KO T

cells (CD90.2+) to WT T cells (CD90.1+/CD90.2+) in the brains and spleens. The ratio in the spleen would function as a control, and the ratio in the brain would indicate whether the lack of CXCR5 expression affects CD8 T cell trafficking to the brain. This would be repeated for CXCR3 KO and WT T cells from donor mice. CD8 T cell infiltration could also be measured in these mice using IF and staining for CD8 T cells in brain sections. Without each of these receptors, I would expect that CD8 T cell recruitment would be disrupted with a reduced number of donor T cells in the brain following CXCR5 or CXCR3 KO T cell transfer. To further test the function of the chemokine and receptor pairs, I could utilize CXCL10 or CXCL13 KO mice and flow cytometry to observe the number of CD8 T cells in the brain following infection as previously described. Alternatively, I could treat WT mice with a neutralizing antibody targeting CXCL10 or CXCL13 (delivered intracerebroventricularly) before infecting the mice with MuPyV. Loss of each chemokine expression would be confirmed using RTqPCR or western blot and IF, and the number of CD8 T cells recruited to the brain would be measured and compared to untreated infected mice using flow cytometry.

It is possible that CXCR3 and CXCR5 and their corresponding chemokine ligands do not function to help recruit virus-specific CD8 T cells into the brain but rather guide them to the correct site of infection once in the brain. To investigate this, I would want to compare the chemokine receptor expression on the CD8 T cells in the blood/serum and in the brain. More or equal numbers of receptor-expressing T cells in the blood compared to the brain could indicate that T cells are using the receptors to move from the circulation into the brain. The T cells may also move into the brain and begin to lose CXCR3 and CXCR5 expression, explaining why not all T cells isolated from the brain express either receptor. I would also observe the chemokine levels within the serum and CSF of MuPyV-infected mice using an ELISA to observe any potential gradients guiding the T cells from the blood and into the brain. If the adoptive transfer experiments reveal there is no difference in the amount of WT and receptor KO T cells

infiltrating the brain, this would indicate that the lack of CXCR3 or CXCR5 does not hinder the ability of the T cells to travel into the brain. However, the chemokine: receptor axis may be more important for guiding the T cells to the site of infection once inside the brain. To test this, I would repeat the adoptive transfer and neutralizing antibody treatment experiments described earlier and observe T cell localization via IF, staining for CD8 T cells, CXCR5- and CXCR3-expressing cells. Many tissue sections would need to be observed, and the T cells localized in the periventricular space would be counted and compared between conditions. T cell localization could also be observed/measured by isolating lymphocytes from resected ventricular-periventricular tissue from mice at different timepoints after i.c. infection. The number of T cells located in the specific area of interest in the brain could be counted using flow cytometry. A higher percentage of chemokine receptor-expressing T cells in this resected area rather than whole brain could implicate the receptors having more of a role in localization within the brain rather than to the brain. These methods would be utilized to compare the localization of T cells in the brain at 8 and 15 d.p.i. in the adoptive transfer system mice, CXCL10 and CXCL13 KO mice, or CXCL10 and CXCL13 neutralizing antibody-treated mice to determine the function and importance of each chemokine and receptor pair in recruitment of T cells to the site of infection during PyV infection.

Loss of one chemokine-receptor pair's signaling may not be enough to observe changes in T cell recruitment as the other pair may compensate for this loss. If this is the case, knocking out only CXCR5 or CXCR3 might result in no difference in the amount of T cells infiltrating the brain or locating around the ventricles. A "double knockout" mouse lacking both receptors could be generated, but doing so won't exclude the potentially confounding variable of a compensatory chemokine assuming an orthogonal effect. Therefore, I would favor using CXCL10 and CXCL13 neutralizing antibodies and treat WT mice with both at the same time. This should dramatically reduce, if not ablate, the signaling for each chemokine. If no other chemokines are responsible for

recruiting T cells to the virus-infected site within the brain or cannot compensate for the loss of two other chemokines, measuring the number of T cells within the brain and in the periventricular zone should show a reduction compared to untreated mice. Another possibility to consider is that there may be more chemokines involved than have been tested in this thesis. To elucidate which other chemokines could be involved, I would sort brain-resident cells from infected and sham infected mouse brains and subject them to RNA sequencing to identify chemokines that are upregulated in the infected mouse cells. Chemokines that were found to be upregulated would become the target of the previously described experiments along with CXCL10 and CXCL13.

