Epigenetic and Environmental Regulation of Myeloid Cells during Inflammatory Demyelinating Disease

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor in Philosophy
(Immunology)
in the University of Michigan
2023

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DEDICATION

To the experiences we never expected,
and the paths that were redirected.
To the friends and family we found along the way.

"When it looks impossible, look deeper. And then fight like you can win."
- *Rost, Horizon Forbidden West.*

ACKNOWLEDGEMENTS

I have many to thank for my professional and personal development during these years. My success is shared by those who guided and supported me along the way.

I have a great appreciation for the guidance, mentorship, and support I received from my research mentor, Dr. Benjamin Segal. He is a thoughtful, rigorous scientist whose ability to think critically is only matched by his endless creativity. He strives for precision in experiments and communication. These values will stay with me for many years to come. Thank you.

I would also like to thank my thesis committee (Drs. Beth Moore, Shannon Carty, Carey Lumeng, Maria Castro, and Mireia Guerau-de-Arellano), the leaders of the Graduate Program in Immunology at UM throughout the years (Drs. Beth Moore, Malini Raghavan, and Kanakadurga Singer) and the program administrators (Zarinah Aquil and Catrece Evans). Their guidance, support, and advice have been invaluable. Many of these individuals supported me during my relocation, for which I will forever be grateful.

I owe many thanks to the members of the Segal lab. Jeff, Patrick, Jerry, Sas, Nicole, Taryn, Calli, Yan, Tom, and Spud, have provided mentorship and encouragement at many different stages of graduate school. They have provided technical assistance, suggestions, and were a constant source of scientific and less scientific discussion (a hot dog is definitely a sandwich, and The Proposal is definitely funny). The other members of the Segal lab were equally as critical to my studies, providing technical assistance and feedback. A special shout out to the wolverine-buckeye hybrids, the move would have been much more difficult without our comradery. Thank you all.

I have many friends I owe so much thanks to. I could not possibly list all the ways you all have changed my life. Thank you:

- To Shannon and Patrick, for your amazing friendship, for the in-person prelim study sessions, and for the virtual complaining/mutual misery/support when it was needed. Also thank you to CB, Mr. Sunshine, Bread, and Toast for joining in on the video calls.
- To Emily, for always being there, for hearing me, and for making me always feel less alone.
- To the squad, Emily, Eli, Mike, Jazib, Vicki, and Julie for hyping me up and always making me smile. I am sad that I wasn't able to attend at all of the hangouts in person, but am happy our friendship is able to cross state lines.
- To the friendships I gained in Columbus, Michaela, Emma, and Holly. Each of you are so important to me. You all have supported me through hard times and been there for some of the best. Thank you for being in my life.
- To my OG friends, Jackie and Lacey. You both are the closest thing I have to sisters. You are always there for me, always have my back, and (perhaps most importantly) will always call me out when I need it. We have lived apart for over 10 years now and I still feel as close to you both as I did the day I left. It has been amazing to be by your side when you got married, and to be there for you as you each became mothers.

Lasting friendships are rare, and I am so lucky to have found such wonderful people to surround myself with.

My parents, Tina Revermann and David Munie, and brother, Aaron Munie, are owed many thanks for their support and encouragement growing up, in college, and onward. My mom helped me with my very first science fair project and my dad bought me so many Encyclopedia

Brown and science experiment for kids books. Aaron always has my back, listens to me complain, and is the best brother one could ask for. Thank you.

I need to spend a few sentences of appreciation for my dog, Copernicus, and my cat, Lox. Cappie is always there to give me all the cuddles, face licks, and laughs that I could ever ask for. Lox has added so much love and sass to my life and has kept my lap warm during the late nights writing my dissertation. I only hope I bring as much joy to their lives as they do to mine.

Finally, I need to thank my husband, Nathaniel Lee Gardner. His patience and understanding is incredible, and his encouragement and support is unmatched. I am so lucky to have found such an amazing partner. Words will never describe the significance he brings to my life. Numbers might: $p<0.00001$.

Thank you all.

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ABSTRACT

Multiple sclerosis (MS) is an incurable inflammatory demyelinating disorder of the central nervous system (CNS). Individuals with MS are burdened by neurological deficits affecting their motor, visual, and sensory function. These symptoms are clinical manifestations of inflammation, axonal swelling and transection, myelin destruction, and neuronal death within lesions of the brain, spinal cord, and/or optic nerve. It is widely accepted that MS is an autoimmune disease driven by autoreactive $CD4^+$ T cells that, following entry into the CNS, recruit and activate other cell types.

Histopathological analyses of CNS tissue from individuals with MS and mice with the murine model experimental autoimmune encephalomyelitis (EAE) have revealed substantial myeloid cell infiltration within inflamed lesions. Recent studies suggests that myeloid cells in the CNS during EAE and MS have pleiotropic functions. Myeloid cells contribute to pathogenesis by producing cytotoxic factors and presenting antigen to encephalitogenic T cells. Conversely, they can mitigate damage through the phagocytosis of harmful debris and release of anti-inflammatory cytokines. Understanding the factors that regulate CNS myeloid cell responses could lead to innovative therapeutic strategies for MS treatment. The aim of my dissertation project was to explore the role of two candidate regulatory pathways during EAE.

The epigenetic profile of myeloid cells influences their phenotype and function in various models of inflammatory disease. Recently, ten-eleven translocation 2 (TET2), an epigenetic modifier that can suppress pro-inflammatory functions in myeloid cells, was identified as a susceptibility locus for MS. TET2 uniquely oxidizes methylcytosine to 5'hydroxymethylcytosine

(5hmC) and facilitates the active demethylation pathway. *TET2* and 5hmC are reduced in MS patient PBMCs compared with healthy controls. The objective of our study was to investigate the hypothesis that TET2 protects against aberrant myeloid cell activation during EAE, and that reductions in TET2 activity are a critical step in the development of a pro-inflammatory myeloid cell response. We found that *Tet2*-deficient mice develop more severe EAE following active immunization with myelin peptide. However, the transfer of myelin-specific $CD4^+$ T cells into *Tet2*-deficient recipients did not result in similarly abrogated disease. This suggests that TET2 does not regulate myeloid cells in a clinically significant manner, but instead may regulate $CD4^+$ T cell function.

Inflammation in EAE and MS is preferentially targeted to certain CNS regions for reasons which are unclear. Regional differences in the intrinsic regulation of resident myeloid cell populations may mediate susceptibility to lesion formation. We have found that Axl and Mer, two members of the TAM family of tyrosine kinases, are highly expressed on microglia in the hindbrain, compared to the spinal cord, both during homeostasis and at EAE onset. In conventional EAE, inflammation localizes to the spinal cord. Blockade of Axl and Mer signaling using a pan-TAM receptor inhibitor (LDC1267) favored the development of an atypical, brain-targeted form of EAE. TAM receptor inhibition did not alter leukocyte populations in the hindbrain during EAE. However, it promoted the migration of leukocytes deeper into the brainstem parenchyma. Our data implicate TAM receptors as region-specific inflammatory regulators during EAE.

Collectively, these findings contribute to our understanding of the regulation of myeloid cells in neuroinflammation. The presented data support a role of TAM receptors, and disputes a

role of TET2, in controlling myeloid cell responses during EAE. Our studies provide insight that supports the development of more efficacious therapeutic options for MS patients.

Chapter 1

Introduction

Portions of this chapter are part of a manuscript in preparation:

Gardner AM, Atkinson JR, Mockus TE, Segal BM, Akkaya B. The Multi-faceted Contribution of Dendritic Cells to Central Nervous System Autoimmunity.

The work presented in this dissertation is the product of a pursuit to further understand the regulation of myeloid cells as an aberrant inflammatory response transpires in the central nervous system (CNS). Over the past three years, the SARS-CoV-2 pandemic has demonstrated that a strong and rapid immune response against infectious agents protects the lives of loved ones. Less commonly appreciated is that when the infection is cleared, health then necessitates rapid immune control to minimize tissue damage. When the immune attack is not directed towards harmful agents, but is instead directed against innocuous self-protein, an individual is burdened with a deleterious immune response, tissue damage, and associated symptoms. In this case, protection from the malady requires interventional immune control. Intervention requires understanding of the dynamic pathways driving and restraining the response; the former in order to oppose and the later to encourage.

The overall goal of my thesis project was to investigate pathways that restrain and regulate myeloid cells, a group of pathogenic effector cells in the presumed autoimmune (self-targeted) disease, multiple sclerosis. Disease modifying therapies for MS patients, particularly those with progressive forms of disease, are limited, and new therapeutic targets may provide more efficacious interventions. To achieve this goal, we used a murine model of inflammatory

demyelinating disease similar to MS, experimental autoimmune encephalomyelitis. The work presented here focuses on two distinct phases of autoimmune pathogenesis – their activation and polarization into pathogenic effectors at disease initiation and the acquisition of an inert, or even immunosuppressive phenotype as inflammation subsides. Two main lines of inquiry were explored, but neither yielded the results we expected. Even so, these findings represent five years dedicated to understanding myeloid cell regulation. The questions asked in this work were valid and well-informed, and the experiments performed yielded important outcomes and answers. Another important outcome, of course, was the growth and development of a young scientist who defines success as finding the *right* answer, even if it is not the exciting one. By these, the project was successful.

The Role of Myeloid Cells in an Antigen-specific Immune Response

An immune response against factors that are recognized as foreign to the body requires a collaboration between the adaptive and innate arms of the immune system. The innate immune system arose early in the evolution of multicellular organisms to provide protection from pathogenic microbes[1]. Pathogen-associated molecular patterns (PAMPs) are conserved elements expressed by large families of microbes that evoke an innate immune response via specialized receptors on the immune cell surface[1]. Related receptors also respond to factors released during cellular stress or injury, termed danger-associated molecular patterns (DAMPs)[2]. The receptors that recognize various PAMPs and DAMPs activate pathways within the cell that appropriately respond to that specific factor. In contrast, innate cells also express inhibiting receptors that bind ligands on the surface of healthy cells, in order to limit aberrant cell activation[1]. Through many million years of evolution, these processes have become more detailed and refined. Even

following the development of adaptive immune cells, these innate systems play a vital role in the immune response.

It is presumed that the adaptive immune system evolved in jawed predators, which were under strong selective pressure due to their larger bodies and smaller brood sizes[3]. Rapid and robust responses were necessary for the species to survive continuous microbial insult. The adaptive system is unique in that it recognizes a myriad of diverse pathogens, mounts an antigen-specific response, and forms an immunological memory that facilitates quick and efficient clearance upon secondary challenge[3]. Three major lymphocyte populations in the vertebrate adaptive system are $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells, and B cells. Each cell expresses a unique T cell receptor (TCR) or B cell receptor (BCR) that is "adapted" to be specific for an antigen expressed by pathogens, yet non-reactive against self protein[3]. While activated lymphocytes play many roles, $CD4^+$ T cells release soluble factors that activate other cell types during infection, $CD8^+$ T cells directly inflict cellular damage and are important in antiviral responses, and B cells secrete antibodies and are central to the humoral immune response against pathogens.

The coordination between adaptive and innate cells begins at the initiation of an antigen-specific response. Antigen-specific T cells are activated via the process of antigen presentation; professional antigen presenting cells (APCs) activate T cells via the engagement of the TCR by antigenic peptide-loaded major histocompatibility complexes MHCII ($CD4^+$ T cells) or MHCI ($CD8^+$ T cells) to the TCR[4]. This process requires the simultaneous binding of costimulatory molecules and the release of pro-survival and differentiation factors[4]. Macrophages and monocyte-derived dendritic cells (mDCs) are innate APCs derived from myeloid progenitor cells (termed "myeloid cells"). Due to the necessity of APCs for an antigen-specific response, myeloid

cells are critical to adaptive immunity. Following the initial activation and clonal expansion, T cells are recruited to the site of inflammation and reactivated. It is then that they can facilitate a coordinated attack, releasing pro-inflammatory, chemotactic, and cytotoxic mediators that recruit and activate other immune and supporting cells[5]. Myeloid cells play an important role in the reactivation of T cells at the site of inflammation and play a critical role in the elimination pathogesn[5].

Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating disease in which an aberrant inflammatory response arises in the central nervous system (CNS). MS lesions are associated with axonal swelling and transection, myelin destruction, and gliosis[6]. In 2020, there was an estimated 2.8 million people living with MS globally, a number that has increased significantly since 1990[7,8]. Patients with MS experience a wide range of neurological symptoms, including paraparesis, paresthesia, ataxia, vision impairment, and urinary tract dysfunction[9]. These symptoms are clinical manifestations of inflammatory demyelinating lesions that form in the brain, spinal cord, and/or optic nerve[9].

Demyelinating lesions in patients with a history of relapsing neurological deficits were first documented in the late 1830's by Robert Carswell and Jean Cruveilhier, independently[10]. The establishment of MS as a distinct disease entity came 30 years later, when Jean-Martin Charcot described a triad of symptoms (known as Charcot's neurological triad) of nystagmus, ataxia, and dysarthria, and linked these neurological deficits with inflammatory demyelinated lesions in post-mortem CNS tissue[11,12]. It was not until the late 20th century that demyelinating white matter lesions could be visualized in living patients using magnetic resonance imaging

(MRI) and quantitative clinical scales were developed for assessment of disability[12,13]. A hallmark of MS is the presence of multiple demyelinating lesions that form at different time points (dissemination in time), in distinct regions of the CNS (dissemination in space)[12,14].

Today, it is appreciated that MS is a heterogenous disease, with respect to the clinical course, lesion load and distribution, and the prominence of different histopathological features[12]. The majority of patients (85%) initially present with relapsing-remitting MS (RRMS), characterized by recurrent, self-limited episodes of neurological dysfunction (relapses) separated by periods of clinical stability (remissions)[15]. RRMS typically onsets in the 20s or 30s[16]. The majority (62%) of individuals with RRMS will ultimately transition into a stage of disease characterized by a gradual, relentless accumulation of disability. This is referred to as secondary progressive MS (SPMS)[16]. Approximately 50% of RRMS patients develop SPMS within 10 years of RRMS onset[15,17]. A minority of patients (10-15%) have primary progressive disease (PPMS), with no initial relapsing-remitting phase[15]. The average age of onset of PPMS patients is 45 years old and the clinical course is similar to that of SPMS, sans the initial relapsing-remitting phase[15,16].

Both genetic and environmental factors can confer an increased risk of MS development. The vast majority of MS genetic susceptibility loci relate to immune function[18,19]. The strongest susceptibility locus lies within the HLA-DRB1 gene[18,19]. However, established risk loci only account for around half of disease risk, though some studies have suggested that the actual genetic contribution is not fully captured by current analysis standards, and that heritability is closer to 68%[20,21]. While studies from monozygotic twins show a concordance rate of approximately 25%, estimates of heritability across studies demonstrates substantial variability; the contribution of genes is speculated to be anywhere from 0.25-0.76 (h^2 calculation,

disregarding wide confidence intervals)[22]. Environmental risk factors include childhood obesity, vitamin D deficiency, smoking, and Epstein-Barr virus (EBV) seroconversion during adulthood, all factors that can influence immune system function[23–26]. In addition to Caucasian populations, MS is more prevalent in women and those born at higher latitudes[6,27–29]. However, MS is generally more severe in non-Caucasian populations and in men, an observation that requires more attention in future studies[30,31].

The pathogenesis of MS is ill-defined and disease-modifying drugs are expensive and not universally effective[32]. According to the National Multiple Sclerosis Society, the annual economic burden of MS in the United States is $85.4 billion a year[33]. The individual impact of MS is even more exigent – symptoms can be pervasive and may significantly decrease a patient's mobility, employment, and quality of life[34,35]. Currently, there are over 20 disease modifying therapies (DMTs) that are FDA approved for the management of RRMS. Most of these drugs target the adaptive immune response and either deplete lymphocytes or curtail their trafficking into the CNS[36]. These therapies are not universally effective in treating RRMS, are generally ineffective in patients with progressive courses, and none are curative[36]. With the number of MS patients rising every year, there is a dire need for more effective DMTs, potentially focused on cell populations currently underutilized as therapeutic targets. An increased understanding of pathogenic and regulatory effector cells in MS will lead to the identification of novel therapeutic strategies.

The Pathological Features of MS

The hallmark features of an active MS lesion are focal inflammation, blood brain barrier (BBB) breakdown, reactive gliosis, demyelination, and axon damage[37]. Though MS was

classically considered a demyelinating disease, it is now appreciated that permanent neurological disability is secondary,in part, to axon damage and subsequent degeneration[38]. The development of inflammatory MRI lesions is not always associated with symptoms[39]. This "clinico-radiological paradox" is explained by lesion location, rather than overall lesion burden, being the determinant of disability[40]. Lesions only cause clinical deficits when they are located in areas where nerve fibers, subserving the same function, converge, and there is little bandwidth for compensation by redundant pathways. Neurological signs correspond to anatomical lesion distribution within the CNS[41–50].

MS lesions preferentially develop in certain regions of the CNS, though the reason for this is unclear[51]. Demyelinating lesions are centered around a central vein, from which peripheral immune cells infiltrate the white matter parenchyma[52,53]. Brain white matter lesions are frequently located adjacent to the cortex (juxtacortical)[54] or the ventricles (periventricular)[55,56]; within the cerebellum, cerebellar peduncles, brainstem[55]; or corpus collosum at the callososeptal interface[57]. White matter located beyond the boundaries of well-defined lesions, sometimes termed "normal appearing white matter", can be abnormal upon histological examination, and contain diffusely activated microglia and scattered mononuclear cells[58]. This pathology is most pronounced in progressive patients[58]. In MS, lesions can also form in the grey matter, including the cortex and deep nuclei, due to the presence of myelinated axons projecting to or from the white matter. Cortical lesions are difficult to visualize using MRI, but are classified histopathologically: intracortical lesions are located within the cortical ribbon, leukocortical lesions are at the grey-white matter junction, and subpial lesions are within the most superficial layers of the cortex[59]. Cortical lesions have been detected at early onset MS, but are most profuse in progressive MS and correlate with disability accumulation[60,61].

MS lesions are classified in post-mortem or biopsied tissue based on myelination status; these characteristics reflect the stage of lesion development[58,62]. Active white matter lesions are populated by lymphocytes and phagocytes and have evidence of blood-brain barrier breakdown, reactive gliosis, and demyelination[58,63]. Myelin degradation byproducts, detected in the cytoplasm of phagocytes within active lesions, can provide clues regarding the age of the lesion[58,64]. Early myelin byproducts are found within phagocytes along the lesion rim, while later myelin byproducts are found closer to the lesion's center, suggesting the inflammatory process expands outward from the center[58]. Chronic active lesions, found in progressive MS patients, have an inactive core surrounded by a rim of activated microglia[65,66]. In these lesions, the lesion center is inactive while activated microglia are found in the rim[67]. In contrast to active and chronic active lesions, inactive lesions are bereft of infiltrating leukocytes or activated microglia[67]. Finally, previously-demyelinated shadow plaques are hypocellular but have thin myelin sheaths surrounding the existing axons, a sign of repair and remyelination[68]. One study showed that dynamic remyelination in the brains of individuals with MS, measured using experimental imaging techniques, was inversely associated with clinical disability[69]. Whether this is a causal relationship or instead reflective of a less efficient immune response has yet to be determined.

Etiology and Pathogenesis of MS

The cause of immune-mediated demyelination in MS patients is unknown. It is clear that neuroinflammation is essential to MS pathogenesis, but the mechanisms that initiate, guide, and sustain the response are poorly understood. Traditionally, organ specific autoimmune diseases have a specific autoantigen or autoantigens[70]. However, no such autoantigen has been

consistently implicated in MS patients[71,72]. This raises the question of whether demyelination and/or axon degeneration are direct targets or a secondary consequence of CNS inflammation. One theory is that immune response against infectious agents, such as Epstein Barr Virus (EBV), with epitopes similar in sequence to myelin protein epitopes, triggers autoimmunity. In support of this theory are several studies observing finding an increased risk of MS following EBV infection and high anti-EBV nuclear agent antibody titers in patients[24,73,74]. Some studies also demonstrated the presence of EBV in some, but not all, MS lesions[75–78]. In addition to EBV, 63% of MS patients have antibodies specific for common vaccine-targeted neurotrophic viruses, such as measles virus, rubella virus, and varicella zoster virus, within the cerebral spinal fluid (CSF)[79]. This finding was quite specific to MS patients, as these antibodies were only present in < 3% of those without MS, including those with the CNS-autoimmune diseases neuromyelitis optica (NMO) and MOG-antibody associated disease (MOGAD)[79]. The vast majority of studies have found no evidence of active viral replication or protein expression in the CNS or CSF of MS patients. These findings suggest that there is polyclonal expansion of a native B cell repertoire during MS. The etiology of an autoinflammatory response is likely multifactorial, and may require an innate inflammatory trigger to initiate CNS inflammation[24,80,81].

An autoimmune etiology of MS is supported by genetic studies. Over 200 risk loci have been identified in MS, and the majority of these lie within genes related to the immune response[21,82,83]. The strongest susceptibility locus, HLA-DRB15:01*, implicates a role of $CD4^+$ T helper (Th) cells in pathogenesis[84–86]. Unlike MHC-I that is expressed by all nucleated somatic cells, MHC-II expression is restricted to professional APCs, including macrophages, DCs, and B cells[87]. Genetic variants of other molecules involved in Th cell stimulation, such as CD80, CD86,

and CD40, are also associated with increased risk of MS, further supporting a role of APC-T cell interactions in MS pathogenesis[88,89].

Treatment of MS

There are over 20 therapies currently approved for the treatment of MS. Some of these drugs deplete lymphocytes (e.g. ocrelizumab and alemtuzumab), while others curtail lymphocyte trafficking to the CNS (natalizumab and fingolimod), or are general immunosuppressants (e.g. cladribine, teriflunomide, and dimethyl fumarate)[36,90]. Unfortunately, many of these drugs come with side effects that range from mild and unpleasant, to severe life-threatening infections such as progressive multifocal leukoencephalopathy (PML)[91]. While these drugs are effective in reducing relapse rates in most RRMS patients, none are curative or induce repair[92,93]. Current therapies have limited, if any, effect in progressive forms of MS[93]. This suggests that CNS infiltration by T and B cells may be less important in driving progressive disease[94]. As previously mentioned, progressive patients have distinctive CNS pathology, with smoldering/chronic white matter lesions characterized by chronic myeloid cell activation at their advancing edge[95–97]. It has thus far been unclear why some patients initially present with PPMS, and why some RRMS patients do not advance to progressive disease[15]. Distinct pathological mechanisms underlying RRMS and progressive MS may be responsible for the discordance in therapeutic responsiveness.

Experimental Autoimmune Encephalomyelitis

Much of our understanding of the pathogenesis of CNS autoimmune disease has come from animal models, most commonly Experimental Autoimmune Encephalomyelitis (EAE).

While EAE is an imperfect model of MS, certain aspects of the pathogenic process, including demyelination, CNS infiltration of leukocytes, and glial cell activation, are recapitulated. The ability to manipulate variables within a controlled system is essential for our understanding of cause and effect during MS development and progression.

EAE has been induced in mice (most commonly), rats, guinea pigs and non-human primates. The first artificial induction of an inflammatory demyelinating disease was unintentional, occurring in individuals that received the early generation rabies vaccine[98]. It was determined that traces of myelin, originating from the hosts used to propagate the virus during vaccine development, contaminated the vaccine and induced a demyelinating syndrome[99]. This phenomenon was replicated in rhesus macaque monkeys immunized with CNS tissue emulsified with an adjuvant, which resulted in MS-like CNS lesions[98]. Since then, an experimental myelin-targeted disease has been induced in several strains of rodents[100]. Similar to MS, inflammation during EAE preferentially targets certain areas of the CNS. Conventional forms of EAE present with ascending paralysis associated with spinal cord inflammation and demyelination[101]. In contrast, ataxia, listing, and hindbrain inflammation occurs in an atypical form of disease that has been observed following immunization of mice genetically deficient in IFN-γ, IFN-γ receptor or SOCS3[102,102,103]. The mechanisms that drive inflammatory infiltration of different CNS regions are poorly understood.

EAE induced by the direct immunization of mice with myelin peptide in combination with adjuvants such as CFA, is commonly referred to as “active” EAE (Figure 1.1.A)[100]. Administration of chemically inactivated Bordetella pertussis toxin on days 0 and 2 post immunization is also required for disease induction in most inbred murine strains. The pathogenesis of active EAE begins with expansion and polarization of myelin-specific Th1 and

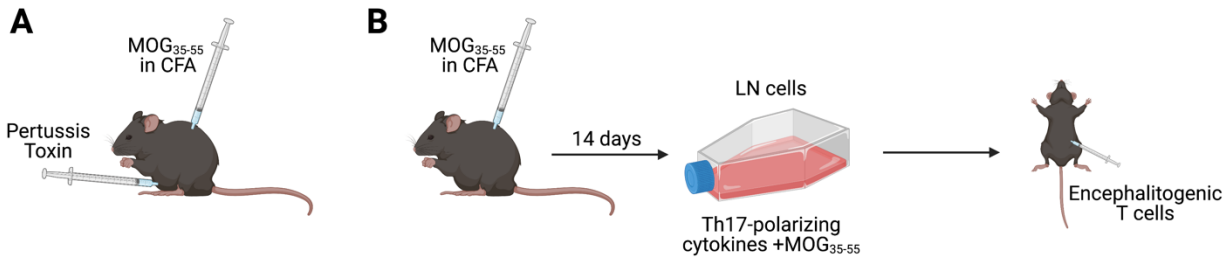

Figure 1.1 - Active immunization and adoptive transfer models of EAE.
EAE can be induced via A) active immunization with MOG_{35-55} peptide emulsified in complete Freund's adjuvant (CFA), followed by pertussis toxin administration on days 0 and 2, or B) via the adoptive transfer of encephalitogenic $CD4^+$ T cells which are harvested from the lymph nodes (LN) of MOG_{35-55} primed mice and expanded and polarized *in vitro* prior to the transfer into naïve syngeneic recipients.

Th17 cells in the draining lymph nodes proximal to the immunization site, via interactions with APCs presenting the immunizing antigen[84]. This is followed by the migration of the antigen experienced, myelin-specific effector Th cells to the CNS, where they are reactivated by a less understood set of interactions with resident and/or infiltrating APCs[84]. An alternative method of EAE induction, termed "passive" or "adoptive transfer" EAE, requires the injection of myelin-specific Th1 or Th17 effector cells, isolated from the draining lymph nodes of immunized mice and expanded and polarized *in vitro*, into naïve syngeneic hosts (Figure 1.1.B) . This protocol does not require the administration of either CFA or pertussis toxin to the T cell recipient. In this model, transferred T cells travel to the CNS where they are reactivated, likely via interactions with APCs similar to those in active EAE[84]. The clinical course varies between animals with different genetic backgrounds[101]. Common models include SJL mice actively immunized with proteolipid protein (PLP) peptide, which presents as a relapsing-remitting disease course, and C57BL/6 mice actively immunized with myelin oligodendrocyte glycoprotein (MOG) peptide, which presents as a monophasic course with chronic neurological deficits[101]. Either phenotype can also be induced in the absence of exogenous peptide or adjuvant by the adoptive transfer of $CD4^+$ T cells with specificity towards the peptides[101]. Though B cells are presumed to be

important for MS development, B cells do not play a critical role in EAE models induced by myelin peptide, but are required in some EAE models induced using whole MOG protein[104,105]. In either the active or adoptive transfer model, inflammatory myeloid cells, such as neutrophils, DCs, macrophages, and microglia, are critical for developing neurological deficits during EAE[106–110]

Myeloid Cell Populations & Functions during EAE

Histopathological analyses of CNS tissue from individuals with MS and mice with EAE have demonstrated substantial myeloid cell infiltration in inflamed lesions and a spatial association with myelin damage[111–113]. Microglia, macrophages, DCs, and neutrophils are particularly important to the clinical course of EAE and make up the majority of cells within active MS lesions[64,95,110,114–117]. There is growing evidence that myeloid cells accumulating in the CNS during EAE and MS have pleiotropic functions and evolve during the clinical course[113]. Here we will review the known functions of each of myeloid cell type as they relate to the pathogenic processes during EAE and MS.

Microglia and Macrophages

Microglia are brain-resident glial cells that arise during development from progenitors in the embryonic yolk sac[118,119]. In the murine adult, they are repopulated from CNS-resident progenitors[120,121]. They make up 5-12% of glial cells in the murine brain, where they are concentrated to the grey matter[122]. In the human brain, more microglia are located within white matter compared to grey matter[123]. Microglia are mononuclear phagocytes and function as a support cell in homeostasis[124]. Their transcriptional profile varies between different regions in

the CNS and suggests functions that are catered towards the needs of a specific CNS compartment: debris clearance in regions with a high degree of neuronal death, synapse engulfment in areas undergoing active neuronal remodeling, neurotransmitter uptake for control of neuronal excitability, trophic support during neurogenesis, and immune surveillance in regions vunerable to infection[124,125]. Interestingly, other glial populations appear to compensate for homeostatic microglial function in their absence[126]. However, during infection or in the presence of toxic, damaging factors, the role of microglia is non-redundant and can be beneficial[127,128].

In contrast to microglia, monocyte-derived macrophages infiltrate the CNS from the bloodstream or skull bone marrow. They arise in response to environmental challenges[129]. Very few peripherally-derived macrophages are present within the healthy murine or human CNS, but large numbers infiltrate through a leaky blood brain barrier (BBB) during inflammation[129,130]. Microglia in a resting state can be easily distinguished from macrophages, morphologically or by the expression level of CD45 or Iba1[131]. During inflammation, the two populations become more similar – activated macrophages can upregulate Iba1, and activated microglia upregulate CD45 and acquire a more ameboid morphology[131]. A separate population of embryonically derived macrophages, termed border-associated macrophages (BAMs) exists in the brain during homeostasis and responds during inflammation. BAMs are located in the subdural meninges, perivascular spaces, and within the choroid plexus[130]. Phenotypically, these macrophages closely resemble microglia and cannot be differentiated using traditional myeloid cell markers (CD45, CD11b, CD68, CX3CR1, F4/80, CSF1R)[132]. Approaches using markers more specific to

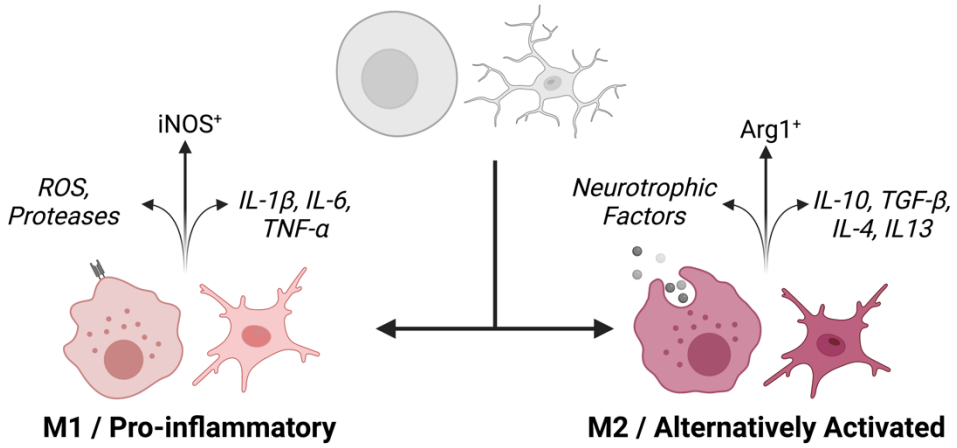

Figure 1.2 - Dynamic myeloid cell phenotypes and functions.
Myeloid cells have dynamic functional phenotypes. Bone marrow derived monocytes can be polarized in vitro towards as "M1"/ pro-inflammatory or M2/ alternatively activated, lineage. Pro-inflammatory "M1" cells (left) express iNOS and can efficiently present antigen, and release inflammatory and/ or damaging factors. Alternatively activated "M2" cells (right) express Arg1 and release neurotrophic factors, regulatory cytokines, and have enhanced phagocytosis capabilities

microglia, such as TMEM119, P2RY12, and SiglecH, will be essential to discern the individual contributions to homeostasis and inflammation[132].

Microglia and macrophages have dynamic phenotypes that are acquired in response to specific environmental signals. Studies *in vitro* have found that activating macrophages with IFNγ, so called "classical activation", enhanced their bactericidal activity[133,134]. In contrast, treatment with IL-4 or IL-13 induced an "alternatively activated" phenotype, with less cytotoxic activity and pro-inflammatory cytokine expression, but a higher expression of mannose receptor[134–136]. Upon further characterization, these activation states were found be related to the Th1/type 1 and Th2/type 2 responses, respectively, and thus were assigned the names M1 and M2[137]. Importantly, the classification of these activation states reflect different cellular phenotypes and functions, rather than the differentiation to a separate lineage.

These patterns have been identified in microglia and replicated *in vivo* to an extent, as many other cytokines were found to differentially activate myeloid cells[138]. The paradigm of an

M1/M2 axis, though over simplified, has been helpful in understanding cellular processes in tumor immunology, autoimmune disease, and infection (Figure 1.2)[139]. M1 cells, commonly defined by the expression of inducible nitric oxide synthetase (iNOS), are generally more pro-inflammatory; they produce IL-1β, IL-6, and TNF-α, in addition to reactive oxygen species[140,141]. M2 cells, expressing arginase-1 (Arg1), release IL-10, IL-4, TGF-β, and IL-13 and have greater phagocytic capabilities[138,140–142]. Accumulation of myelin and cellular debris inhibits oligodendrocyte progenitor differentiation and remyelination, thus clearance of this debris promotes CNS repair[143].

In EAE, there is a transition in CNS myeloid cell phenotype and function as disease progresses. Classically activated macrophages and microglia predominate during acute lesion formation and putatively inflict damage to CNS tissues by producing pro-apoptotic factors, free oxygen radicals, and proteases, and by presenting antigen to encephalitogenic T cells[113,115,116,144,145]. In chronic active MS lesions, the ratio of classically activated to alternatively activated myeloid cells is higher in the lesion rim (a site of ongoing demyelination) compared to the inactive lesion core (where inflammation has already subsided)[113,146,147]. This suggests that myeloid cells transition towards an alternative phenotype during MS lesion evolution, analogous to what was observed in EAE. CNS-infiltrating, $iNOS^+$ macrophages are believed to be responsible for the majority of myelin damage, as blocking the recruitment of these cells abrogates EAE[108,148,149]. Similarly, increasing the number of pro-inflammatory $Ly6C^+$ monocytes in circulation exacerbates EAE[115]. These cells can transition, on a single-cell level, to an alternatively activated phenotype as EAE progresses[113]. In fact, 33% of $Arg1^+$ myeloid cells present at peak disease previously expressed iNOS[113].

Leukocytes expressing Arg1 are more frequent immediately prior to and during EAE remission, and may be protective[113]. Bone marrow chimeric mice, in which irradiated wild-type (WT) mice received bone marrow from mice expressing the diphtheria toxin receptor under control of the Arg1 promoter (Arg1-DTR), were treated with diphtheria toxin (DT) to deplete alternatively activated cells prior to the adoptive transfer of Th17-polarized MOG_{35-55}-specific T cells. Compared to controls, these mice had more severe EAE and a greater number of pro-inflammatory myeloid cells (Segal lab, unpublished). This phenotype was not due to the loss of Arg1 itself, as *Arg1*$^{-/-}$ mice do not have an altered disease course (Segal lab, unpublished). Other studies have shown that introducing alternatively activated macrophages during EAE suppresses ongoing inflammation[150,151]. The specific mechanism through which these cells mediate protection is a focus of ongoing studies, but Arg1^{+} cells are known to have a variety of beneficial functions during EAE. Arg1^{+} myeloid cells have been demonstrated to release the immune suppressing cytokines IL-10 and TGF-β, to release neurotrophic factors that support CNS repair, and to have enhanced phagocytic ability[113,138,144,152,153].

These studies demonstrate that the phenotype of microglia and macrophages during EAE is dynamic and changes in response to the CNS environment[113,154]. Given the regulatory properties of alternative activated microglia and macrophages, as well as the understanding that their phenotype can be induced by the CNS environment, these cells are a prime candidate to target for therapies focused on dampening an ongoing immune response within the CNS.

Dendritic cells

Various subtypes of DCs exist in both mice and humans, each with their own roles in inflammatory processes. Two major types of myeloid DCs have been identified, distinguished by

their cellular origins. Classical or conventional dendritic cells (cDCs) differentiate within tissues from a pre-cDC precursor[155–159]. Monocyte-derived dendritic cells (moDCs or mDCs) arise from circulating monocytes that infiltrate tissue during inflammation[155–157,159]. DCs can sometimes be differentiated from other murine myeloid populations by expression of CD11c[117]. Murine cDC and mDC populations have overlapping protein expression, but can be differentiated using transcription factor expression (e.g. ZBTB46), fate mapping (e.g. CLEC9A), or surface marker expression (CD26 and CD88/C5a receptor, respectively)[160–163]. In humans, CD209 (DC-SIGN) is used to distinguished DCs from other populations, as both human monocytes and macrophages can express high levels of CD11c[164].

Recent high-dimensional mass cytometry and fate mapping of the murine brain, spinal cord, and meninges indicate that DCs contribute up to 2% of total leukocytes in the naïve CNS[155,164–166]. The majority of CD11c-expressing cells have been found in the pia mater, subarachnoid space, and dura mater of the meninges, with very few in parenchymal tissue[110,167,168]. Similar to the CNS of mice, the human brain and spinal cord parenchyma are normally devoid of DCs, but CD209-expressing cells are present in the meninges and perivascular spaces[169].

When an immune response is initiated in the CNS, murine resident DCs are important in reactivating encephalitogenic $CD4^+$ T cells upon their arrival in naïve CNS[109]. Meningeal whole mounts have demonstrated that CD11c-expressing cells make up a substantial percentage of $MHCII^+$ cells in the pia and dura mater (23% and 62%, respectively), and *in vitro* assays have demonstrated that CNS $CD11c^+$ cells are competent APCs[170,171]. Following interactions with APCs in border associated regions, myelin-specific $CD4^+$ T cells access the spinal cord

parenchyma[172]. This indicates a key role for DCs, particularly cDCs, in immune surveillance and initiating autoimmunity in the naïve CNS.

During the evolution of EAE , 30-40% of the immune cells accumulating in the CNS are DCs, and they can play multifaceted roles[155]. $CD11c^+$ cells accumulation in the CSF, meninges, perivascular space, and white matter parenchyma is an early detectable event during EAE progression, occurring prior to the onset of symptoms[110,173,174]. Within the inflamed spinal cord, perivascular $CD11c^+$ MHCII-expressing cells spatially associate with $CD4^+$ T cells, and antigen presentation by these cells alone are sufficient to reactivate encephalitogenic $CD4^+$ T cells in the CNS[109,172]. Further, recipients in which H2-Ab1 expression is restricted to CD11c-expressing cells are susceptible to induction of EAE, indicating that antigen presentation by $CD11c^+$ cells is sufficient for the reactivation of encephalitogenic T cells in the CNS[109]. In addition to antigen presentation, DCs have a propensity to polarize $CD4^+$ T cells to the pathogenic Th1 and Th17 cells through the release of IL-12 and IL-23[175–177]. These studies evidence the ability of DCs to drive and sustain CNS autoimmunity by promoting encephalitogenic T cell activation and polarization in animal models.

However, in addition to their pathogenic functions, DCs have been shown to mediate tolerance in the CNS via either direct cell-to-cell interactions or via modulation of the CNS microenvironment through cytokines and chemokines[178,179]. DCs can directly limit T cell differentiation into Th1 and Th17 lineages through the release of IL-27 and TGF- β[180–183]. DCs can further support tolerance by releasing IL-10 and promoting proliferation, activity, and differentiation of T_{reg} cells during EAE through various mechanisms[184–187]. The protective function of DCs extends past the modulation of $CD4^+$ T cells, as they can assist microglia and macrophages in efficiently phagocytose myelin and cellular debris[110,188,189]. Similarly to

microglia and macrophages, the classically activated versus alternatively activated nomenclature can be applied to iNOS$^+$ and Arg1$^+$ DCs, respectively[113]. Arg1$^+$ DCs are inefficient antigen presenters but do not actively suppress T cells, suggesting these cells represent a population of exhausted DCs that may be inertly contributing to inflammation resolution[113]. The multifaceted roles of DCs demonstrate the importance of this cell type to initiation and suppression of inflammation during EAE and establish a need to understand how these findings translate to MS patients.

Neutrophils

Neutrophils are a pathogenic polynuclear myeloid cell in EAE. In infectious models, neutrophils are important to the clearance of pathogens by the release of pro-inflammatory cytokines and chemokines, ROS, and nuclear extracellular traps (NETs), as well as direct removal through phagocytosis[190]. These functions can be damaging to tissue if inappropriately directed or not properly suppressed, as is the case in autoimmunity.

Neutrophils infiltrate the spinal cord early in EAE, but are rare later in disease[191]. Depleting neutrophils from the circulation at early stages of EAE, through the administration of antibodies specific for CXCR2 or Ly6G, delays onset and reduces the severity of neurological deficits[106,192,193]. Similarly, mice deficient in granulocyte activating and mobilizing factor (G-CSF) are highly resistant to EAE development[194]. Neutrophils perform multiple functions throughout the course of EAE including the release of factors that are potentially harmful. At the clinical onset of EAE, neutrophils release pro-inflammatory cytokines such as IL-1β, IFN-γ, TNF-α, and IL-6[195,196]. The release of these cytokines promotes the recruitment and activation of APCs[195,196]. CXCR2$^+$ neutrophils are also important for the disruption of the blood brain

barrier[193]. In addition to cytokine release, scanning electron microscopy has detected neutrophil phagocytosis of myelin, but this has yet to be confirmed in other studies[197]. In addition, direct neuronal cell death by neutrophils has been suggested by *in vitro* studies showing neutrophil migration across brain endothelium activates NET production and results in neuronal cell death, but there is no evidence of this occurring *in vivo*[198].

CNS-infiltrating neutrophils are particularly prevalent in brain-targeted forms of EAE, termed "atypical" EAE. Hyperactivated neutrophils that overexpress ROS are found in the cerebellum and brainstem of certain genetic knockouts that develop atypical disease[102,199–202]. One study found neutralization of either ROS or G-CSF reduces the severity and incidence of atypical EAE[200]. Interestingly, neutrophil-specific SOCS3 deficiency results in the development of atypical EAE and associated cerebellar infiltration of neutrophils[203]. These data underscore the possibility that neutrophil overactivation can drive the development of CNS inflammation in specific CNS compartments.

Myeloid Cell Regulation & Potential Therapeutic Targets

Myeloid cells are currently an underutilized target of DMTs. This is may be particularly pertinent to progressive MS, for which current therapies are minimally effective[93]. Myeloid cells make up the majority of cells within MS lesions and are important in the development and progression of EAE[64,95,110,114–117]. Our lab and others have shown that depletion of myeloid cells during acute EAE abrogates disease[108,115,149]. However, this approach can cause sustained immunosuppression and does not distinguish between conventional pathogenic versus alternatively-activated and beneficial myeloid cell subsets[113]. A more effective strategy may be to modulate the dynamic myeloid cell phenotypes. This would require elucidating the pathways

that drive myeloid cell polarization towards different lineages. It is also important to recognize that myeloid cells play a key role in protection against infectious agents[204]. Therapies that disarm or deplete pathogenic myeloid cells, without affecting protective myeloid cells, would be ideal.

Previous literature suggests a specific signal or signals that trigger the transition of inflammatory myeloid cells to a regulatory phenotype[113]. Two functional pathways that regulate myeloid phenotype and function will be discussed in the current section: phagocytosis and the TAM receptors; and DNA methylation, hydroxymethylation, and demethylation.

Phagocytosis and the TAM receptors

During CNS autoimmunity, phagocytosis contributes to both disease pathogenesis and resolution. The primary phagocytes in conventional EAE and MS are macrophages, DCs (mDCs and cDCs), and microglia. Phagocytes containing engulfed myelin, so called "foamy" phagocytes, are found in the CNS of MS patients within active lesions and chronic active lesion rims, and constitute the majority of myeloid cells in these areas[205]. During EAE, phagocytosis by these cells is facilitated by various receptors and receptor families, including TAM (Tyro3, Axl, Mer) receptors, Fc receptors, complement receptors, and scavenger receptors, as well as TREM2[188,206,207]. Phagocytosis is essential for the uptake of antigenic peptides for presentation to $CD4^+$ T cells. In this way, it is pathogenic. As damage accumulates in the CNS, myelin and apoptotic cell debris can promote inflammation, by activating DAMP receptors, and inhibit repair and remyelination, by inhibiting oligodendrocyte differentiation and function[143]. In this way, phagocytosis of damaging debris promotes CNS repair.