Identifying the chemokines responsible for recruiting CD8 T cells to the site of infection in the brain is only a piece of the puzzle. We know that IFN signaling plays a part in regulating both CXCL10 and CXCL13 expression in other tissues, with both being stimulated by type I IFNs and CXCL10 being stimulated by IFN-γ. Here I show that in the brain, STAT1 signaling is required for the full expression of both CXCL10 and CXCL13. Expression of CXCL13 seems to be more affected by the loss of STAT1 than CXCL10 expression. This could be due to other signaling molecules and cytokines inducing CXCL10 expression, such as TNF-α. TNFα signals through its receptors TNFR1 and TNFR2 and the NFκB pathway. It is also possible that IFN signaling may be using another pathway besides JAK/STAT that compensates for the loss of STAT1. To further test if type I IFN signaling is as important for CXCL13 expression as my data suggests, I would utilize IFNAR KO mice and infect them i.c. with MuPyV. After 7 d.p.i., I would carve out the periventricular region of their brains, isolate RNA, and perform RTqPCR to measure chemokine expression and compare with WT infected mice. If type I IFN signaling is important, CXCL13 expression should be significantly reduced if not ablated, showing that type I IFN is the main signal following MuPyV virus infection to induce downstream CXCL13 expression. For CXCL10, I would use IFNγR KO mice and repeat the experiment above. If IFN-γ

is the major signal inducing CXCL10 expression, then a majority of the CXCL10 expression as detected by RTqPCR should be ablated compared to WT mice.

In this thesis, I show that global STAT1 signaling is required for CXCL10 and CXCL13 expression, but only certain cells in the periventricular zone express each chemokine. CXCL13 appears to be expressed by the ependymal cells and SVZ astrocytes or microglia, and CXCL10 appears to be expressed by the choroid plexus and the SVZ astrocytes. My data using the Dcdc2Cre+ *Stat1*$^{fl/fl}$ mice shows that STAT1 expression in the ependyma may only partially contribute to CXCL10 and CXCL13 expression. As mentioned in the Results section, we discovered a sexual dimorphism in the Dcdc2Cre expressing mice where Cre expression seems to be lacking in the male mice. At this point, we have no explanation for this sex difference, and it is not mentioned in the mouse listing for JAX laboratories. Both male and female mice were used in the experiments, potentially skewing the results. Future experiments to measure chemokine expression differences using these mice would need to be done using exclusively female mice. This could explain the variation I observed in chemokine expression in the Dcdc2Cre+ *Stat1*$^{fl/fl}$ mice and why CXCL10 and CXCL13 expression in the periventricular zone of these mice was not as affected by the loss of STAT1. This could also be due to STAT1 expression being more important in the other cells, such as the CXCL10 and CXCL13 expressing astrocytes. To confirm that STAT1 signaling is important for astrocytes expressing each chemokine, I would utilize GFAP-Cre mice crossed to *Stat1*$^{fl/fl}$ mice to delete STAT1 expression only in astrocytes. I would then use IF and RTqPCR of the periventricular area and sorted cells from the brain to detect and measure CXCL10 and CXCL13 expression. If chemokine expression occurs mostly in the astrocytes, or STAT1 signaling is important for chemokine expression in the astrocytes, then I would expect the GFAPCre+ x *Stat1*$^{fl/fl}$ mice to have reduced expression of both CXCL10 and CXCL13.

Conclusions

In summary, I demonstrated that the chemokines CXCL10 and CXCL13 are induced in the brain following MuPyV infection. These chemokines are localized particularly in the area of infection surrounding the ventricles. Brain-infiltrating CD8 T cells express the corresponding chemokine receptors, CXCR3 and CXCR5, and localize near the CXCL10 and CXCL13 expressing brain-resident cells. I also showed that STAT1-mediated IFN signaling is required for full expression of both CXCL10 and CXCL13 in the brain after MuPyV infection. This suggests that IFN signaling is required for efficient recruitment of CD8 T cells to the site of infection, which plays a crucial role in protection against localized PyV infection.

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