Recognition of myelin debris by certain phagocytosis receptors, namely TREM2 and the TAM receptors Axl and Mer, not only mediate clearance, but also induce an anti-inflammatory

phenotype in myeloid cells and microglia[208–212]. *In vitro*, myelin can dampen a pro-inflammatory response and induce an anti-inflammatory phenotype, though outcomes can vary depending on the mechanism of activation and the timing of myelin administration[209,213]. Activation of phagocytosis receptors, such as Mer, by myelin can facilitate greater receptor expression, a positive feedback loop that strengthens the regulatory response to environmental signals[210]. However, phagocytosis of myelin and the polarization state of the cell are subject to reciprocal regulation[188]. Alternative activation of human microglia and monocyte-derived macrophages with IL-4 and IL-13 leads to greater phagocytosis of myelin than classical activation with LPS[214]. Within MS lesions, myelin-containing phagocytes express anti-inflammatory molecules, such as mannose receptor, IL-10, TGF-β, IL-4, and IL-1α[213]. It is unclear whether the activation status of the cells promotes myelin phagocytosis, or if myelin phagocytosis drives the observed activation status. their activation status is in response to myelin phagocytosis[213].Together, these studies suggests that cytokines and myelin debris in the CNS environment collaborate to dictate the circumstances which require immune suppression.

The most well studied mediators of the aforementioned relationship of myelin and myeloid cell phenotype are the TAM receptor tyrosine kinases Mer and Axl. TAM receptors are expressed by many cell types in many tissues and facilitate phagocytosis of molecules with extracellularly-exposed phosphatidylserine (PS), including apoptotic cells and myelin[215]. PS binds to the TAM receptors through a linker molecule, most notably grow arrest-specific 6 (Gas6) or protein S (ProS). Upon binding to the linker molecules, TAM receptors upregulate phagocytosis machinery through the PI3K/AKT pathway[215]. Within myeloid cells and microglia, TAM receptor engagement signals for immune suppression in parallel through JAK/STAT induction of cytoplasmic SOCS1/SOCS3 expression[216].

The regulatory nature of TAM receptors may be useful for suppressing latent inflammatory reactions during homeostatic phagocytosis[217,218]. During disease, mechanisms which recognize inappropriate inflammation or tissue damage and respond by suppressing inflammatory pathways prevent further damage. In a model of traumatic brain injury, signaling through the TAM receptors promotes the expression of M2-like genes and alleviates inflammation[219]. In cuprizone-induced demyelination model, TAM receptor signaling facilitates the improvement of motor-coordinative dysfunction following therapeutic Electroacupuncture[220]. In EAE, loss of Axl leads to greater neurological disability and a buildup of myelin debris following active immunization with MOG_{35-55} peptide[221]. Myelin debris builds up during in neuroinflammatory models such as EAE can promote leukocyte recruitment and impede remyelination, making TAM receptors ideal candidates for major immune regulators during disease[222,223].

DNA methylation, hydroxymethylation, and demethylation.

The activation status and effector functions of myeloid cells are regulated, in part, by the epigenetic profile of the cell, including DNA methylation[224]. The specificity and stability of myeloid cell responses can only be accomplished with a diverse repertoire of transcriptional control[225]. DNA methylation, produced and sustained by DNA methyl transferases (DNMTs), is complemented by DNA hydroxymethylation and DNA demethylation, both catalyzed by the ten-eleven translocase (TET) family of dioxygenases (Figure 1.3)[226]. The modification of cytosine residues on DNA regulates the accessibility of the genetic element to DNA-binding proteins, which can activate or suppress gene expression in a loci-dependent manner[227].

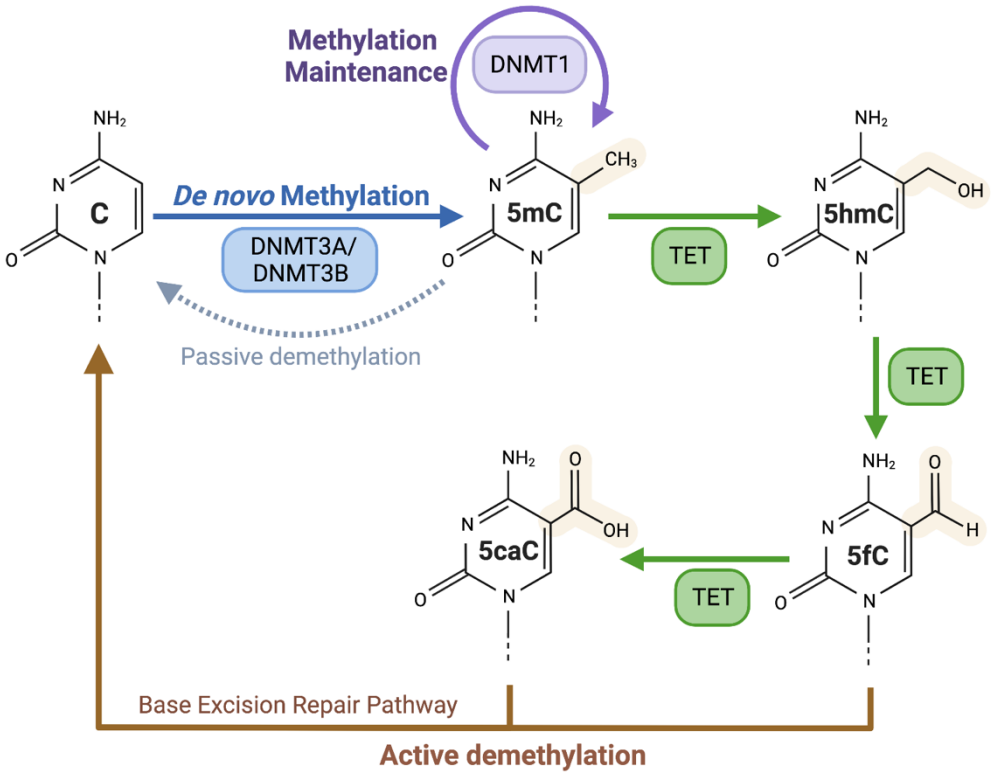

Figure 1.3 - Dynamic regulation of DNA methylation.
DNA methyltransferases (DNMTs) establish and maintain methylation on cytosine (C) residues. Ten-eleven translocation (TET) enzymes catalyze the oxidation of methylcytosine (5mC) to hydroxymethylcytosine (5hmC), and subsequent conversions to formylcytosine (5fC) and carboxycytosine (5caC). These later two can be converted to unmethylated cytosine through the base excision repair pathway.

The active demethylation pathway is facilitated by TET enzymes through the sequential oxidation of 5'methylcytosine (5mC) to 5'-hydroxymethylcytosine (5hmC), which is then further oxidized to 5'-formylcytosine (5fC), and finally to 5'-carboxycytosine (5caC) (Figure 1.3)[228–230]. 5fC and 5caC are thought to be "committed" to demethylation and can be reverted to unmodified cytosine via the base excision repair pathway[231]. 5hmC is more abundant and serves as a stable epigenetic mark[232]. In the genetic environment, 5hmC and 5mC share structural similarities which allow for overlapping binding of some chromatin-modifying enzymes[233]. However, upon conversion of 5mC to 5hmC, methyl-CpG binding domain (MBD) proteins dissociate and other DNA binding proteins are allowed to bind[232,234]. Because of the relative abundance and stability

of 5hmC in the mammalian genome, 5hmC content is often used as a readout of TET protein activity.

It is important to note that, while the active demethylation pathway has been the subject of much research since its discovery in 2002[228], there is still uncertainty regarding how this process is regulated at specific loci – not all methylated cytosine residues are hydroxymethylated, and not all hydroxymethylated cytosine residues continue through the active demethylation pathway. Interestingly, 5fC and 5caC do not exist in large quantities throughout the genome, but instead are found in specific genomic areas, implicating loci-specific regulation of demethylation[235]. Independent of the active demethylation pathway, TET proteins modulate activity of other gene-regulating or chromatin-modifying enzymes via direct binding or dioxygenase activity, a lesser studied function of TET enzymes[236,237].

Interestingly, both DNMTs and TET proteins are differentially expressed in myeloid cells with a pro-inflammatory versus regulatory phenotype[238–240]. Similarly, deficiencies of these enzymes have dramatic effects on the polarization and effector function of myeloid cells[238–241]. While the TET family of proteins are universally expressed in mammalian tissues, TET2 is the predominant TET protein in terminally differentiated hematopoietic cells, including myeloid cells[226,242]. Reports of individuals with biallelic TET2 deficiencies developing autoimmunity in addition to hematopoietic malignancies is highly suggestive of an important role of TET2 in maintaining homeostasis[243,244]. Much of the current data on TET2 and 5hmC in patients with autoimmune disease is descriptive. Mutations of *TET2* are commonly found in rheumatoid arthritis (RA) patients[245]. Similarly, histological analysis of the salivary glands from patients Sjögren's syndrome (SS) have demonstrated reduced methylation and enriched *TET2* and 5hmC within inflammatory cells[246]. Murine models have been helpful in providing more mechanistic

insights. B cell-specific *Tet2*/*3* double knockout mice develop spontaneous systemic lupus erythematosus (SLE)-like autoimmunity[247]. In *Ldlr*-deficient mice, a model of atherosclerosis, *Tet2*-deficiency in murine hematopoietic cells results in accelerated disease[248]. TET2 is also required for a model of autoimmune diabetes induced by IFN-α expression in pancreatic β cells[249]. These observations suggest a role of TET2 in protection against autoimmunity, but more studies are needed to elucidate specific mechanisms leading to pathology in human disease.

Alterations in DNA methylation patterns are characteristic of disease states, including autoimmunity, including systemic lupus erythematosus (SLE)[250], rheumatoid arthritis (RA)[250], inflammatory bowel disease (IBD)[251], and MS[252]. Studies of epigenetic changes in MS have predominately focused on T cells[253–255]. However, in a study of genetic sequence variation in T cells isolated from MS-discordant monozygotic twin pairs, there was no evidence for genetic, epigenetic, or transcriptomic differences that explained disease discordance[254]. Relatively few studies on the epigenetic regulation of myeloid cells have been conducted in EAE and MS[256,257]. Differential methylation in MS patients, compared to healthy controls, is more pronounced in $CD14^+$ monocytes than in $CD4^+$ or $CD8^+$ T cells[252]. Monocytes isolated from RRMS patients have 95-124 sites of differential methylation compared to healthy controls, the majority of which are hypomethylated, suggesting TET protein activity[252,258]. Of particular interest, the methylation status of the HLA-DRB1 locus in circulating monocytes affects the impact of the HLA-DRB1*15:01 risk allele on MS development[259]. Some methylation patterns are unique to RRMS, when compared to SPMS[252]. Many of these differentially methylated sites are within genes associated with myeloid function, further evidence that the progression of MS is associated with stable changes within myeloid cells[252]. When interpreting these studies, it is important to consider that the traditional bisulfite sequencing does not distinguish between methylated

cytosine and hydroxymethylated cytosine, which has been found to be reduced in MS patient PBMCs[260,261]. These data implicate DNA methylation as a mechanism of myeloid cell regulation during CNS autoimmunity.

Rationale and Specific Aims

There are significant gaps in our understanding of how myeloid cell responses are regulated during EAE and MS. Most of the disease modifying agents currently used in the treatment of MS deplete lymphocytes or block their trafficking to the CNS[262]. While these agents decrease MS relapse rates in many individuals with MS, none are universally effective, and none are curative. Drugs that specifically target myeloid cells might be effective in MS patients refractory to current treatments. This is particularly relevant to patients with progressive forms of MS, which is characterized by wide spread microglial activation and smoldering white matter lesions with a rim of activated microglia at their advancing edge[95–97]. Our lab and others have shown that global depletion of myeloid cells during acute EAE reduces disease burden[108,115,149]. However, this approach can cause sustained immunosuppression and does not distinguish between functional myeloid cell subsets[113]. Investigation of the factors that control the phenotype and effector functions of myeloid cells within the CNS could provide insights into innovative therapeutic strategies for the treatment of MS. The studies presented here interrogate two candidate pathways of myeloid cell regulation within the CNS autoimmune model EAE:

Aim 1: *Chapter 2:* Determining the role of myeloid-expressed TET2 in regulating myeloid cell regulation during EAE. Our hypothesis was that TET2 limits the activation and pro-inflammatory functions of myeloid cells during EAE, and that downregulation of TET2 activity

in myeloid cells is essential for clinical EAE development. We induced EAE via active immunization or adoptive transfer of MOG_{35-55} $CD4^+$ T cells in *Tet2*$^{-/-}$ and *Tet2*$^{-/-}$ bone marrow chimera recipients, or control mice.

Aim 2: *Chapter 3:* Investigating the role of TAM receptors in regulating myeloid cell differentiation and function during EAE. While some studies have examined the role of the TAM receptor Axl during EAE, data are lacking regarding the role of Mer, highly expressed on myeloid cells, or potential redundancies in the function of TAM receptors in regulating myeloid cells during EAE pathogenesis. We used an inhibitor highly specific for all three TAM receptors to block signaling at the onset of adoptive transfer EAE. Our hypothesis was that TAM receptor signaling induces a regulatory or anti-inflammatory phenotype in CNS myeloid cells during EAE.

The results of this research provide new insights into the immunopathogenesis and regulation of neuroinflammation in diseases such as MS. These studies highlight the significance of myeloid cells in determining clinical outcomes of EAE. Further studies are needed to understand the role of soluble CNS factors or cellular interactions in determining the contexts in which TET2 and TAM receptors function as regulatory factors.

Chapter 2

Loss of TET2 Does Not Augment the Clinical Course of Adoptive Transfer Experimental Autoimmune Encephalomyelitis

Abstract

Myeloid cells play a prominent role in the pathogenesis of central nervous system (CNS) autoimmune disease, contributing to both pathogenesis and resolution. ten-eleven translocation 2 (TET2), an epigenetic regulator facilitating active demethylation, has been implicated in the suppression of myeloid cell activation and pro-inflammatory functions and was recently identified as a susceptibility locus in multiple sclerosis (MS). Alterations in *TET2* expression and activity have been associated with a number of hematopoietic and neurodegenerative diseases, including a variety of cancers characterized by aberrant myeloid proliferation, differentiation, and activation. Previous studies found expression of *TET2* and its enzymatic product, 5'-hydroxymethylcytosine (5hmC), was reduced in peripheral blood mononuclear cells from MS patients compared to healthy volunteers. Based on this data, we hypothesized that downregulation of endogenous TET2 is a critical step in the transformation of myeloid cells into pathogenic effectors during CNS autoimmunity. To investigate this hypothesis, we used both active immunization with myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) and the adoptive transfer of MOG_{35-55}-specific $CD4^+$ T cells for the induction of EAE. We observed augmented neurological disability in immunized *Tet2*-deficient mice and immunized bone marrow chimeric mice which lack TET2 specifically within the hematopoietic compartment. Following the transfer of wild-type (WT) MOG_{35-55}-specific Th17 cells into syngeneic recipients,

Tet2 transcript levels and 5hmC content were decreased in spinal cord-infiltrating leukocytes at peak neurological disability. Contrary to our hypothesis, we did not observe an exacerbated disease course or enhanced CNS infiltration when EAE was induced in *Tet2*-deficient recipients via adoptive transfer of *Tet2*-sufficient encephalitogenic T cells. This effect was not likely due to differences in antigen presentation between *Tet2*$^{-/-}$ and WT antigen presenting cells (APCs) in the priming stage of disease, as both populations facilitated similar levels of T cell proliferation and activation *in vitro*. Our data suggest that, while there are alterations in *Tet2* expression and activity during adoptive transfer EAE, myeloid-expressed TET2 does not independently limit neurological disability. Future efforts should focus on understanding the protective function of TET2 within CD4^{+} T cells or exploring a detrimental role within microglia, an observation recently made in models of systemic inflammation and Alzheimer's disease.

Introduction

Multiple sclerosis (MS) is a debilitating inflammatory demyelinating disease of the central nervous system that affects 2.8 million people globally[7,8]. Genetic evidence and studies in animal models indicate MS is driven by a CD4^{+} T cell response, but growing evidence implicates myeloid cells as important inflammatory mediators of disease[84–86]. Myeloid cells, including macrophages and dendritic cells (DCs), constitute the majority of infiltrating immune cells within MS lesions and are spatially associated with myelin damage[111–113]. Studies in the murine model experimental autoimmune encephalomyelitis (EAE) have demonstrated that myeloid cells have pleiotropic functions that evolve during a monophasic clinical course[113]. During the initial stages of EAE, they contribute to pathogenicity by presenting antigen to encephalitogenic T cells and by producing pro-apoptotic factors, free oxygen radicals, and proteases that inflict damage to

cells[113,115,116,144,145]. As disease progresses, myeloid cells transition, individually and as a population, to an alternatively activated phenotype with functions that may limit disability or resolve inflammation, such as phagocytosis of cellular debris and release of anti-inflammatory factors[113,144,145]. A deeper understanding of the factors that suppress pro-inflammatory functions in favor of more protective functions is needed.

The activation status and effector functions of myeloid cells are regulated, in part, by the epigenetic profile of the cell, including post-translational histone modifications and DNA methylation[224,257]. Single-nucleotide polymorphisms that are associated with MS risk or age of clinical onset include genes relevant to methylation pathways, suggesting that epigenetic regulation contributes to MS pathogenesis[253]. Many studies on epigenetic changes in MS have focused on T cells[253–255]. However, $CD14^+$ monocytes from MS patients have distinct methylation patterns in functionally relevant pathways when compared to healthy controls[252].

A recently identified MS susceptibility locus, ten-eleven translocation 2 (TET2), is an epigenetic modifier that negatively regulates myeloid cell proliferation, activation, and cytokine expression[226,240,263–266]. TET2 belongs to a family of epigenetic modifying enzymes uniquely responsible for the active demethylation of 5'-methylcytosine (5mC) and the production of 5'-hydroxymethylcytosine (5hmC). TET2 is important in maintaining homeostasis; loss-of-function *Tet2* mutations and reductions in genomic 5hmC content are associated with myeloid malignancies, such as acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML), neurodegenerative diseases, and autoimmunity in both humans and in mice[245,267–271]. In murine myeloid cells, *Tet2*-deficiency results in enhanced pro-inflammatory responses, including elevated IL-6 and IL-1β, and reduced PD-L1[240,264,272].

In MS patients, *TET2* transcripts and 5hmC levels are reduced in peripheral blood mononuclear cells (PBMCs) when compared to healthy controls[260]. *TET2* expression was inversely correlated with disease duration, suggesting TET2 may contribute to inflammation resolution[260]. The role of TET2 in $CD4^+$ T cells has been explored in EAE[273,274]. These studies have found that TET2 suppresses pro-inflammatory cytokine expression in T cells and is protective during EAE[273,274]. Surprisingly, there are no published studies on the role of TET2 in myeloid cells during EAE. In this study, we expand upon the understanding of the participation of TET2 in CNS autoimmunity by examining a potential role for TET2 as a regulator of myeloid cell function. We found that *Tet2*$^{-/-}$ and *Tet2*$^{-/+}$ mice, actively immunized with myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) peptide emulsified in complete Freund's adjuvant, had more severe neurological disability than wild-type (WT) controls. Through bone-marrow chimera experiments, we determined that this augmented disease was due to *Tet2* deficiency in the hematopoietic compartment. To further isolate this effect to myeloid cells, we utilized the adoptive transfer model of EAE, where MOG_{35-55}-specific $CD4^+$ T cells are transferred into naïve syngeneic recipients. In WT recipients, *Tet2* expression and 5hmC levels were reduced in myeloid cells at peak disease, when compared to other timepoints. The transfer of WT $CD4^+$ T cells into *Tet2*-deficient recipient mice resulted in a similar clinical course to WT recipients. Further experiments *in vitro* suggested that TET2 loss does not impact the ability of myeloid cells to present antigen or activate encephalitogenic T cells by myeloid cells. Collectively, our data does not support a role for TET2 in intrinsically regulating myeloid cell function during EAE and diverts the attention of future studies to other cell types.

Materials and Methods

Mice. WT C57BL/6, *Tet2*$^{-/-}$, *Tet2*$^{-/+}$, and 2D2 transgenic mice were obtained from Jackson Laboratories (strain #: 023359) at six to eight weeks of age. Mice were housed under specific pathogen-free conditions in microisolator cages. Bone marrow chimeric mice were generated as described previously[275]. CD45.1 congenic mice were irradiated with 1300 rad, delivered in two 650 rad doses spaced 3 hours apart. Bone marrow was isolated from donor mice by harvesting bones from the hind limbs, cutting the ends of each bone, and cleaning the bone cavity with 3mL 2% FBS in PBS. Donor bone marrow was injected intravenously into irradiated recipient mice at $2x10^6$ cells per mouse. Bone marrow chimeric mice were housed under sterile conditions in microisolator cages and given antibiotic water for two weeks following irradiation. Chimeras were bled after six weeks to check for proper reconstitution. All animal studies were approved by the Institutional Animal Care and Use Committee.

Antibodies and reagents. The following antibodies were obtained from eBioscience: PerCP-Cy5.5 anti-CD11c [N418], PE anti-CD44 [IM7]. The following antibodies were obtained from BD Biosciences: BUV737 anti-CD45 [30-F11], PE anti-Ly6G [1A8], BV786 anti-CD11b [M1/70], PE-Cy5 anti-CD3 [145-C11], BUV563 anti-CD88 [20/70], and BV421 anti-I-A/I-E [2G9]. The following antibodies were obtained from Biolegend: APC anti-CD26 [DPP-4]. The polyclonal 5hmC primary antibody was obtained from Active Motif (39791). The secondary antibody used for 5hmC staining was goat anti-rabbit 488 (Invitrogen A11008). Carboxyfluorescein succinimidyl ester staining was performed using CellTrace CFSE Cell Proliferation Kit (C34554).

Induction and scoring of EAE. EAE was induced as previously described[110,275]. Mice were immunized with an emulsion consisting of 100 μg of MOG_{35-55} peptide (MEVGWYRSP-

FSRVVHLYRNGK; Biosynthesis) emulsified in CFA (Sigma [F5881], supplemented with BD Difco [BD 231141]), at four sites over the flanks. To induce active EAE, 300 ng of pertussis toxin (List biological) was administered at days 0 and 2. To induce EAE via adoptive transfer, inguinal, axial, and brachial lymph nodes were harvested 10-14 days after immunization, homogenized, and passed through a 70 μm strainer to achieve a single-cell suspension. The dissociated cells were cultured with $MOG_{35–55}$ peptide (50 μg/mL; Biosynthesis), in RPMI media (Gibco) supplemented with HEPES buffer (Gibco, 12.3mM) MEM non-essential amino acids (Gibco, 1X), sodium pyruvate (Gibco, 1X), β-mercaptoethanol (Gibco 0.5mM), L-glutamine (Gibco, 1X), and Pen Strep (Gibco, 1:100 dilution), under Th17-polarizing conditions: 8 ng/mL recombinant murine IL-23 (R&D), 10 ng/mL recombinant murine IL-1α (Peprotech), and 10 μg/mL anti–IFN-γ (Bio X Cell). After 96 hours of culture, $CD4^+$ T cells were purified via L3T4 magnetic bead sorting (Miltenyi Biotec) and injected into naïve syngeneic recipients ($5x10^6$ $CD4^+$ T cells per mouse i.p.). Recipient mice were observed daily for signs of EAE by an examiner blinded to the experimental groups. Mice were scored using a 0-5 scale: 0=no abnormality, 0.5=distal limp tail, 1=complete limp tail, 1.5=difficulty righting from supine position, 2=inability to right from a supine position, 2.5=overt gait abnormality, 3=difficulty elevating body while walking, 3.5=unilateral hind limb paralysis, 4=bilateral complete hind limb paralysis, 4.5=moribund, 5=death.

Cell isolation. Immune cells were isolated as previously described[275]. Cardiac perfusion was performed under isoflurane anesthesia with 10 mL 1X PBS. The spinal cord was isolated by pushing 10 mL of 2% FBS in 1X PBS through the spinal column. The spinal cord and brain were processed by homogenizing the tissue in 3 mL of collagenase A (1 mg/mL) and DNase I (1 mg/mL) in HBSS with calcium and magnesium and incubated in a 37˚C water bath for 20

minutes. Samples were pelleted, brought up in 27% Percoll (GE Healthcare), and spun at 800xg for 10 minutes. The myelin/debris layer was removed, and the pelleted cells were used for downstream analyses. The spleen was processed by homogenizing the tissue through a 70 μm strainer. Red blood cells from the spleen and blood were lysed using ACK lysis buffer (Quality biologicals) and reaction was quenched using 2% FBS in 1X PBS. Pelleted cells were then used for downstream analyses.

Flow cytometry. Flow cytometry was performed as previously described[275]. Cells were resuspended in PBS with 2% FBS, Fc Block (anti–CD16/32; 100 ng/mL), and Fixable viability dye (eBioscience, 1:500) before 1:2 dilution with fluorochrome-conjugated Abs, listed in the "Antibodies and reagents" methods section above. 5hmC staining was performed with the BD Cytofix/Cytoperm kit following the provided instructions. Cells were fixed and incubated with DNAse 1 (300 μg/mL) at 37˚C for 1 hour prior to staining for 5hmC. Cells were stained with anti-5hmC primary antibody (1:50), washed, and stained with anti-rabbit secondary (1:125). Data were collected with a FACSymphony or FACSCanto flow cytometer using FACSDiva software (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star).

Digital droplet PCR (ddPCR) analysis. Immune cells were isolated from the brain, spinal cord, and spleen and stained for flow cytometry as described above. $1x10^4$ $CD45^{hi}CD11b^{+}CD26^{-}$ myeloid cells were isolated from the CNS (combined brain and spinal cord) or spleen of individual mice. For ddPCR analysis, sorted CNS cells and splenocytes from three individual mice and pooled by tissue, for a total number of $3x10^4$ cells in 3-4 samples / tissue / timepoint. RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) according to manufacturer's instructions. 2 μL of cDNA was used for

ddPCR analysis using the QX200 Droplet Digital PCR system (Bio-Rad) using the manufacturer's protocol for mouse *Tet2* ddPCR gene expression assay (Bio-Rad). Readouts were analyzed using QuantaSoft Software (Bio-Rad).

T cell stimulation assay. Mononuclear cells were harvested as described above from the spleens and skin-draining lymph nodes of *Tet2*-deficient or WT mice. Myeloid APCs were isolated through magnetic-activated cell sorting (MACS, Miltenyi Biotec) of $CD11b^+$ cells. For isolation of MOG_{35-55}-specific T cells, the spleen and skin draining lymph nodes of naïve 2D2 transgenic mice were harvested and $CD4^+$ cells were isolated using the mouse CD4 MACS kit (Miltenyi Biotec). T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) using CellTrace CFSE Cell Proliferation Kit following the manufacturer's instructions. APCs and T cells were cultured at a 1:20 ratio in the presence of MOG_{35-55} peptide (50 µg/mL) for 96 hours. After culture, cells were stained with antibodies directed against T cell extracellular markers and analyzed using flow cytometry.

Statistical analysis. All statistical analyses were performed with GraphPad 9.0. The Wilcoxon Rank Sum test was used to compare clinical courses. One way ANOVA analysis was used to compare disease metrics, ddPCR analysis, and flow cytometry analysis of time course data and T cell stimulation assay. The unpaired Student's *t*-test with Welch's correction was used to compare flow cytometry analysis at a single timepoint. using GraphPad 9.0 (Prism). AUC was calculated using GraphPad 9.0. A p-value of 0.05 was considered significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Results

Tet2-*deficient mice develop more severe EAE following active immunization*

To confirm a role of TET2 in regulating the severity of EAE, we immunized 8-week-old *Tet2*$^{-/-}$ or WT mice with MOG_{35-55} emulsified in CFA to induce disease. *Tet2*$^{-/-}$ mice displayed a more severe clinical course compared to WT controls (Figure 2.1.A). Area under the curve (AUC) analysis showed that *Tet2*$^{-/-}$ animals trended towards a greater disease burden over the course of EAE (Figure 2.1.B). Homozygote TET2 knockouts can exhibit enhanced and inappropriate myeloid cell accumulation in the bone marrow and spleen as early as 8 weeks of age[276]. To control for an overabundance of myeloid cells influencing the disease course, we also immunized 8-week-old *Tet2*$^{-/+}$ mice, which do not display this phenotype[277]. Similar to *Tet2* mice, *Tet2*$^{-/+}$ mice displayed more severe disease and trended towards greater accumulated disability compared to WT mice (Figure 2.1.A-B). In both *Tet2*-deficient strains, the affected animals had a similar day of onset and peak clinical score compared to WT controls, demonstrating the greatest change in the disease course was the speed at which the mice accumulated disability (Figure 2.1.C-D).

Because TET2 is not exclusively expressed in hematopoietic cells, we generated bone marrow chimeric mice in which *Tet2*-deficient bone marrow was transferred into irradiated WT recipients. MOG_{35-55}-immunized recipients of *Tet2*$^{-/-}$ and *Tet2*$^{-/+}$ bone marrow had an earlier onset and greater disease burden than recipients of WT bone marrow (Figure 2.1.E-G). No differences were observed between mice that received *Tet2*$^{-/-}$ bone marrow and mice that received *Tet2*$^{-/+}$ bone marrow. The peak clinical disability was comparable between all groups (Figure 2.1.H). These data indicate that *Tet2*-deficiency within leukocytes results in greater disease burden following active induction of EAE.

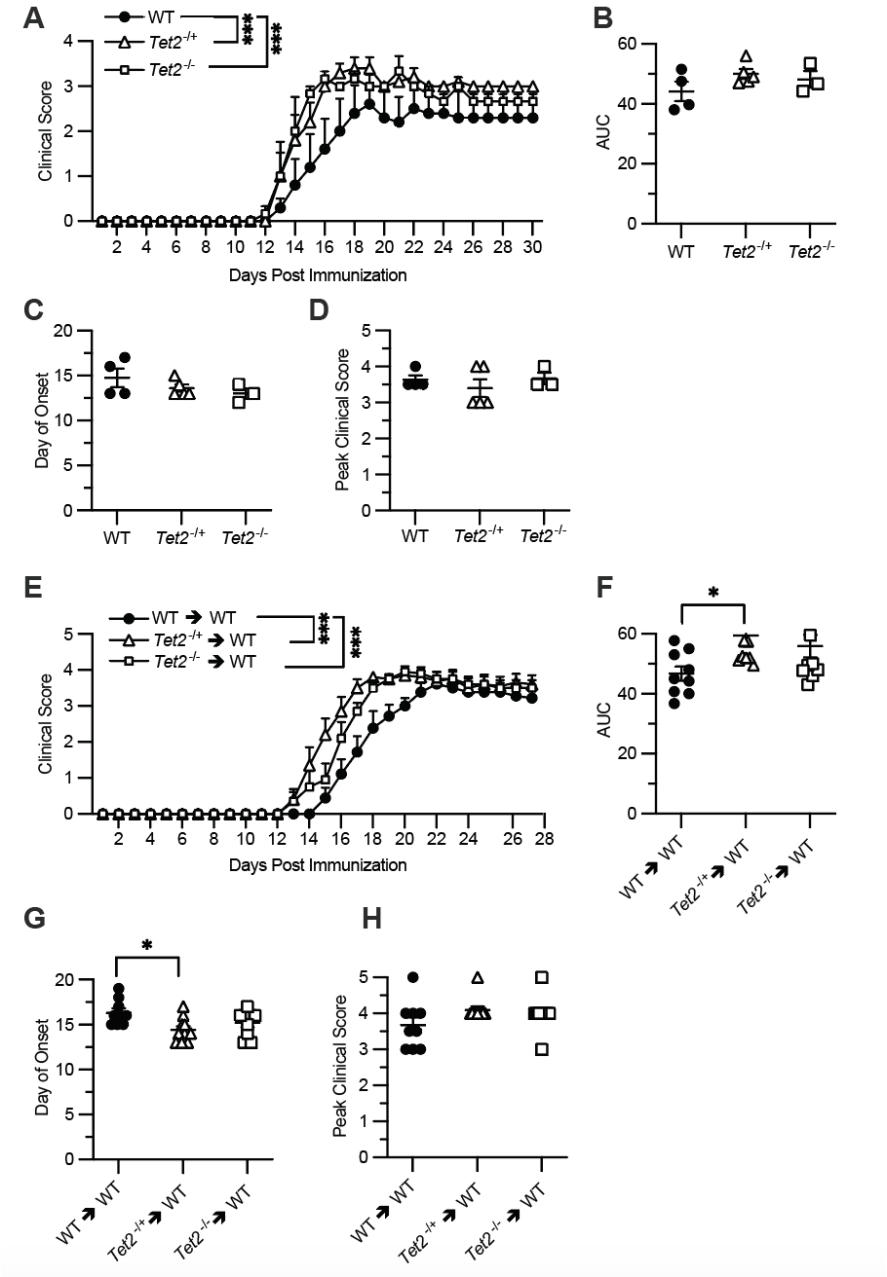

Figure 2.1 - *Tet2*-deficient mice and recipients of *Tet2*-deficient bone marrow present with more severe EAE following immunization with MOG35-55/CFA.

A-D) Tet2$^{-/-}$, Tet2$^{-/+}$, and WT mice were immunized with MOG_{35-55} emulsified in CFA. **A)** Disease course, **B)** total disease burden, **C)** the day of onset, and **D)** the peak clinical score of immunized mice with disease. [n=3-5/group, representative of two experiments] **E-H)** Bone marrow from Tet2$^{-/-}$, Tet2$^{-/+}$, and WT were transferred into irradiated WT recipients. Following 8 weeks of reconstitution, mice were immunized with MOG_{35-55}/CFA. **E)** Disease course, **F)** total disease burden, **G)** the day of onset, and **H)** the peak clinical score of immunized chimeric mice with disease. [n=7-9/group, representative of two experiments] * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Tet2 *transcripts and 5hmC are downregulated in myeloid cells during adoptive transfer EAE*

Previous studies have demonstrated that mice with T-cell specific *Tet2* deficiency have a more severe clinical course following immunization with MOG_{35-55} peptide/CFA[273]. To focus on non-$CD4^+$ T cell populations within the effector phase of disease, we used an adoptive transfer model of EAE. Encephalitogenic $CD4^+$ T cells from WT MOG_{35-55}/CFA primed mice were isolated, expanded and polarized *in vitro*, and transferred to naïve recipients. To measure the patterns of *Tet2* expression throughout adoptive transfer EAE, we isolated myeloid cells ($CD45^+CD11b^+CD26^-$) from the CNS and spleen of WT recipients and performed droplet digital PCR (ddPCR) for *Tet2* transcripts throughout EAE. In the CNS, *Tet2* expression in myeloid cells was significantly reduced at peak disease compared to disease onset (Figure 2.2.A). This reduced expression corresponds to the timepoint at which large numbers of activated myeloid cells are found within the CNS[275]. At later stages of EAE, few peripheral leukocytes are found in the CNS and, while the mice are left with chronic neurological deficits, inflammation has mostly resolved. At this timepoint, *Tet2* expression in myeloid cells isolated from the CNS was similar to the observed levels at onset (Figure 2.2.A). This upregulation may play a role in restraining myeloid responses as disease resolves. In splenocytes, *Tet2* was consistently expressed at low levels in naïve mice and through peak inflammation during EAE but was significantly increased at late stages.

5hmC is a stable DNA methylation derivative that is uniquely produced by TET enzymes and can be used as a measure of TET protein activity[278,279]. Using flow cytometry to measure 5hmC in myeloid cell populations isolated from the spinal cord, we found that the patterns of 5hmC in the CNS throughout EAE followed similar trends of *Tet2* expression patterns. In spinal

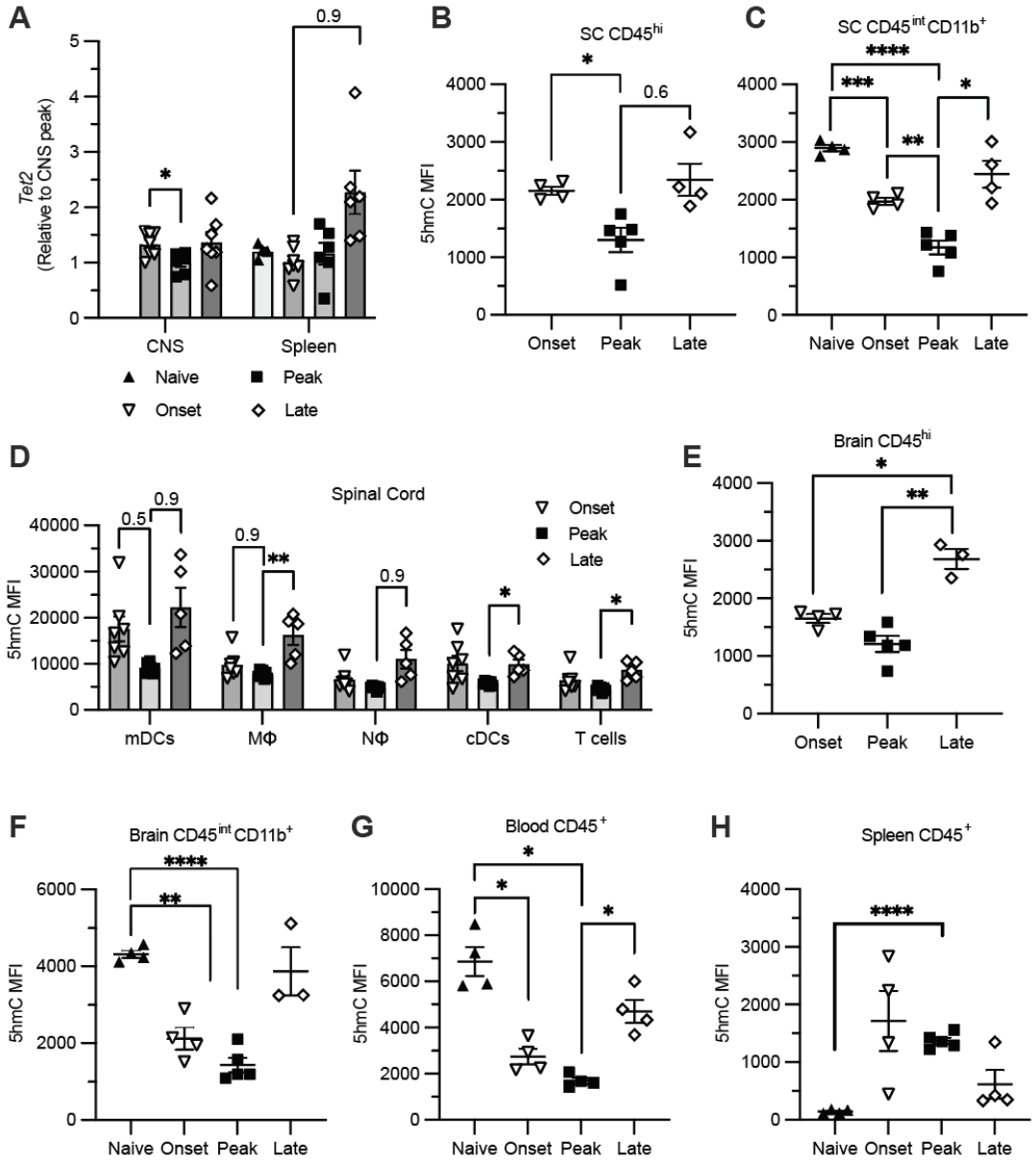

Figure 2.2 - *Tet2* and 5hmC are reduced in the CNS at the peak of inflammation during EAE. **A)** Tet2 transcripts were measured by doplet digital PCR (ddPCR) in $CD45^{hi}CD11b^{+}CD26^{-}$ myeloid cells isolated from the CNS (combined brain and spinal cord) or spleen of WT mice at various timepoints of EAE. Data was pooled from two independent experiments with n=6-7/timepoint. **B-H)** Cells were isolated from naïve mice and at various timepoints of EAE. 5hmC content was analyzed by flow cytometry. Data is representative of three independent experiments with n=4-7/timepoint. **B-C)** 5hmC expression in **B)** spinal cord (SC) infiltrating leukocytes ($CD45^{hi}$) and **C)** spinal cord microglia ($CD45^{int}CD11b^{+}$) at various time points. **D)** 5hmC expression in spinal cord $CD45^{hi}$ populations: monocyte-derived dendritic cells (mDCs, $CD11c^{+}CD88^{+}$), macrophages (MΦ, $CD11b^{+}CD11c^{-}Ly6G^{-}$), neutrophils (NΦ, $CD11b^{+}Ly6G^{+}$), conventional dendritic cells (cDCs, $CD11c^{+}CD26^{+}$), and T cells ($CD3^{+}$). **E-H)** 5hmC expression in **E)** brain-infiltrating leukocytes, **F)** brain microglia, **G)** circulating leukocytes, and **H)** splenic leukocytes. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

cord-infiltrating CD45^{hi} cells, as well as in CD45^{int}CD11b^{+} microglia, the lowest levels of 5hmC were observed at the peak of EAE when the spinal cord is most inflamed (Figure 2.2.B-C)[275].

Late in disease, elevated 5hmC content in these cell populations corresponded to increased levels of *Tet2* (Figure 2.2.A-C). The levels of 5hmC in microglia isolated at late stages of EAE were similar to the levels in naïve microglia, suggesting that TET2 expression can return to baseline following inflammation (Figure 2.2.C).

The patterns of 5hmC in CD45^{hi} cells during EAE are reflected within individual myeloid and non-myeloid populations, indicating changes in TET2 activity are not specific to myeloid cells (Figure 2.2.D). Monocyte-derived DCs (mDCs, CD45^{hi}CD11c^{+}CD88^{+}) displayed the highest expression of 5hmC early in disease and exhibited the most significant reduction at the peak of inflammation (Figure 2.2.D). All cell populations saw a significant increase in 5hmC late in disease (Figure 2.2.D). At this timepoint, expression was again highest in mDCs, followed by macrophages (CD45^{hi}CD11b^{+}CD11c^{-}) (Figure 2.2.D). Macrophages and mDCs have a shared monocyte progenitor and undergo a similar phenotypic transition during EAE, thus it is unsurprising that they may utilize the same regulatory pathways as inflammation resolves[113]. Similar to spinal cord-infiltrating cells, brain-infiltrating CD45^{hi} cells displayed significantly higher 5hmC levels at late stages of EAE than at earlier timepoints (Figure 2.2.E). Brain resident microglia displayed similar patterns to spinal cord resident microglia, with a sharp reduction in 5hmC content at the onset of neurological disability, followed by rebounded levels at late disease (Figure 2.2.F). These patterns were also reflected in CD45^{+} circulating leukocytes (Figure 2.2.G). Patterns of 5hmC in the spleen did not correspond to *Tet2* expression, suggesting that 5hmC levels may be affected by the activity of DNA methyltransferases (DNMTs) and other

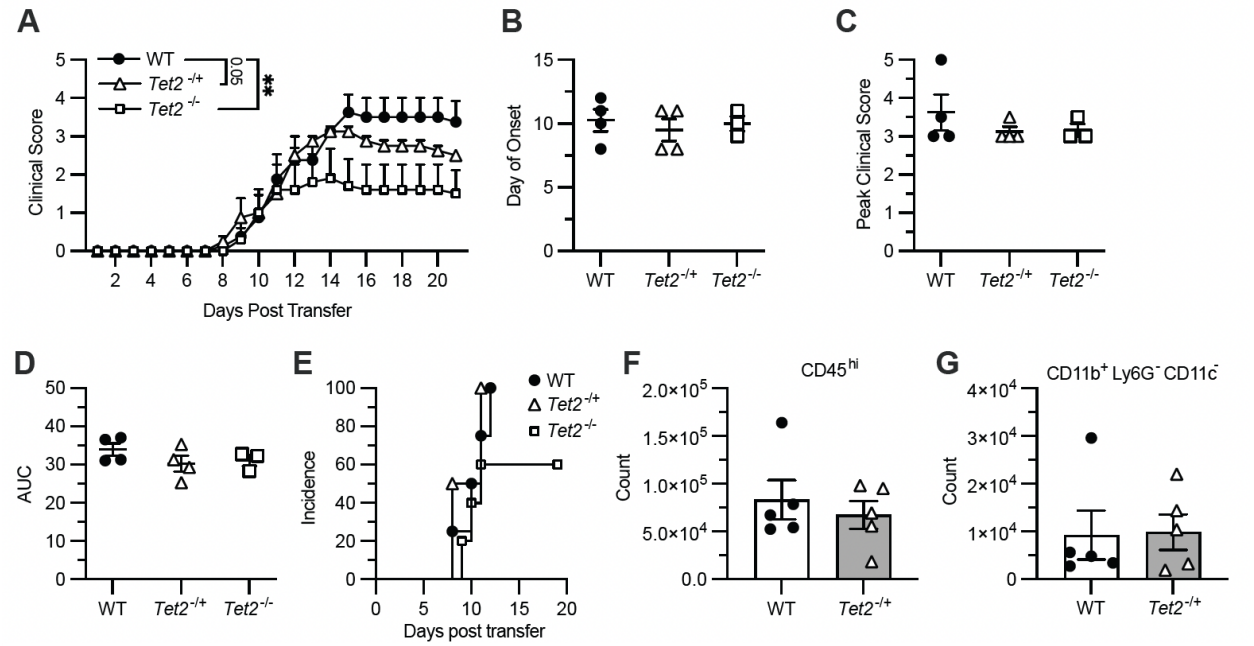

Figure 2.3 - EAE induced by the adoptive transfer of MOG_{35-55} $CD4^+$ T cells is not augmented in *Tet2*-deficient mice.
WT MOG_{35-55}-specific Th17 cells were transferred into $Tet2^{-/-}$, $Tet2^{-/+}$, and WT recipients to induce EAE. Data are representative of two independent experiments with similar results. **A)** Disease course, **B)** day of onset, **C)** peak clinical score, and **D)** total disease burden of mice with disease. **E)** Incidence of EAE in recipient mice. [n=3-4/group] **F)** The number of total infiltrating leukocytes ($CD45^{hi}$) and **G)** infiltrating macrophages ($CD45^{hi}CD11b^+CD11c^-Ly6G^-$) within the spinal cord of *Tet2*$^{-/+}$ and WT adoptive transfer recipients at the onset of EAE. [n=5/group] ** $p<0.01$

TET enzymes (Figure 2.2.H). Together, these data indicate that there is a dysregulation of *Tet2* expression and activity in microglia and infiltrating leukocyte populations in the inflamed CNS, and in circulation, during adoptive transfer EAE.

Tet2 deficient mice do not develop worse EAE following adoptive transfer

Following our evaluation of *Tet2* expression and activity during adoptive transfer EAE in WT mice, we assessed neurological disability in *Tet2*-deficient recipients of WT MOG_{35-55}-specific $CD4^+$ T cells. Contrary to our hypothesis, we found *Tet2*$^{-/+}$ and *Tet2*$^{-/-}$ recipients experienced less clinical disability that WT controls (Figure 2.3.A). There were no significant differences in the day of onset, peak clinical disability, or total disease burden between the groups (Figure 2.3.B-D). There appeared to be a lower incidence of disease in *Tet2*$^{-/-}$ mice

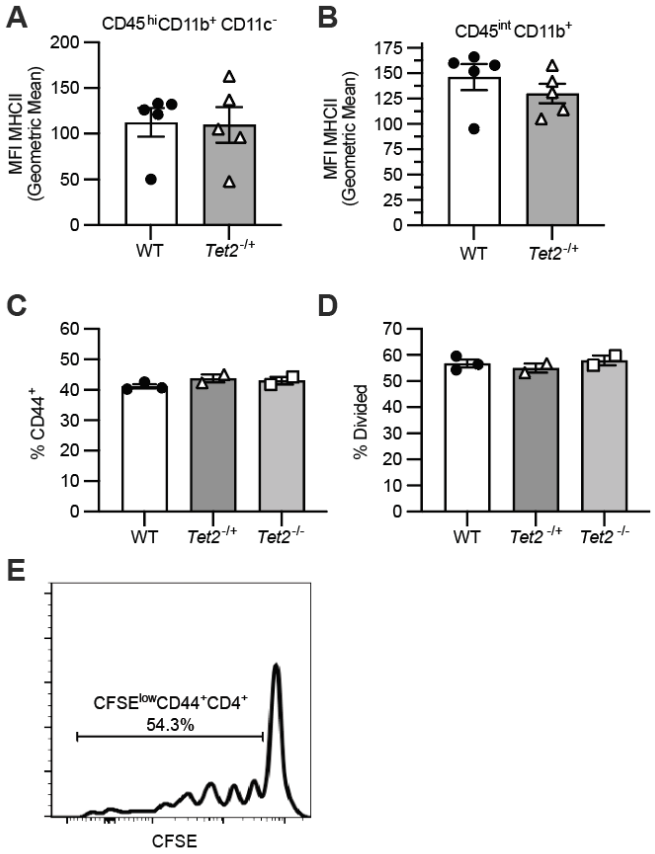

Figure 2.4 - Tet2-deficient myeloid cells efficiently present antigen to MOG_{35-55}-specific 2D2 T cells.

A,B) Infiltrating leukocytes were isolated from the spinal cord of $Tet2^{-/+}$ and WT mice and analyzed for MHCII expression by flow cytometry. [n=5/group] **A)** MHCII on infiltrating macrophages ($CD11b^{+}CD11c^{-}$) and **B)** microglia ($CD45^{int}CD11b^{+}$). **C-E)** $CD11b^{+}$ cells, isolated from the lymph nodes and spleens of naïve $Tet2^{-/-}$, $Tet2^{-/+}$, and WT mice, were co-cultured with CFSE-stained MOG_{35-55} $CD4^{+}$ T cells from 2D2 mice, in the presence or absence of MOG_{35-55}. After 4 days in culture, cells were analyzed by flow cytometry. [n=2-3/group] **C)** Percentage of $CD4^{+}$ T cells expressing CD44 and **D)** percentage of $CD4^{+}CD44^{+}$ T cells that were $CFSE^{low}$, indicative of cell division. **E)** Representative histogram of CFSE staining of $CD4^{+}CD44^{+}$ T cells cocultured with APCs from $Tet2^{-/-}$ mice.

compared to WT controls, but this was not statistically significant (Figure 2.3.E). To assess if loss of TET2 impacted CNS infiltration during EAE, leukocytes were isolated from the spinal cord of *Tet2*$^{-/+}$ and WT mice at the onset of disease and analyzed by flow cytometry. We found similar numbers of total $CD45^{hi}$ cells, as well as a similar number of infiltrating $CD11b^{+}CD11c^{-}$ macrophages, suggesting there was no impact of TET2 on myeloid infiltration (Figure 2.3.F-G).

Though our sample size was limited, our data suggests that *Tet2*-deficiency in non-T cell populations does not augment the clinical course of adoptive transfer EAE or significantly affect spinal cord infiltration.

TET2 in myeloid APCs does not affect MHCII expression during adoptive transfer EAE or antigen presentation in vitro

Our results thus far suggest that TET2 does not regulate myeloid cells in the effector stage of disease, following the transfer of encephalitogenic $CD4^+$ T cells. A key difference between the EAE models we used is that in active immunization model, $CD4^+$ T cell priming to MOG_{35-55} occurs in the immunized mouse, while in adoptive transfer models, transferred $CD4^+$ T cells are primed prior to injection. Because *Tet2* deficiency only augmented EAE induced by active immunization, we then questioned if TET2 regulates myeloid cell antigen presentation and priming of $CD4^+$ T cells. Within the CNS of *Tet2*$^{-/+}$ animals, expression of MHCII on microglia and macrophages, isolated at the onset of disability, was not significantly different than WT controls (Figure 2.4.A-B). To examine the direct impact of *Tet2* deficiency on the activation of $CD4^+$ T cells, we cultured carboxyfluorescein succinimidyl ester (CFSE)-labeled, MOG_{35-55}-specific $CD4^+$ T cells, isolated from the lymphoid tissue of mice with a transgenic T cell receptor (2D2 mice), with *Tet2*-deficient $CD45^+CD11b^+$ macrophages in the presence or absence of MOG_{35-55}. We found no difference in T cell activation, measured by CD44 expression, or proliferation, measured by CFSE dilution, between *Tet2*-deficient and -sufficient groups in the presence of MOG_{35-55} (Figure 2.4.C-E). No proliferation was detected in the absence of MOG_{35-55} (data not shown). This suggests TET2 is not required for antigen presentation by myeloid cells.

Discussion

Previous studies have suggested a role of TET2 in MS pathogenesis. Analysis of single nucleotide polymorphisms (SNPs) of various immune-related genes using a custom ImmunoChip genotyping array identified TET2 as a genetic susceptibility loci for MS[265]. The SNP that was associated with MS risk (rs2726518) was correlated with the proportion of monocytes within the PBMC sample[280]. A study of MS patient PBMCs demonstrated reduced *Tet2* transcript expression and lower 5hmC content compared to healthy PBMCs, but did not examine cell populations individually[260]. These studies informed our initial hypothesis, that decreased TET2 activity within myeloid cells was necessary for a substantial pro-inflammatory response during CNS autoimmunity. Our goal was to investigate the biological consequences of *Tet2* expression specifically in myeloid cells during EAE. We demonstrated that actively immunized *Tet2*$^{-/-}$ and *Tet2*$^{-/+}$ mice presented with more severe neurological disability than WT controls. Statistical significance was only achieved when analyzing the overall disease course, and metrics such as day of onset, peak clinical score, and total disease burden were not significantly different. This may be because the immunized control mice presented with a substantial degree of neurological disability, displaying deficits that placed them at the top of the scoring scale used for EAE, leaving little room for measurement of exacerbated disease. While an appreciable disease burden renders a more reliable model, it makes it difficult to distinguish more severe disease. Greater disease burden was also observed in a larger cohort of irradiated *Tet2*-deficient recipients of WT bone marrow, with greater significance. These findings are not surprising given that a previous study demonstrated that mice with a T cell-specific *Tet2*-deficiency had more a severe EAE clinical course following active immunization with myelin peptide[273]. One potential reason that *Tet2*-deficient chimeric mice had an earlier onset, while *Tet2*-deficient non-chimeric mice did

not, is that irradiated mice have a greater susceptibility to severe disease (Segal lab, unpublished observation).

Because this model involved a *Tet2*-deficiency in all hematopoietic cells, including $CD4^+$ T cells, we used an adoptive transfer model to further delineate the role of TET2 in other cell types. The *Tet2*-deficient recipients of WT myelin-specific Th17 cells had similar or less severe disability and disease metrics as WT recipients, a finding that was reproducible across multiple experiments. The main disparity between our adoptive transfer experiments and our active immunization experiments, in which *Tet2*-deficient mice had more severe disease than *Tet2*-proficient mice, was the presence of *Tet2*-proficient $CD4^+$ T cells in the adoptive transfer model. We demonstrated that *Tet2*-deficient APCs could stimulate T cells similarly to WT APCs, with comparable levels of T cell proliferation and activation. This suggested that TET2 did not affect myeloid cell's ability to prime T cells in the active immunization model. Therefore, the differences are likely due to the known intrinsic differences between *Tet2*-deficient and *Tet2*-proficient $CD4^+$ T cells. This finding is seemingly in contrast to the previously mentioned MS patient study, that demonstrated a change in the frequency of circulating monocytes in association with a *Tet2* risk allele[280]. We did not observe any difference in monocyte numbers or frequency between $Tet2^{-/+}$ and WT mice at the onset of adoptive transfer EAE. One possible explanation is that the change in monocyte frequency in MS patient PBMCs is an indirect effect of altered TET2 function in other cell types, such as $CD4^+$ T cells. The authors did not specify whether monocytes were more or less frequent in individuals with the *Tet2* risk allele or identify the effects of the allele on TET2 expression or activity, information which would aid in developing a more specific hypothesis.

In addition to T cells, recent studies have identified a surprising pro-inflammatory role of TET2 in microglia in models of systemic inflammation and Alzheimer's disease through the regulation of metabolic reprograming[241]. Considering we observed less severe disability in *Tet2*-deficient adoptive transfer recipients, we speculate that TET2 may also regulate pro-inflammatory responses in microglia during EAE. This is a reasonable idea, as the functional outcomes of methylation changes are highly context-dependent[232]. The interpretation of results from mice with a global deletion of *Tet2* are complicated by this possibility, and future studies in neuroinflammatory models should consider utilizing cell-specific knockout animals.

We observed a dysregulation of *Tet2* expression and 5hmC content in myeloid cells during adoptive transfer EAE, despite *Tet2*-deficient recipients having a similar or less severe disease course compared to WT recipients. This may reflect redundancy in the pathways which regulate myeloid cell phenotypes and responses. TET2 is the predominant TET protein in terminally differentiated hematopoietic cells, including myeloid cells, however, the TET family of proteins (TET1, TET2, and TET3) are universally expressed in mammalian tissues[226,242]. RNA sequencing of individual myeloid cell populations isolated at the peak timepoint of adoptive transfer EAE revealed comparable expression of both *Tet2* and *Tet3* transcripts, with little expression of *Tet1* (data not shown). While our findings suggest that TET2 alone does not regulate myeloid cell responses, it is possible that TET3 functions in parallel and compensates for TET2 in its absence. Indeed, previous studies have demonstrated that inducing TET3 deletion in $Tet2^{-/-}$ mice, using an MX1-Cre promoter and Poly(I:C) treatment, resulted in a more rapid-onset, aggressive, and penetrant myeloid leukemia than what is seen in $Tet2^{-/-}$ alone, suggesting overlapping regulatory roles[263].

The presented data argues against a critical role of TET2 in regulating myeloid cell responses or phenotypes during EAE in a biologically relevant manner, though some technical constraints limited the depth of our investigations and the statistical significance of our results. Data from MS patients argues for an important role of TET2 in the development of MS. Our findings suggest that focus of future research should be directed at investigating the overlapping roles of TET2 and TET3 in myeloid cells or studying the role of TET2 in other cell types, such as $CD4^+$ T cells and microglia, during EAE.

Chapter 3

TAM Receptor Signaling Dictates Lesion Location and Clinical Phenotype During Experimental Autoimmune Encephalomyelitis

Gardner, A.M., Atkinson, J.R., Wilkinson, N.M., Jerome, A.D., Bellinger, C.E., Sas, A.R,. Segal, B.M. TAM Receptor Signaling Dictates Lesion Location and Clinical Phenotype During Experimental Autoimmune Encephalomyelitis. (2023). Journal of Neuroimmunology.

Abstract

Experimental autoimmune encephalomyelitis (EAE), induced by the adoptive transfer of Th17 cells, typically presents with ascending paralysis and inflammatory demyelination of the spinal cord. Brain white matter is relatively spared. Here we show that treatment of Th17 transfer recipients with a highly selective inhibitor to the TAM family of tyrosine kinase receptors results in ataxia associated with a shift of the inflammatory infiltrate to the hindbrain parenchyma. During homeostasis and preclinical EAE, hindbrain microglia express high levels of the TAM receptor Mer. Our data suggest that constitutive TAM receptor signaling in hindbrain microglia confers region-specific protection against Th17 mediated EAE.

Introduction

In multiple sclerosis (MS), lesions form throughout the central nervous system (CNS) axis, including the optic nerves, cerebrum, brainstem, cerebellum, and spinal cord. Experimental autoimmune encephalomyelitis (EAE), widely used as an animal model of MS, typically presents with ascending paralysis secondary to inflammatory demyelination of the thoracolumbar spinal

cord[281]. CNS-specific lymphocytes initiate lesion development during EAE and, putatively, during MS[282]. However, myeloid cells are the most prevalent inflammatory cells in established EAE and MS infiltrates and have been strongly implicated in mediating demyelination and axonal damage. We and others have previously shown that depletion of circulating monocytes, or blockade of their recruitment to the CNS, prevents clinical EAE[108,115]. Disease modifying therapies (DMTs), currently used in the clinic to reduce MS relapse rates, primarily target lymphocytes. Therapeutic targeting of myeloid cells is an untapped, alternative approach for the management of MS, particularly germane to those individuals who do not respond to currently available DMTs. As EAE and MS lesions evolve, monocyte-derived cells, as well as microglia, transition from a pro-inflammatory state, evident during the preclinical stage and at clinical onset, to a quiescent, or anti-inflammatory/ reparative state, evident during lesion resolution[113,146]. An increased understanding of the endogenous signaling pathways that regulate CNS myeloid cells, and that drive their transition from a destructive to an innocuous, or even beneficial, phenotype could be informative with regard to the design of myeloid specific DMTs.

The TAM family of tyrosine kinase receptors, consisting of Tyro3, Axl and Mer, are pleiotropic inhibitors of pro-inflammatory myeloid cell activation[211]. The TAM receptors have two known ligands, grow arrest-specific 6 (Gas6) and protein S (ProS), and normally mediate phagocytosis of apoptotic cells and clearance of debris. Loss of function of all three TAM receptors results in systemic immune dysregulation. *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mer*$^{-/-}$ triple mutant mice (TAM TKOs) develop spontaneous inflammation in essentially all tissues, including the CNS; inflammatory infiltration of CNS tissues is accompanied by blood-brain barrier breakdown, glial cell activation, and neuronal damage[283,284]. Immune dysregulation in TAM TKOs is nonautonomous with respect to lymphocytes and is believed to be secondary to the loss of TAM

signaling in antigen presenting cells (APCs), particularly macrophages and monocyte-derived dendritic cells (mDCs)[285]. This suggests that deficient TAM signaling in myeloid cells could be a predisposing and/ or exacerbating factor in CNS autoimmune disease.

There is increasing evidence that TAM receptor expression influences MS risk and prognosis. Both common and low frequency polymorphisms in the *Mer* gene are independently associated with susceptibility to MS, and certain variants may be associated with an increased probability of transitioning from a relapsing-remitting (RR) to a secondary progressive (SP) disease course[286,287]. In two independent cohorts, baseline plasma levels of free ProS were reduced in female RRMS patients compared with age- and sex-matched healthy controls, and low levels of circulating ProS correlated with higher disability severity scores[288]. These observations led the authors to speculate that TAM receptor signaling might be protective in MS. Consistent with that conjecture, an independent study found that relapsing MS patients with high cerebrospinal fluid concentrations of Gas6 had milder neurological deficits and recovered more quickly[289]. Furthermore, soluble forms of Axl and Mer, known to act as decoy receptors, are elevated in homogenates of chronic MS lesions compared with control brain homogenates[290].

A regulatory role of TAM receptor signaling in CNS autoimmunity is supported by experiments with animal models. Prophylactic treatment of myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) peptide-immunized C57BL/6 mice with either an anti-Axl agonistic antibody via intraperitoneal injection, or recombinant Gas6 via intracerebral infusion, ameliorates the later stages of EAE[291,292]. However, neither intervention alters the day of onset or the early clinical course. Such a delay in therapeutic impact is consistent with the fact that Axl protein is expressed at very low levels in the naïve CNS but is upregulated in the context of neuroinflammation[293]. Corroborating the above findings, mice that are deficient in Axl develop a

relatively severe form of EAE, but they divergence in their clinical scores from those of wild-type (WT) counterparts only occurs once the disease is established, approximately one week following clinical onset[221]. In contrast to Axl, Mer is readily detected on microglia in unmanipulated mice during homeostasis[293], suggesting that modulation of Mer could influence susceptibility to, and/or the initial clinical presentation of EAE. There are no published studies that specifically address the role of Mer, or potential redundancies in the function of TAM receptors, in EAE pathogenesis and regulation.

In the current study we investigate the impact of global TAM receptor inhibition on Th17 cell-mediated EAE. We chose to employ an adoptive transfer model in order to focus on the role of TAM receptors during the effector phase of EAE, beyond encephalitogenic T cell priming. Unexpectedly, a large percentage of the TAM inhibitor-treated adoptive transfer recipients presented with an atypical form of disease, characterized by ataxia and neutrophil-rich inflammatory infiltrates in the hindbrain white matter. These results indicate that constitutive TAM signaling can be protective against the initial establishment of CNS parenchymal inflammation in a region-specific manner.

Materials and Methods

Mice. Female C57BL/6 mice were obtained from the National Cancer Institute Frederick, Charles River Laboratories, or Jackson Laboratories at eight to twelve weeks of age. Mice were housed under specific pathogen-free conditions in microisolator cages. All animal studies were approved by the Institutional Animal Care and Use Committee.

Antibodies and reagents. The following antibodies were obtained from eBioscience and used for flow cytometry: PE anti-Axl [MAXL8DS], PE-Cy7 anti-Mer [DS5MMER], PerCP-

Cy5.5 anti CD11c [N418]. The following antibodies were obtained from BD Biosciences and used for flow cytometry: BUV805 anti-CD45 [30-F11], PE-Cy7 and BUV563 anti-Ly6G [1A8], BUV395 and BUV661 anti-CD11b [M1/70], BUV737 anti-CD11c [N418], PE-Cy5 anti-CD3 [145-C11], and BV786 anti-I-A/I-E [M5/114.15.2].The following antibodies were obtained from Biolegend and used for flow cytometry: PE and APC anti-CD88 [20/70], APC anti-I-A/I-E [M5/114.15.2], and FITC anti-CD26 [H194-112]. The TAM receptor kinase inhibitor LDC1267 (Millipore Sigma) was given daily via intraperitoneal injection at 20 mg/kg in 5% DMSO. The following primary Abs were used for IHC: rat anti-CD45 (IBL-5/25, Millipore Sigma), rabbit anti-IBA1 (Wako), rat anti-Ly6G (1A8 eBioscience), FITC anti-CD11b (M1/70 eBioscience), hamster anti-CD3 (500A2 BD), and mouse anti-myelin basic protein (MBP, SMI99, Biolegend). The following secondary Abs were used for IHC: goat anti-rat Alexa Fluor 488 (Invitrogen) and Alexa Fluor 594 (Invitrogen), goat anti-rabbit Alexa Fluor 647 (Invitrogen), goat anti-hamster Alexa Fluor 647 (Life Technologies) and goat anti-mouse Alexa Fluor 594 (Invitrogen)

Induction and scoring of EAE. EAE was induced as previously described[275]. Mice were immunized with an emulsion consisting of 100 μg of MOG_{35-55} peptide (MEVGWYRSP-FSRVVHLYRNGK; Biosynthesis) emulsified in CFA (Sigma [F5881], supplemented with BD Difco [BD 231141]), at four sites over the flanks. Inguinal, axial, and brachial lymph nodes were harvested 10-14 days later, homogenized, and passed through a 70μm strainer to achieve a single-cell suspension. The dissociated cells were cultured with $MOG_{35–55}$ peptide (50 μg/mL; Biosynthesis) under Th17-polarizing conditions (8 ng/mL recombinant murine IL-23 [R&D], 10 ng/mL recombinant murine IL-1α [Peprotech], and 10 μg/mL anti–IFN-γ [Bio X Cell]), in RPMI media (Gibco) supplemented with HEPES buffer (Gibco, 12.3mM) MEM non-essential amino acids (Gibco, 1X), sodium pyruvate (Gibco, 1X), β-mercaptoethanol (Gibco 0.5mM), L-

glutamine (Gibco, 1X), and Pen Strep (Gibco, 1:100 dilution). After 96 hours of culture, $CD4^+$ T cells were purified via L3T4 magnetic bead sorting (Miltenyi Biotec), and injected into naïve syngeneic recipients ($5x10^6$ $CD4^+$ T cells per mouse i.p.). Recipient mice were observed daily for signs of EAE by an examiner blinded to the experimental groups. Mice with conventional EAE were scored using a 0-5 scale: 0=no abnormality, 0.5=distal limp tail, 1=complete limp tail, 1.5=difficulty righting from supine position, 2=inability to right from a supine position, 2.5=overt gait abnormality, 3=difficulty elevating body while walking, 3.5=unilateral hind limb paralysis, 4=bilateral complete hind limb paralysis, 4.5=moribund, 5=death. Mice with atypical EAE were scored using an alternative 4 point scale: 0=no abnormality, 1=slight listing/difficulty righting, 2=obvious imbalance but able to ambulate, 3=severely impaired balance/ambulation, 4=incapacitated due to inability to maintain upright posture/spinning.

Cell isolation. Immune cells were isolated as previously described[275]. Cardiac perfusion was performed under isoflurane anesthesia with 10mL 1X PBS. The spinal cord was isolated by pushing 10mL of 2% FBS through the spinal column. The optic nerve was dissected from the eye. The spinal cord, hindbrain, and forebrain were processed separately by homogenizing the tissue in 1 mL PBS containing protease inhibitor (Roche). After pelleting, the supernatant was collected for protein analysis. 3 mL of collagenase A (1 mg/mL) and DNase I (1 mg/mL) in HBSS with calcium and magnesium was added to the cellular layer and optic nerves and incubated in a 37˚C water bath for 20 minutes. The optic nerves were then homogenized through a 70 µm strainer. Samples were pelleted, brought up in 27% Percoll (GE Healthcare), and spun at 800xg for 10 minutes. The myelin/debris layer was removed, and the pelleted cells were used for downstream analyses. The spleen was processed by homogenizing the tissue through a 70 µm strainer. Red blood cells from the spleen and blood were lysed using ACK lysis buffer (Quality

biologicals) and reaction was quenched using 2% FBS in 1X PBS. Pelleted cells were then used for downstream analyses.

Flow cytometry. Flow cytometry was performed as previously described[275]. Cells were resuspended in PBS with 2% FBS, Fc Block (anti–CD16/32; 100 ng/ml), and Fixable viability (ebioscience, 1:500) before 1:2 dilution with fluorochrome-conjugated Abs, listed in the "Antibodies and reagents" methods section above. Data were collected with a FACSymphony flow cytometer using FACSDiva software (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star).

Measurement of CNS cytokine and enzyme expression. Cytokine levels were measured in CNS homogenate supernatants, as described previously[275], via Luminex multiplex bead-based analysis (Millipore) using the Bio-Plex 200 system, following the manufacturer's protocols. Myeloperoxidase was measured using the MPO DuoSet ELISA kit (R&D Systems) and Leukotriene B4 was measured using the LTB4 Parameter Assay kit (R&D Systems), according to the manufacturer's protocols. Total protein, measured via a Bradford assay (Thermo Scientific), was used to normalize analyte concentrations.

Immunohistochemistry. Immunohistochemistry was performed as previously described[275]. Following intracardiac perfusion with 10mL of PBS and 10mL of 4% paraformaldehyde, brain and spinal cords were removed, postfixed in 4% paraformaldehyde for one day at 4˚C, cryoprotected in 30% sucrose for 3-5 days at 4˚C, and embedded and frozen in OCT (Fisher Health Care) at -80˚C. 30 µm sections were cut with a Leica CM1950 cryostat, placed on charged slides, and frozen at -20˚C. For staining, slides were brought to room temperature, washed twice with PBS, and twice with PBST (PBS + 0.5% triton X, Thermo Fisher), prior to antigen retrieval treatment with EDTA (anti-Ly6G) or sodium citrate (anti-MBP) for 20 minutes

at 95˚C. Slides were blocked with 5% goat serum (Sigma) in PBST for 1 hour prior to incubation with primary antibody overnight at 4˚C. After washing twice with PBST, slides were incubated with a secondary antibody for two hours at room temperature. Slides were washed twice with PBST and stained with DAPI (1:1000). Slides were mounted using Fluoromount-G (SouthernBiotech). Images were acquired on an Olympus IX83 light microscope or an Olympus IX83 confocal microscope and processed using CellSens. The meningeal-parenchymal border of spinal cord and brain cross-sections was outlined manually using the Surface function in Imaris (version 9.0). The total number of $CD45^{+}IBA1^{-}$ cells in the white matter parenchyma was counted using the Cells function in Imaris, and normalized to the total parenchymal white matter area, which was calculated using Fiji software (Image J 2.3 distribution). The shortest distance between each $CD45^{+}IBA1^{-}$ cell and the meningeal-parenchymal border was determined by Imaris. Cells were grouped into 50 μm sequential segments from the border and counted.

Statistical analysis. The Wilcoxon Rank Sum test was used to compare clinical courses. A mixed-effects analysis test for trend was used to compare Mer and Axl expression in leukocytes through the course of EAE. The unpaired Student's *t*-test with Welch's correction was used to compare other clinical metrics, cytokine levels, IHC quantification, and cell counts. Statistical tests were performed in GraphPad 9.0 (Prism). A p-value of 0.05 was considered significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Results

The frequency of Mer$^+$ microglia is elevated in the brain compared with the spinal cord during homeostasis and early EAE.

The TAM receptors predominantly expressed on myeloid cells, including microglia, are Axl and Mer. We measured the levels of both receptors on CD45^{int}CD11b$^+$ microglia, isolated separately from the forebrain, hindbrain, and spinal cord of naïve mice by flow cytometry. Consistent with previous published data[293], Mer was detectable on the majority of microglia in the forebrain and hindbrain during homeostasis (Figure 3.1.A). Interestingly, the frequency of Mer$^+$ microglia varied markedly between the brain and spinal cord. While 60-70% of forebrain or hindbrain microglia in individual mice consistently expressed Mer, less than 25% of microglia in spinal cord tissue harvested from the same mice were Mer$^+$. Axl was sparsely expressed by microglia (2-18%), irrespective of the CNS compartment. The mean fluorescent intensity (MFI) of TAM receptor staining on the receptor positive microglial cells was comparable across all three CNS compartments (Figure 3.1.B).

Next, we analyzed the expression of Mer and Axl on microglia, as well as CD45^{hi}CD11b$^+$ hematogenous myeloid cell subsets, during sequential stages of EAE (Figure 3.1.C-D, 3.2.A-C). At the pre-clinical stage (day 6 p.t.) and afterwards, the percentage of Mer$^+$ microglia in the forebrain and hindbrain dropped substantially from baseline, varying between 20-45% through late EAE The percentage of Mer$^+$ spinal cord microglia remained at 20% or below throughout the clinical course (Figure 3.1.C, upper left panel). Very few microglia, in either the brain or spinal cord, expressed Axl at any stage of the clinical course. With regard to Infiltrating subsets, relatively few CD45^{hi}CD11b$^+$CD11c$^-$ monocytes/ macrophages or CD26$^+$CD11c$^+$ conventional dendritic cells (cDC) were Mer$^+$ at any timepoint (Figure 3.1.C, upper and lower right panels).

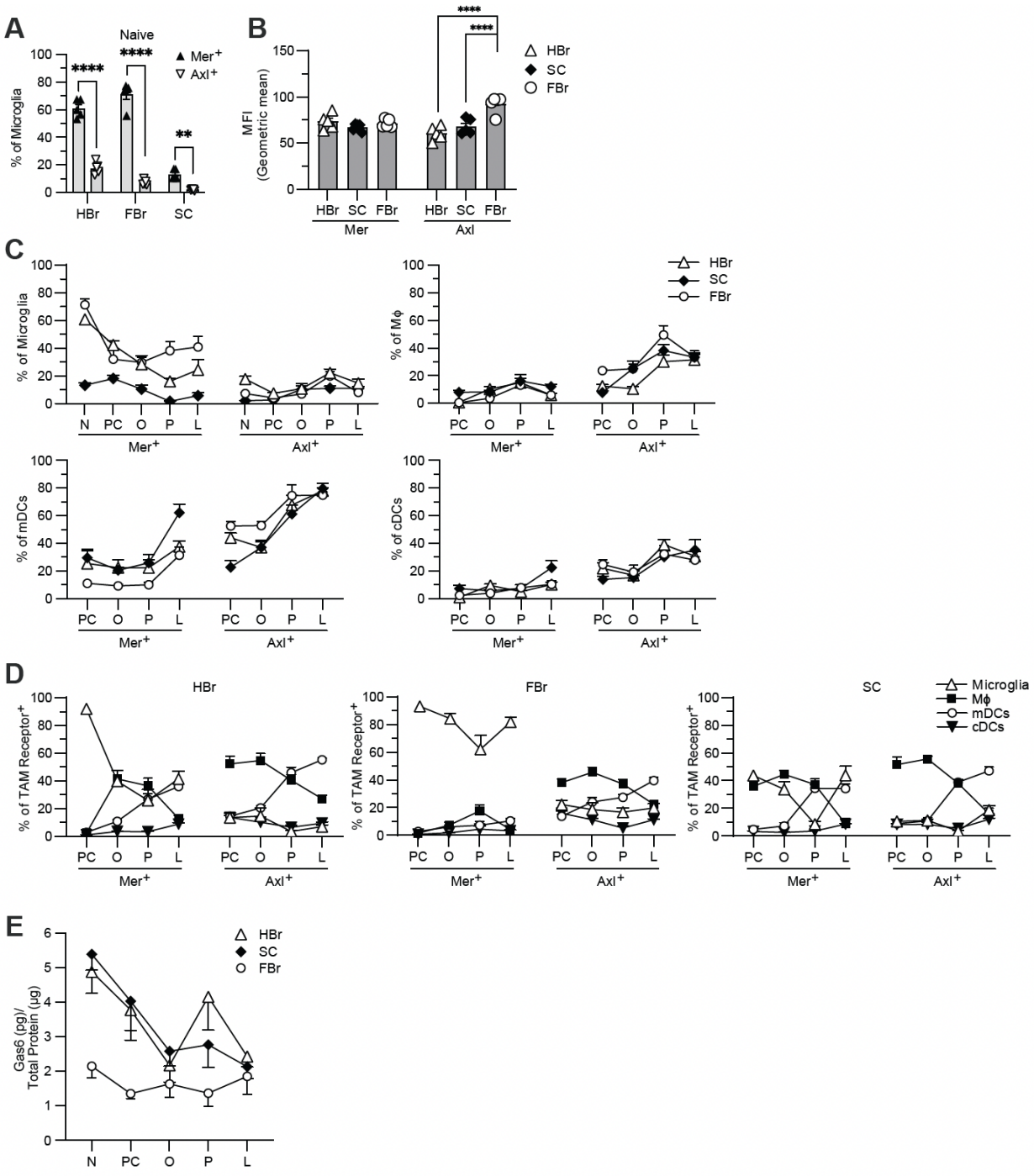

Figure 3.1 - Mer is highly expressed on brain microglia during homeostasis and immediately prior to EAE onset.

The hindbrain (HBr), forebrain (FBr), and spinal cord (SC) were harvested from Th17-adoptive transfer recipients at the preclinical (PC), onset (O), peak (P), and late (L) stages of EAE, or from naïve mice. Inflammatory cells were isolated from each compartment separately, and surface stained to detect Axl and Mer. The cells were co-stained with antibodies specific for leukocyte subset markers in order to distinguish microglia ($CD45^{int}CD11b^{+}$), and $CD45^{hi}$ macrophages (Mϕ,

continued… $CD11b^+Ly6G^-CD11c^-$), monocyte-derived dendritic cells (mDCs, $CD88^+CD11c^+Ly6G^-$), and conventional dendritic cells (cDCs, $CD11c^+CD26^+Ly6G^-$). **A)** Frequency of Mer^+ or Axl^+ cells among naïve microglia. **B)** The geometric mean fluorescence intensity (MFI) of each TAM receptor on Mer^+ or Axl^+ naïve microglia. **C)** The frequency of Mer^+ or Axl^+ cells among microglia (upper left panel), macrophages (upper right panel), mDCs (lower left panel), and cDCs (lower right panel) isolated from the hindbrain, forebrain, and spinal cord, respectively, of EAE adoptive transfer recipients at the indicated time points. **D)** The cellular composition of $CD45^+Mer^+$ or $CD45^+Axl^+$ cells in individual CNS regions during serial stages of EAE. **E)** Gas6 protein levels in CNS homogenate supernatant, measured by ELISA. Data were pooled from two independent experiments with similar results with n=5-10/ group. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Although the percentage of Mer^+ $CD45^{hi}CD88^+CD11c^+$ monocyte-derived dendritic cells (mDC) were also relatively low during pre-clinical and acute EAE, it rose in all three compartments during late EAE (Figure 3.1.C, lower left panel). The percentage of Axl^+ macrophages, mDCs, and cDCs rose in all three compartments during peak and late EAE (Figure 3.1.C, right panel and lower left panel). We did not detect Mer or Axl on $CD11b^+Ly6G^+$ neutrophils in any of the samples (data not shown).

During pre-clinical EAE, microglia constituted the vast majority of Mer^+ myeloid cells in the hindbrain and the forebrain (>90%, Figure 3.1.D, left and middle panels). In contrast, at that time point, approximately half of the Mer^+ myeloid cells in the spinal cord were macrophages, and the other half were microglia (Figure 3.1.D, right panel). Mer^+ cells in the hindbrain were evenly divided between microglia and macrophages at clinical onset and peak EAE, while microglia remained the predominant Mer^+ cell in the forebrain throughout the clinical course. The majority of Axl^+ myeloid cells in all three compartments were macrophages during the pre-clinical stage and at the onset of clinical EAE (Figure 3.1.D, all panels). We did not detect Mer or Axl on infiltrating T cell, B cell, and neutrophil populations, nor on circulating leukocytes (data not shown). Further, we did not detect Tyro3 on the cell surface of any $CD45^+$ population (data not shown).

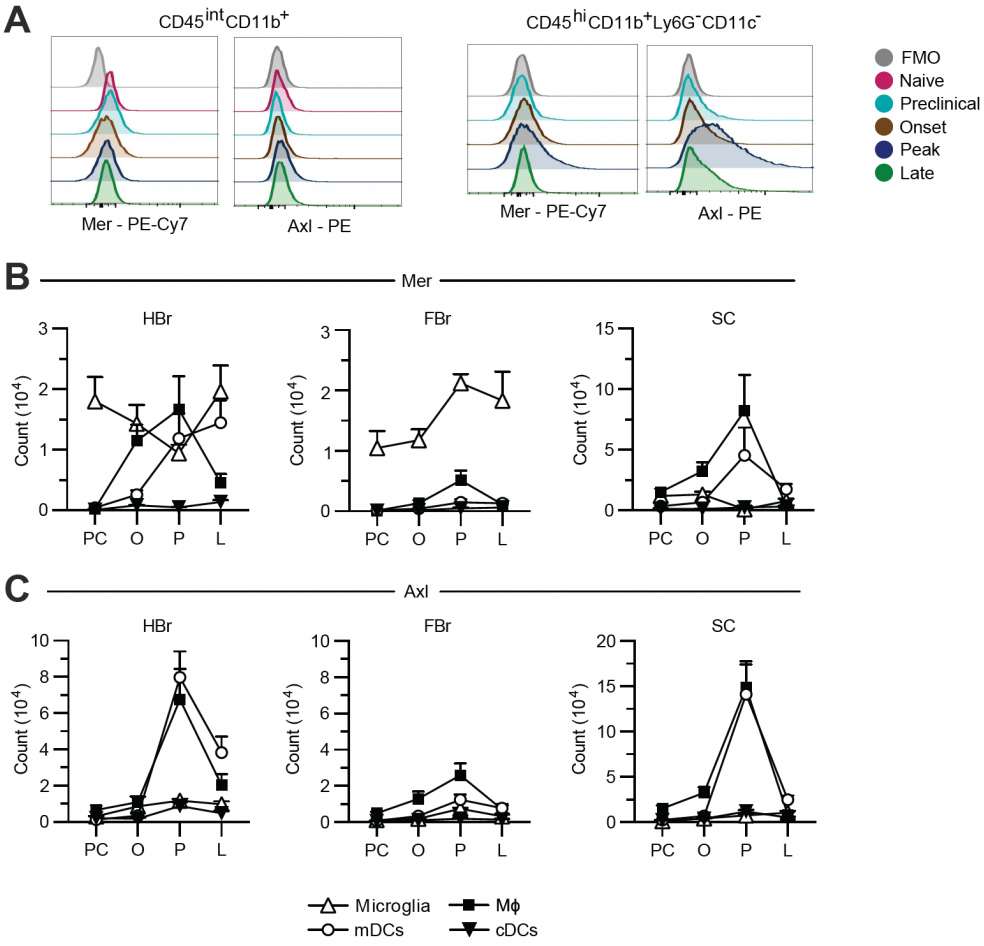

Figure 3.2 - Mer and Axl expressing cell populations in the CNS during EAE.
A-C) The CNS was harvested from Th17-adoptive transfer recipients at the preclinical (PC), onset (O), peak (P), and late (L) stages of EAE. Mer and Axl expression were assessed via flow cytometry on microglia ($CD45^{int}CD11b^{+}$), macrophages (Mϕ, $CD45^{hi}CD11b^{+}$ $Ly6G^{-}CD11c^{-}$), monocyte-derived dendritic cells (mDCs, $CD45^{hi}CD88^{+}CD11c^{+}Ly6G^{-}$), and conventional dendritic cells (cDCs, $CD45^{hi}CD11c^{+}CD26^{+}Ly6G^{-}$) [n=5/ time point]. **A)** Representative flow plots of Mer (left panel) and Axl (right panel) in $CD45^{int}CD11b^{+}$ microglia (left group) and $CD45^{hi}CD11b^{+}CD11c^{-}$ macrophages (right group) throughout disease. **B,C)** The number of B) Mer^{+} and **C)** Axl^{+} cell populations in the hindbrain (HBr, left), forebrain (FBr, middle), and spinal cord (SC, right) throughout EAE. [n=5/ time point].

Gas6 is the primary TAM receptor ligand expressed in the CNS[294]. We measured its level in CNS homogenate supernatants taken from naïve mice and at various time points during EAE. Gas6 was expressed at relatively high concentrations in the hindbrain and the spinal cord of naïve mice (Figure 3.1.E). Its levels fell progressively in both of those compartments until late

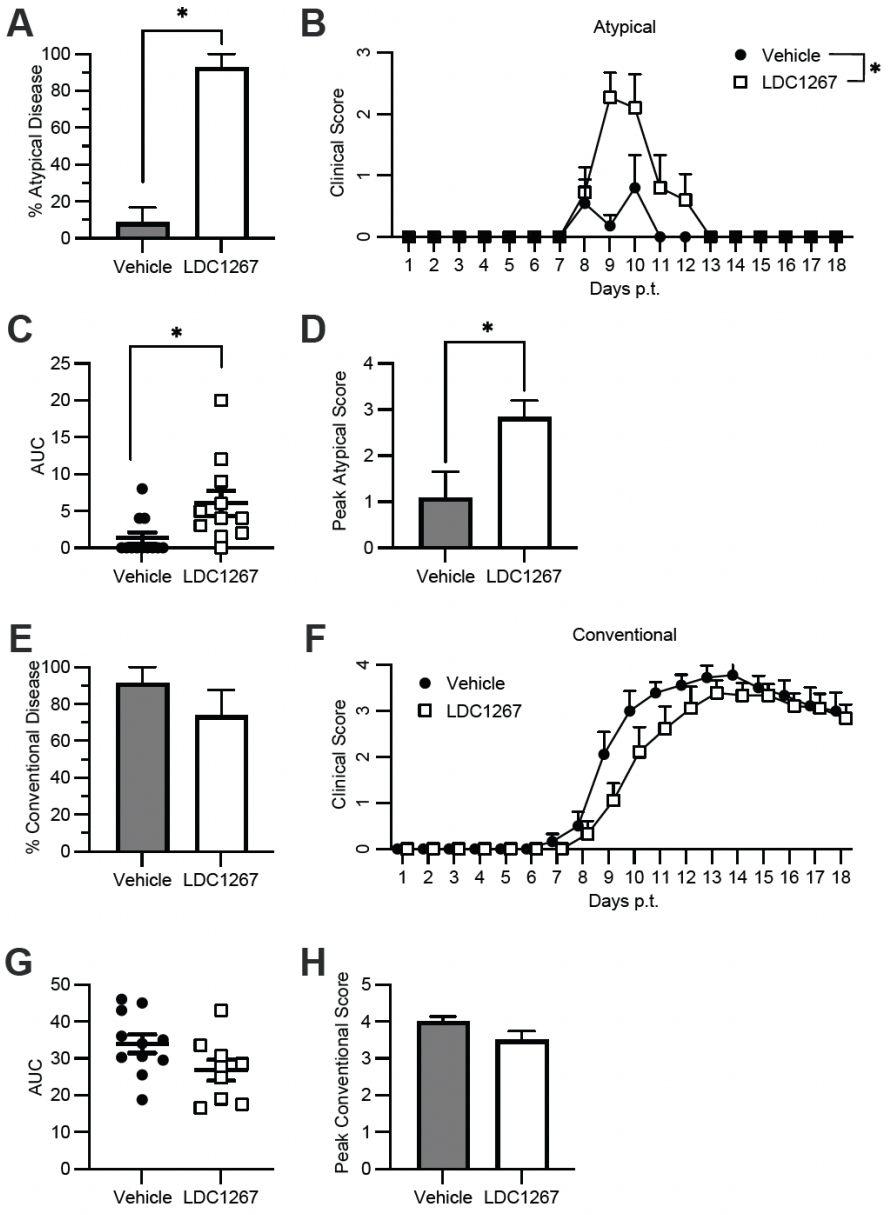

Figure 3.3 - TAM receptor inhibition promotes the development of atypical EAE in Th17 recipients.

C57BL/6 mice were injected with encephalitogenic Th17 cells on day 0, and treated with the TAM tyrosine kinase inhibitor LDC1267 (n=11), or vehicle control (n=11), daily from day 6 to day 14. Mice were assessed for severity of neurological deficits by rater blinded to the experimental groups. Data were pooled from two independent experiments with similar results. **A)** Percentage of mice that developed atypical disease. **B)** Atypical disease scores over time. Comparison of the groups during the time the animals had deficits (d8-12) demonstrated a significant difference. **C)** Area under the curve (AUC) reflecting collective clinical scores, and **D)** peak atypical score of individual mice in each group. **E)** Percentage of mice that developed conventional disease. **F)** Conventional disease score of the treated mice that developed conventional EAE. **G)** AUC analysis and **H)** peak conventional score for individual mice in each group. * $p<0.05$

stage, with the exception of a transient upregulation in the hindbrain at peak EAE. Gas6 was expressed at relatively low levels in the forebrain at baseline and throughout the course of EAE.

Inhibition of TAM receptors in Th17 adoptive transfer recipients triggers a high incidence of atypical EAE

In order to assess the role of TAM receptor signaling during the effector phase of EAE, we treated Th17 adoptive transfer recipients with LDC1267, a highly selective global TAM receptor kinase inhibitor[220], or with vehicle alone, starting on day 6 post-transfer. Surprisingly, a high percentage of LDC1267-treated mice presented with atypical neurological signs, characterized by gait imbalance and listing, as opposed to the usual ascending paralysis (Figure 3.3.A). Approximately 90% of LDC1267-treated mice initially developed atypical disease, compared with 10% of their vehicle treated counterparts (Figure 3.3.A-B). Among the mice that developed ataxia, those treated with LDC1267 developed more severe deficits and cumulative disability compared to those treated with vehicle (Figure 3.3.C-D). The majority of LDC1267-treated mice transitioned from an atypical into a conventional course within several days of clinical onset (Figure 3.3.E-F). Despite the fact that conventional EAE was slightly delayed in LDC1267-treated mice, they ultimately reached a degree of hindlimb weakness comparable to the control group (Figure 3.3.G-H).

The number and composition of brain- and spinal cord-infiltrating cells are comparable in LDC1267- versus vehicle-treated adoptive transfer recipients

Atypical EAE has previously been described in the setting of SOCS3 and IFN-γ receptor deficiency[102,201,295–297]. In those instances, the atypical clinical presentation is associated with

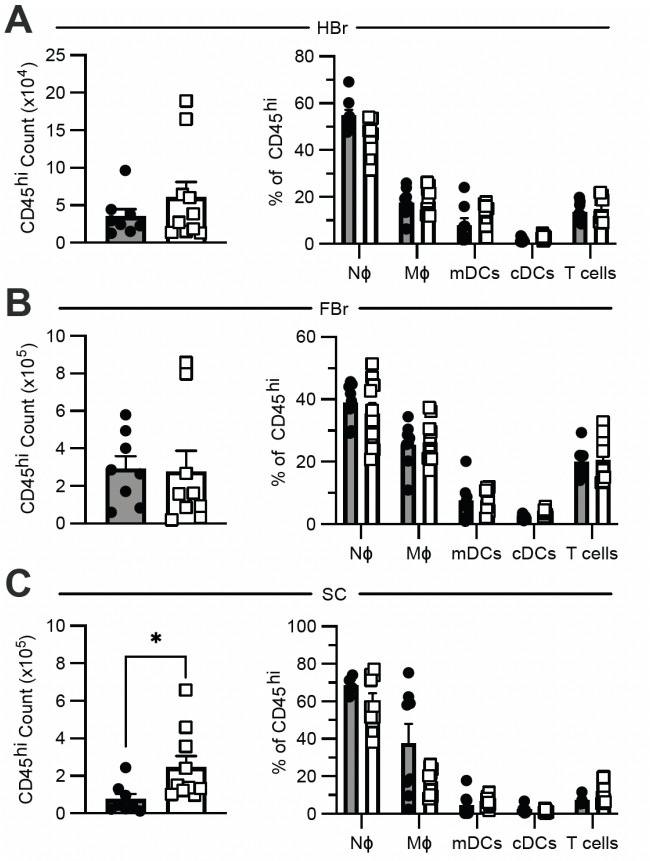

Figure 3.4 - LDC1267 treatment does not alter the number or composition of CD45^{hi} populations.
Leukocytes were isolated from the **A)** hindbrain (HBr), **B)** forebrain (FBr), and **C)** spinal cord (SC) of LDC1267- and vehicle-treated mice at the onset of atypical or conventional EAE, respectively. Isolated cells were surface stained for leukocyte markers to distinguish neutrophils (Nϕ, CD45^{hi} CD11b^{+}Ly6G^{+}) macrophages (Mϕ, CD45hi CD11b^{+}Ly6G^{-} CD11c^{-}), monocyte-derived dendritic cells (mDCs, CD45^{hi} Ly6G^{-}CD11c^{+}CD88^{+}), conventional dendritic cells (cDCs, CD45^{hi} Ly6G^{-}CD11c^{+}CD26^{+}), and T cells (CD45^{hi}CD3^{+}). Data were pooled from two independent experiments [n=6-10/group]. * $p<0.05$

enhanced hindbrain inflammation. Inflammatory cells isolated from LDC1267-treated mice at the onset of atypical EAE, and from vehicle-treated mice at the onset of conventional EAE, were subjected to flow cytometric analysis. Unexpectedly, the absolute numbers of hindbrain- and forebrain-infiltrating CD45^{hi} cells were comparable in LDC1267- and vehicle-treated mice, and the number of spinal cord-infiltrating CD45^{hi} cells was elevated in LDC1267-treated mice (Figure 3.4.A-C, left panel). The optic nerve is another region of white matter that displays

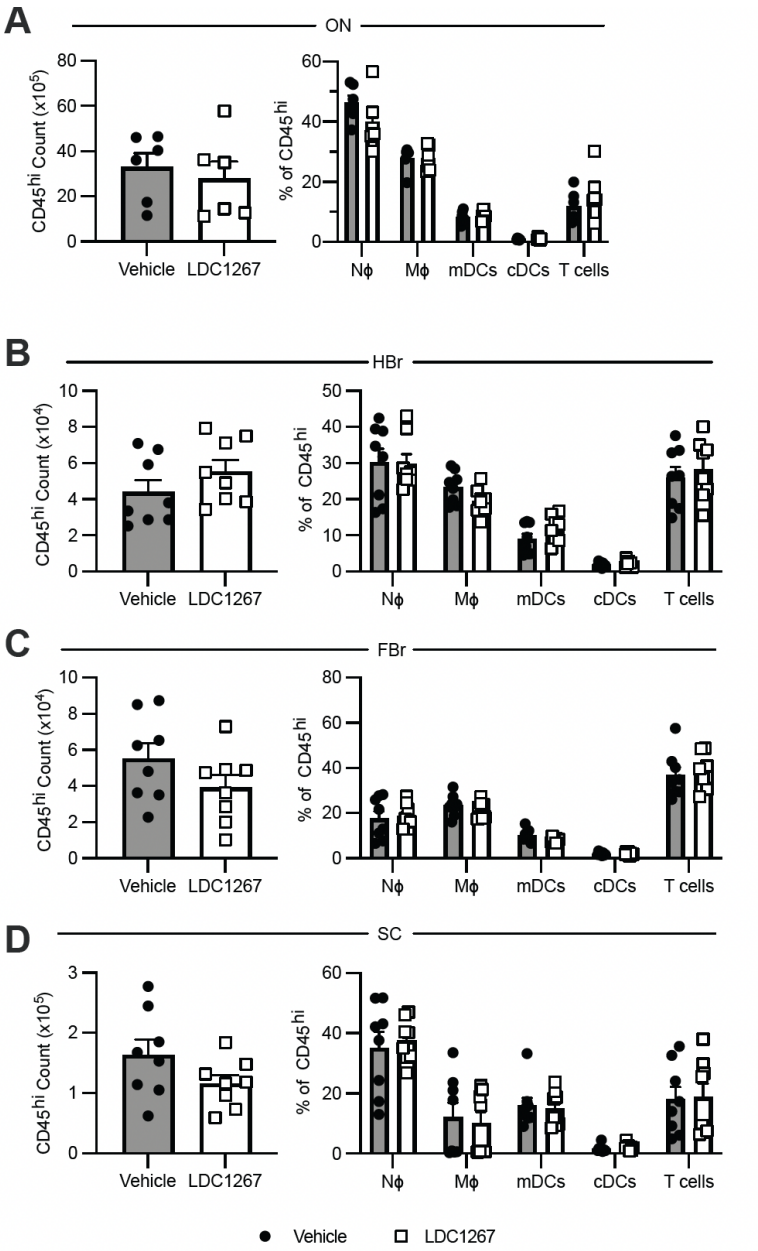

Figure 3.5 - CNS infiltrate number and composition is not altered in LDC1267-treated mice during or after EAE onset.

Tissue was harvested from LDC1267-treated mice with an atypical EAE course and vehicle-treated mice with a conventional EAE course. Leukocytes were isolated from the **A)** optic nerve (ON) of at EAE onset [n=6/group], or the **B)** hindbrain (HBr), **C)** forebrain (FBr), and **D)** spinal cord (SC) 2-3 days post onset [data pooled from two independent experiments, n=7-8/group]. Flow cytometry was used to quantify the number (left) of $CD45^{hi}$ cells and the frequency (right) of neutrophils (Nϕ, $CD45^{hi}$ $CD11b^{+}Ly6G^{+}$) macrophages (Mϕ, CD45hi $CD11b^{+}Ly6G^{-}$ $CD11c^{-}$), monocyte-derived dendritic cells (mDCs, $CD45^{hi}$ $Ly6G^{-}CD11c^{+}CD88^{+}$), conventional dendritic cells (cDCs, $CD45^{hi}$ $Ly6G^{-}CD11c^{+}CD26^{+}$), and T cells ($CD45^{hi}CD3^{+}$).

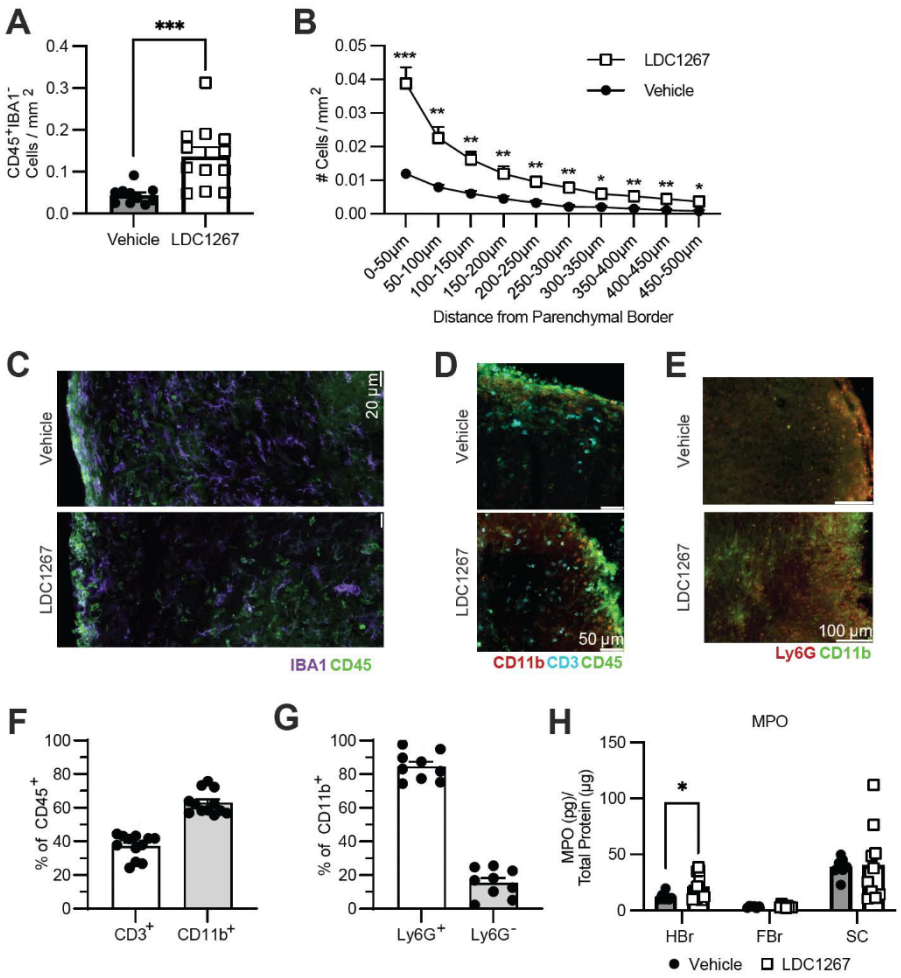

Figure 3.6 - Inhibition of TAM receptor signaling in Th17 recipients causes a redistribution of inflammatory cells within the hindbrain.

The brains and spinal cords were harvested from LDC1267- and vehicle-treated mice at the onset of atypical or conventional EAE, respectively, and processed for immunohistochemistry. **A)** Density of $CD45^{+}IBA1^{-}$ within the brainstem parenchymal tissue [2-3 brainstem sections per mouse, 4 mice/group]. **B)** The distribution of $CD45^{+}IBA1^{-}$ in the brainstem, quantified at serial distances from parenchymal border, and normalized to total parenchymal area. **C)** Representative brainstem images of mice treated with vehicle control (upper panel) or LDC1267 (lower panel) and stained with antibodies directed against CD45 (green) and IBA1 (purple). **D, E)** Representative images of lesions within the brainstem of vehicle- (upper) or LDC1267-treated (lower) mice. Sections were stained with antibodies directed against **D)** CD45 (red), CD11b (green), and CD3 (blue) or **E)** CD11b (green) and Ly6G (red). **F, G)** The composition of cells found within brainstem parenchyma lesions of LDC1267-treated mice. [2-3 sections per mouse, 3-4 mice/group] F) The frequency of T cells ($CD3^{+}$) and myeloid cells ($CD11b^{+}$) among the $CD45^{+}$ cells within brainstem parenchymal white matter. **G)** The frequency of neutrophils ($Ly6G^{+}$) among the $CD11b^{+}$ cells brainstem parenchymal white matter. H) Levels of myeloperoxidase (MPO) were measured in brainstem homogenates via ELISA, and normalized to total protein. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

inflammatory pathology at the onset of EAE[298]. There was no difference in the number of optic-nerve infiltrating leukocytes between the groups (Figure 3.5.A, left panel). We observed no significant difference in the composition of the infiltrating myeloid cells in the CNS tissues from LDC1267-treated versus vehicle treated mice, irrespective of region (Figure 3.4.A-C, 3.5.A, right panel). We also examined cellular infiltration of the hindbrain, forebrain, and spinal cord of recipients 2-3 days post disease onset. The number and composition of inflammatory infiltrates were similar between LDC1267- and vehicle-treated mice (Figure 3.5.B-D).

TAM receptor inhibition promotes neutrophil infiltration of the hindbrain parenchymal white matter during Th17 mediated EAE

We next performed immunohistochemical studies of brain and spinal cord sections, harvested from mice in both treatment groups, to assess the spatial distribution of infiltrating inflammatory cells. $CD45^{+}Iba1^{-}$ cells, consistent with infiltrating leukocytes, were largely confined to the meninges in the hindbrains of control mice but migrated deep into the hindbrain white matter of LDC1267-treated mice (Figure 3.6.A-C). The majority of the $CD45^{+}Iba1^{-}$ cells that had infiltrated the hindbrain white matter were $CD11b^{+}Ly6G^{+}$, indicative of neutrophils (Figure 3.6.D-G). Consistent with that observation, myeloperoxidase (MPO), an enzyme primarily expressed in neutrophils, was elevated in hindbrain homogenates of the LDC1267-treated compared with vehicle-treated mice (Figure 3.6.H). The spatial distribution of infiltrates and the demyelination in spinal cord sections were similar between the treatment groups; sparse infiltrates were found in the cerebellum and corpus callosum parenchyma in all the experimental animals (Figure 3.7).

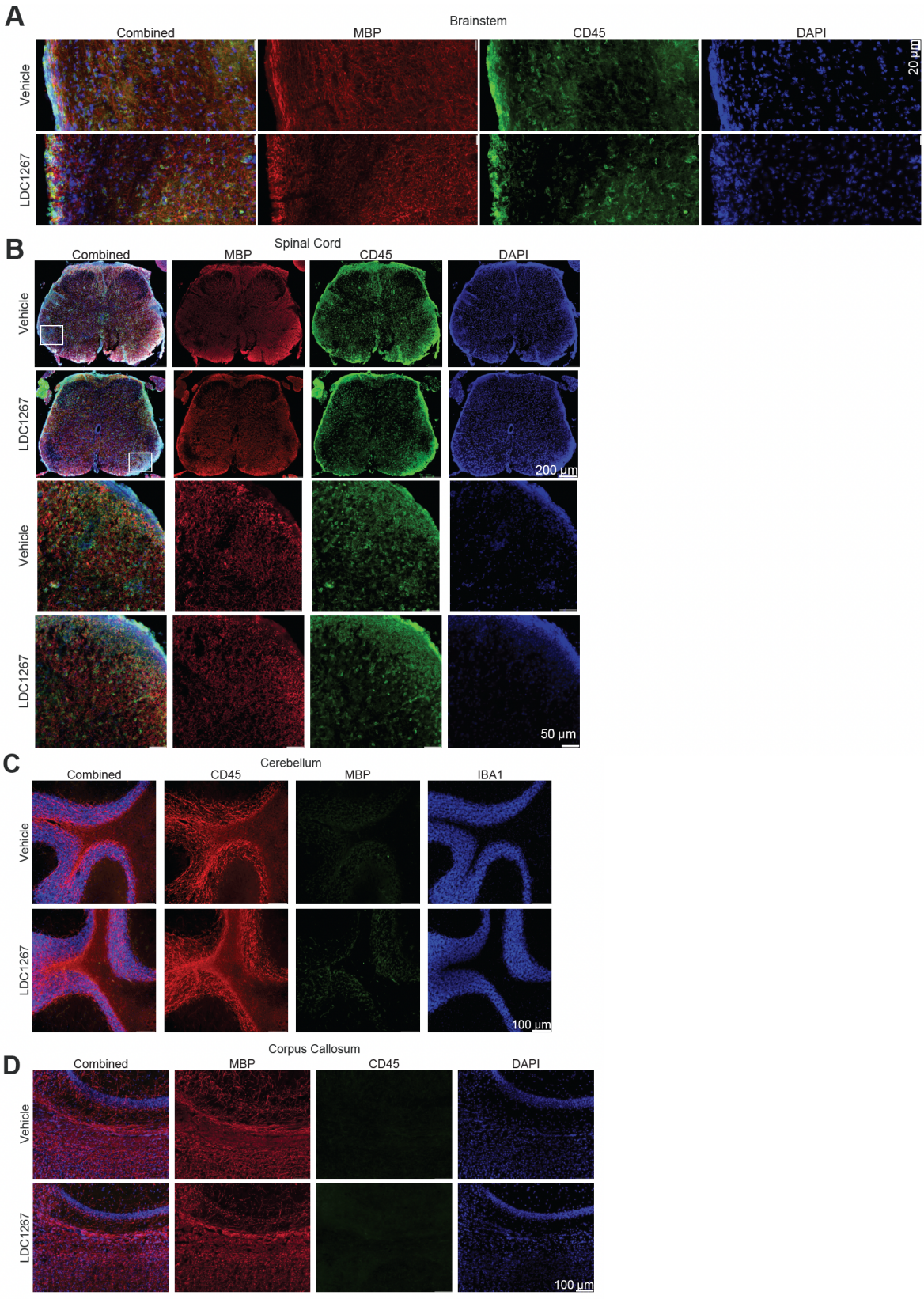

Figure 3.7 - Representative figures of CNS inflammation and demyelination in LDC1267- and vehicle-treated mice.

continued… Brains and spinal cords were harvested from LDC1267- and vehicle-treated mice at the onset of atypical or conventional EAE, respectively, and processed for immunohistochemistry. **A)** Representative image of myelin basic protein (MBP, red) and CD45 (green) staining in the brainstem of vehicle- (upper) or LDC1267-treated (lower) mice. **B)** Representative image of the distribution of $CD45^+$ (green) cells in the spinal cord of vehicle- (first and third rows) or LDC1267-treated (second and fourth rows) mice and their association with demyelination (MBP, red). The images in the third and fourth rows are taken at a greater magnification within the areas of the spinal cord indicated in the first and second rows. **C-D)** Representative images of the sparse inflammatory infiltrate (CD45, green) and patterns of myelination (MBP, red) in the **C)** cerebellum, **D)** corpus callosum of vehicle- (upper) and LDC1267-treated (lower) mice.

To determine whether the inflammatory milieu in the hindbrain is altered by LDC1267 treatment, we measured a panel of cytokine and chemokine proteins in CNS homogenates at clinical onset. Levels of granulocyte-colony stimulating factor (G-CSF), IL-1β, TNF-α, and IL-6 were elevated in hindbrain homogenates from LDC1267-treated versus vehicle-treated mice, but not in forebrain or spinal cord homogenates (Figure 3.8.A-D). Conversely, there were no significant differences between the treatment groups in expression of chemotactic molecules CCL2, CXCL2, CCL3, CCL4, CCL11, CXCL1, CXCL5, CXCL9, CXCL10, and Leukotriene B4 (Figure 3.8.E-F, data not shown), or Th-released cytokines IFN-γ and IL-17 (Figure 3.8.G-H).

Discussion

In the current study, we show that acute blockade of all three TAM receptors, shortly following the adoptive transfer of encephalitogenic Th17 cells, triggers an atypical clinical presentation of EAE that is suggestive of hindbrain dysfunction. Surprisingly, this altered clinical phenotype is not associated with gross differences in the absolute number, or cellular composition, of the hematogenous myeloid cells that traverse the CNS vasculature and invade the border-associated regions of the brain. Rather, TAM receptor blockade skews the spatial distribution of the infiltrating myeloid cells within the hindbrain, such that they more readily

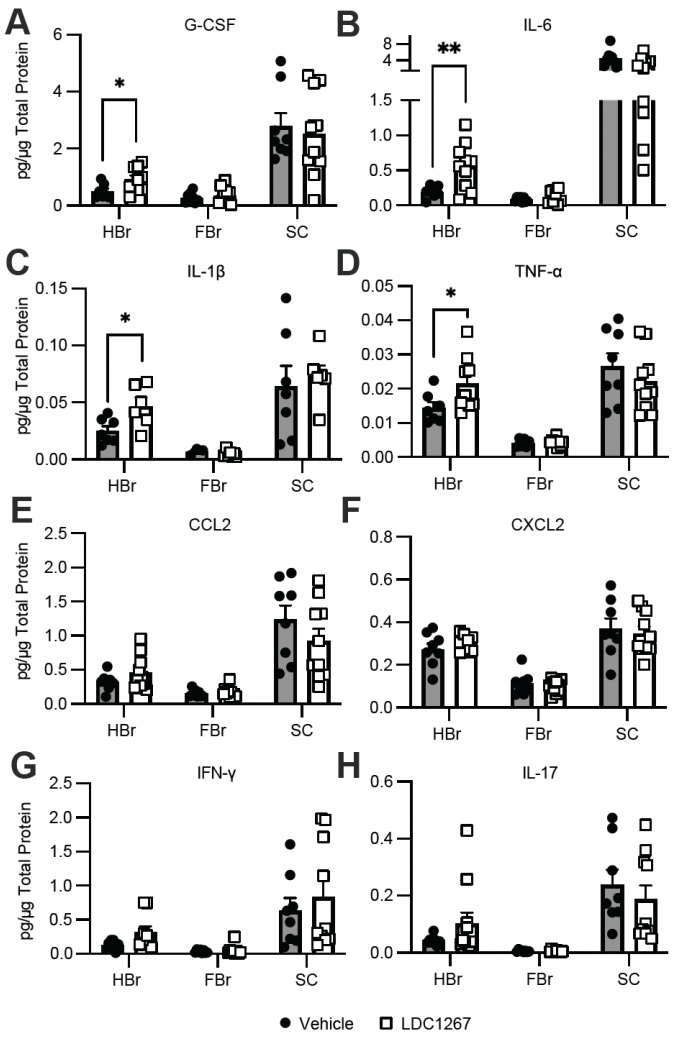

Figure 3.8 - Pro-inflammatory cytokines are elevated in the hindbrain of LDC1267-treated Th17 transfer recipients.

A-H) Supernatants of hindbrain (HBr), forebrain (FBr), and spinal cord (SC) homogenates were collected from mice treated with LDC1267 (n=11) or vehicle control (n=8) at the onset of atypical or conventional EAE, respectively. Multiplex analysis was performed to measure the expression of a panel of chemokines and cytokines. Analyte levels were normalized to total protein. * $p<0.05$

migrate from the surrounding meninges into the parenchyma. Reminiscent of these findings, the numbers of CNS-infiltrating cells are comparable in MOG-sensitized *Axl*$^{-/-}$ versus WT mice, as per flow cytometry, but infiltrating CD11b^{+} cells extend deeper into the spinal cord white matter of the knock-out mice, which develop more severe hindlimb weakness[221]. Collectively, these data suggest that in a number of experimental paradigms, TAM receptor signaling in the CNS deters the movement of infiltrating myeloid cells from border-associated regions into

parenchymal tissue, but it does not impede the early recruitment of leukocytes across the blood-meningeal or blood-brain barrier.

Tyro3$^{-/-}$ and WT mice have comparable EAE clinical courses[221]. *Axl*$^{-/-}$ mice exhibit more severe conventional EAE than WT mice, but the exacerbated course only becomes apparent several days following clinical onset[221]. This delay likely reflects the fact that Axl is expressed at low levels in the naïve CNS and is upregulated in response to early inflammation. In contrast, prophylactic blockade of all three TAM receptors alters the clinical phenotype of EAE at its initial presentation. We propose that a critical target of the TAM receptor inhibitor in the naïve CNS is Mer, which is expressed at relatively high levels on hindbrain microglia during homeostasis. The Mer ligand, Gas6, is also expressed at high levels in the naïve hindbrain. Our data suggest that Gas6-mediated activation of Mer in microglia during the pre-clinical stage of EAE increases resistance of the hindbrain white matter to inflammatory infiltration. Since Mer signaling in hindbrain microglia is likely constitutive, blockade of that receptor in Th17 adoptive transfer recipients should have an immediate effect on the distribution of neuroinflammatory infiltrates and the clinical presentation of EAE, consistent with our results. Our finding that Mer is preferentially expressed on hindbrain microglia, compared with microglia in other CNS compartments, adds to a growing body of data demonstrating that spatial diversity of glial subpopulations (with regard to transcriptome, phenotype, and intrinsic biological properties), can help determine region-dependent susceptibility to environmental insults or disease, including inflammatory demyelination[299].

The detailed mechanism by which TAM receptors regulate leukocyte trafficking in the CNS remains to be elucidated. TAM receptor signaling might directly inhibit microglia (and perhaps CNS-infiltrating myeloid cells), from producing chemoattractants and/or growth factors

that normally facilitate white matter tissue invasion by hematogenous myeloid cells. In fact, Gas6/TAM signaling has been shown to negatively regulate production of GM-CSF, TNF-α, IL-1β and IL-6 by activated microglia *in vitro* and *in vivo*[300–302]. Alternatively, TAM signaling could induce microglia to secrete anti-inflammatory or chemorepellent factors[303]. Microglia interact with astrocytes during EAE, polarizing them towards a pro-inflammatory, neurotoxic phenotype[304]. TAM receptor signaling in microglia might curb such interactions, thereby indirectly suppressing astrocyte production of factors that attract or activate myeloid cells. In the current study, levels of G-CSF, IL-1β, TNF-α, and IL-6 were elevated in hindbrain homogenates of TAM receptor inhibited mice with EAE. Each of these factors has been implicated in the recruitment of myeloid cells to brain parenchymal tissue[305–307]. In future studies we plan to determine the cellular source and role of each of the pro-inflammatory factors in our experimental system. Interestingly, transgenic expression of IL-6 in cerebellar astrocytes predisposes MOG-sensitized mice to develop atypical EAE[103].

Atypical forms of EAE, characterized by ataxia and hindbrain inflammation, have previously been described in myeloid cell specific IFNγ receptor (IFNγR) and SOCS3 conditional knock-out (cKO) mice[102,200,295,296]. Similar to our TAM receptor inhibited mice, EAE in these genetically engineered mice is characterized by ataxia and neutrophil-rich hindbrain white matter infiltrates. The neutrophil attracting chemokines CXCL1 and CXCL2 are highly upregulated in the hindbrains of IFNγR and SOCS3 cKO mice with atypical EAE, and blockade of their cognate receptor, CXCR2, ameliorates or prevents clinical disease in both models[102,295]. The major cellular sources of CXCL1 and CXCL2 were microglia and infiltrating myeloid cells. We did not find CXCL1 or CXCL2 levels to be elevated in hindbrain homogenates from TAM receptor-inhibited compared to vehicle-treated mice. In future studies we will determine whether the

spatial patterns of CXCL1 and/or CXCL2 expression in the brainstem differ between the groups. We did find that neutrophil mobilizing/survival factor, G-CSF, was enhanced in hindbrain homogenates from TAM receptor inhibited mice. Neutralization of G-CSF significantly reduced the incidence and severity of atypical EAE in SOCS3 cKO mice[200], and may be consequential in TAM receptor inhibited mice as well. Although Mer signaling in hindbrain microglia may be protective against the initial presentation of atypical EAE, that does not rule out a synergistic effect of TAM receptor engagement on infiltrating myeloid cells following clinical onset that slows or aborts white matter infiltration and disease progression. TAM receptors suppress production of pro-inflammatory cytokines in myeloid cells through regulation of SOCS3[308], providing a possible explanation for the similar EAE phenotypes exhibited by TAM receptor inhibited and SOCS3 cKO mice.

The data presented in this paper demonstrate a role of TAM signaling in restraining hindbrain inflammation during Th17-mediated EAE. Our findings suggest that TAM receptor activation during EAE can limit the expression of G-CSF, as well as other pro-inflammatory cytokines, and the subsequent recruitment of neutrophils into parenchymal white matter. Our data illustrate how spatial heterogeneity in microglial phenotypes can determine the susceptibility of certain CNS regions to autoimmune inflammation.

Chapter 4

Discussion and Future Directions

Despite many efforts to elucidate the etiology and pathogenesis of MS, significant gaps in the understanding of immune regulation still exist. Currently, over 20 therapeutic options are FDA approved for the treatment of RRMS, yet 20-50% of patients still have breakthrough disease activity[309,310]. Progressive forms of disease are still majorly untreatable, in part due to a lack of understanding of the cellular mechanisms that facilitate the development of progressive disease. Developing therapeutics that enhance the regulatory function of myeloid cells or prevent the acquisition of a pro-inflammatory phenotype is a viable strategy to improve MS symptoms in patients.

The ability of myeloid cells to initiate or sustain inflammation during MS is the focus of many current studies. Macrophages are the predominant cell type within active lesions of RRMS patients and display an activated phenotype[311]. In EAE, they are responsible for antigen presentation and tissue damage[197]. Myeloid cells are likely even more essential to the pathogenesis of progressive MS. In these patients, CNS lesions are often devoid of lymphocytes and show signs of chronic microglial activation[312]. Areas of the CNS not affected by lesions also display activated microglial phenotypes and neuronal death, presentations not observed in the normal appearing white matter of RRMS patients[312,313]. Additionally, DMTs that target lymphocytes, commonly used to treat RRMS, are not effective in treating progressive disease[313].

Overall, determining the factors that control the phenotype and effector functions of CNS myeloid cells may provide innovative therapeutic strategies for the treatment of MS patients. It is

unclear whether myeloid cells are simply active participants in recovery or if they drive immune suppression and repair. With the work presented in this dissertation, we sought to understand the role of two pathways implicated in the regulation of myeloid cell phenotypes during inflammation. We hypothesized these pathways could prevent the acquisition of a pro-inflammatory phenotype or, alternatively, promote the acquisition of a suppressive or reparative phenotype.

Major Findings, Implications, and Future Directions from Chapter 2

The loss of TET2 in naïve mice results in aberrant myeloid cell responses. Small hairpin RNA-mediated knockdown of TET2 in hematopoietic precursors induces myeloid cell expansion and enhances stem cell self-renewal, and complete loss of TET2 leads to myeloid malignancies[267,268]. *Tet2*-deficient murine myeloid cells have an enhanced pro-inflammatory signature in response to stimulation *in vitro* and *in vivo*, including elevated IL-6 and IL-1β expression, and reduced PD-L1 expression[240,264,272]. In humans, *TET2* is commonly mutated in a variety of myeloproliferative and myelodysplastic cancers, leading to a loss of 5hmC in the genome that correlates to disease pathogenesis[260,269,314–316]. This indicates TET2 has regulatory roles that are directly relevant to human inflammatory disease[260,269,314–317].

Recently, *TET2* was identified as a genetic susceptibility locus for MS, a disease in which pathology is mediated by myeloid cells[265]. The risk-associated SNP was correlated with the number of monocytes in circulation, though the authors do not specify the direction of association[280]. Additionally, PBMCs from MS patients demonstrate lower *TET2* transcript and 5hmC content compared with healthy controls, and was negatively correlated with disease duration[260]. Our initial hypothesis was that TET2 within myeloid cells restrained pro-

inflammatory functions during EAE. An alternative hypothesis, which was not mutually exclusive with the first, was that this regulation aided in the resolution of inflammation during the later stages of EAE. In either case, loss of TET2 within myeloid cells would result in an augmented disease course. We found that *Tet2*$^{-/-}$ and *Tet2*$^{-/+}$ mice, as well as WT recipients of *Tet2*-deficient bone marrow, developed more severe EAE following immunization with MOG_{35-55}/CFA, compared to controls. However, when EAE was induced in *Tet2*-deficient animals via the transfer of WT encephalitogenic $CD4^+$ T cells, the clinical course was not augmented. We further confirmed there was no role of TET2 in regulating the antigen presentation ability of myeloid cells *in vitro*, suggesting myeloid-expressed TET2 did not play a role in activating T cells during the priming phase of EAE. While we cannot eliminate the possibility that the biological functions of TET2 vary between EAE disease models, resulting in different outcomes in knockout mice, the more likely possibility is that TET2 does not play a critical role in non-T cell populations and does not independently regulate disability in a clinically relevant manner.

There are a few explanations regarding why our data did not support our hypothesis. The most unambiguous reason is that TET2 simply does not regulate myeloid cell function in EAE as it does in other disease models. The CNS microenvironment is unique due to the presence of neuronal and glial cell populations. The interactions of these populations with infiltrating immune cells could induce or necessitate alternative pathways of regulation. Additionally, the specific inflammatory factors regulating TET2 expression and activity are not well understood. EAE could diverge from other models in the expression of these factors.

A likely explanation for our findings is that TET2 and TET3 have overlapping functions in myeloid cells during EAE. In preliminary studies, we found both *Tet2* and *Tet3* were expressed in myeloid cells during EAE. Previous studies have demonstrated that deficiency of

both *Tet2* and *Tet3* results in a more significant reduction of bone marrow 5hmC levels than *Tet2* alone[318]. Additionally, aberrant hematopoiesis and preferential differentiation to the myeloid lineage is observed in both *Tet2* and *Tet3* individual knockouts, but more severe hematopoietic malignancy is a penetrant phenotype when neither are present[263]. To evaluate if TET3 is compensating for TET2 loss in myeloid cells during adoptive transfer EAE, WT encephalitogenic $CD4^+$ T cells can be transferred into whole body *Tet2*/*Tet3* double knockout mice, and the clinical disease course measured. The number of circulating myeloid cells and possible myeloid malignancies should be continuously evaluated as compounding factors. If redundant roles exist and the combined functioning is clinically relevant, these mice should present with more severe disease than $Tet2^{-/-}$ or WT mice.

Regarding the finding that TET2 protects against severe EAE induced by active immunization, our data suggest future studies should focus on T cells. TET2 regulates differentiation and cytokine expression in $CD4^+$ T cells and, in collaboration with TET3, stabilizes FoxP3 expression in regulatory T cells within the thymus[273,319]. Previous studies in actively-induced EAE have demonstrated that TET2 is functionally relevant in T cells, as T cell-specific *Tet2*-deficiencies leads to greater neurological disability[273]. We show that *Tet2*-deficincy in recipient cell populations does not enhance severity of adoptive-transfer EAE. The main disparity between our active model, where *Tet2*-deficient mice had augmented disease, and our adoptive transfer model, where *Tet2*-deficienct recipients did not display that phenotype, was the presence of *Tet2*-proficient $CD4^+$ T cells. This suggests that it is the presence of TET2 within these cells which mediate protection during active EAE. In our studies, we did not definitively show that TET2 in $CD4^+$ T cells could mediate protection in adoptive transfer EAE, as well. The transfer of *Tet2*-deficient or control encephalitogenic $CD4^+$ T cells into WT recipients may

provide evidence supporting a critical role of $CD4^+$ T cell-expressed TET2 in multiple models of CNS autoimmunity. An evaluation of proper T cell priming and functional studies of *Tet2*-deficient $CD4^+$ T cells *in vitro* would determine what mediates any observed clinical effect. TET2 could regulate encephalitic T cell differentiation and/or function through enzyme-dependent or independent mechanisms. Assessing methylation patterns (using MeDIP-Seq and hMeDIP-Seq or Tet-assisted pyridine borane sequencing [TAPS]) and chromatin accessibility (using ATAC-Seq) would aid in this determination.

A detailed analysis of TET2 and 5hmC in MS patient samples may help narrow the relevant cell type in human disease. Identifying cell-specific differences in TET2 activity and expression, rather than differences in bulk PBMCs, could ideally be accomplished by flow cytometry. However, there is currently a lack of reliable anti-TET2 antibodies for use in flow cytometry. Instead, transcript expression and methylation sequencing that differentiates between 5hmC and 5mC (rather than bisulfide methylation sequencing) could be performed on sorted cell populations. Additionally, direct analysis of TET activity in leukocyte populations could be accomplished using *in vitro* assays. Given that RRMS and progressive MS are believed to have diverging pathogenic mechanisms, these experiments should be performed using samples collected at various time points of both clinical courses.

Independent of peripheral cells, some data suggests TET2 regulates the inflammatory responses of CNS resident microglia and neurons. A recently published study reported that TET2 promotes pro-inflammatory metabolic reprograming within microglia[241]. The authors also found that TET2 is upregulated in microglia that are spatially associated with Aβ plaques in a mouse model of Alzheimer's disease[241]. This deleterious role of TET2 contrasts the regulatory functions commonly seen in peripheral myeloid cells. We observed a reduction in EAE severity in *Tet2*$^{-/-}$

and *Tet2*$^{-/+}$ adoptive transfer recipients. If TET2 promotes the inflammatory response of microglia during EAE, this slight clinical improvement could be due to the loss of microglial TET2. Initial experiments that compare the disease course, microglia activation status, and CNS infiltrate of *Tet2*$^{-/-}$ and WT recipients of WT bone marrow would identify if this is a viable route of investigation. TET enzymes have also been suggested to regulate neuronal expression of neurotrophic factors. During EAE, 5hmC levels are reduced within the brain-derived neurotrophic factor (BDNF) gene, a pro-repair molecule released by neurons that promotes remyelination[320]. The level of 5hmC within this locus was positively correlated with *Bdnf* transcript expression, suggesting functional relevance of TET activity to neuronal survival and/or repair during EAE[320]. We did not examine neuronal outcomes or remyelination following inflammation, an interesting future route of investigation.

Another potential role of TET2 that we did not explore was in regulating inflammatory resolution during EAE. In models of peripheral inflammation, *Tet2*-deficient mice show sustained inflammation, compared to controls, and enhanced expression of IL-6 in macropahges[321]. While we did not see clinical signs of ongoing inflammation in the late stage of EAE, C57BL/6 mice have sustained neurological deficits following peak inflammation and continued inflammation may be more difficult to identify using the EAE scoring scale. We did not assess cytokine expression, CNS infiltration, or cell activation in late stages of adoptive transfer or active immunization EAE. Interestingly, we did find a stark upregulation of 5hmC, indicative of TET enzymatic activity, in infiltrating immune cells at this late timepoint. In the endotoxin shock model of peripheral inflammation, TET2 repressed macrophage IL-6 expression through the binding of the *Il6* promoter and recruitment of histone deacetylase 2 (HDAC2), a mechanism independent of its enzymatic activity[321]. If the loss of TET2 results in sustained

inflammation during EAE, the necessity of enzyme function to disease resolution should be assessed.

Due to the lack of clinical significance, we did not explore the role of TET2 in regulating cytokine expression by myeloid cells during EAE. In addition to the previously mentioned regulation of IL-6, loss of TET2 is associated with increased expression of *Il12b*, *Il6*, and *Tnf* in TET2-deficient tumor-associated macrophages, as well as IL-1β in murine models of atherosclerosis and endotoxin shock[240,248,322]. TET2 deficiency also results in reduced expression of regulatory markers, such as *Il4*, *Mgl2*, and *Arg1*, in tumor-associated macrophages, although other studies report that *Arg1* is suppressed by TET2 and expression is positively associated with TET2 mutations in patients with myelodysplastic syndrome and chronic myelomonocytic leukemia (CMML)[240,322,323]. Analysis of the cytokine levels within the CNS of *Tet2*$^{-/-}$ and WT adoptive transfer recipients, or *ex vivo* cytokine production by myeloid cells isolated from the CNS of these mice, would determine if TET2 regulates production of these molecules during EAE. If *Tet2*$^{-/-}$ recipients would have more pro-inflammatory cytokine expression than WT mice, it is relevant that this excess of cytokines does not change the clinical outcome.

TET2 may be relevant as a therapeutic target for CNS autoimmunity. In two independent studies, decitabine (5-aza-2'-deoxycytidine), an FDA-approved chemotherapeutic agent that inhibits DNA-methyltransferases and induces TET2 expression and activation, ameliorated EAE induced by active immunization with MOG_{35-55}[274,324]. Decitabine treatment reduced the expression of MHCII on bulk myeloid cells and the transcription of myeloid-associated chemokines and cytokines, including *Il6*, *Il1b*, *Tnf*, and *Nos2*[274]. There were also significant differences in the levels of T-cell associated cytokines within the CNS, such as IFN-γ and IL-17[274,324]. There were opposing observations regarding the effect of decitabine treatment on the

number of regulatory FoxP3$^+$ T cells[274,324]. Currently, decitabine is used to improve outcomes in patients with myelodysplastic syndromes, underscoring the potential impact of the drug on myeloid cell populations[325]. However, both lymphoid and myeloid compartments were affected by decitabine treatment, making it unclear if either are a primary target. Despite this, decitabine represents a potential therapeutic option for MS patients.

In summary, previous studies show that TET2 can regulate the function of both myeloid and non-myeloid populations in numerous inflammatory models. The data presented in chapter 2 suggest that TET2 alone does not regulate myeloid cell function during EAE in a clinically relevant manner, and instead support a protective role of TET2 in CD4$^+$ T cells in disease. These findings add to the growing body of literature demonstrating that the contribution of TET2 to cell responses in inflammatory disease is context dependent. Further studies are needed to understand the relevance of our findings to MS and determine the potential of TET2 as a target of therapeutic intervention.

Major Findings, Implications, and Future Directions from Chapter 3

Previous studies in MS and EAE support a regulatory role of TAM receptors during CNS autoimmunity. However, no prior studies have identified an association between TAM receptor activity and region-specific protection within the CNS. In our studies, we observed a high frequency of Mer$^+$ microglia in the naïve murine brain, greater than that of the spinal cord. We induced EAE via the adoptive transfer of MOG_{35-55}-specific, Th17-polarized CD4$^+$ T cells. Prior to the onset of neurological deficits, microglia are the primary cell type expressing Mer in the forebrain and hindbrain. This is in contrast to Axl$^+$ cells, which were comprised majorly of macrophages at preclinical timepoints. The percentage of microglia that expressed Mer in the

spinal cord, hindbrain, and forebrain was reduced at peak disease compared to naïve mice (~1.5-3 fold). At this timepoint, all three CNS regions displayed a similar percentage Mer^+ or Axl^+ infiltrating myeloid cells. Less than 20% of microglia expressed Axl at any timepoint during disease. These expression patterns do not correspond to observations in post-mortem analysis of chronic white matter lesions within the cerebellum of progressive MS patients[290]. Within these lesions, Mer and Axl are upregulated on astrocytes and microglia, respectively, and are only expressed at low levels non-inflamed control tissue[290]. While these findings challenge the hypothesis that TAM receptors are protective, chronic lesions also express elevated levels of soluble Mer and Axl, which bind TAM ligands and act as decoy receptors to the membrane-bound proteins[290]. The high levels of soluble Mer and Axl within MS lesions may lead to low accessibility of Gas6 and ProS in the CNS tissue, limiting the engagement of membrane-bound receptors[290]. Importantly, these studies measured TAM expression only in cerebellar white matter and did not compare expression to lesions within other CNS regions.

High Gas6 levels in cerebrospinal fluid and high ProS levels in plasma were associated with less severe relapses in MS patients[288,289]. These data are in partial agreement with our findings, as Gas6 is reduced in the spinal cord at the timepoint in which mice present with the most severe spinal cord inflammation and disability. However, we see low Gas6 expression in the forebrain throughout EAE, a region that does not have large numbers of CNS infiltrates. This could be explained by the small number of TAM-receptor expressing cells in the forebrain requiring only low levels of Gas6.

Previous studies found *Axl*$^{-/-}$ and *Gas6*$^{-/-}$ mice present with more severe EAE following immunization with MOG_{35-55}/CFA[221,292]. This phenotype was associated with a buildup of myelin debris and/or increased axonal swelling in the spinal cord[221,292]. Conversely, Axl

activation via systemic administration of an anti-Axl antibody or intracerebroventricular treatment with Gas6 abrogates EAE disability and reduces demyelination in the spinal cord[291,292]. In models of cuprizone-induced demyelination, mice deficient in Mer or Axl signaling demonstrate enhanced microglia activation and delayed remyelination[326–328]. Interestingly, *Tyro3*$^{-/-}$ and WT mice have comparable EAE clinical courses[221]. To our knowledge, no studies have examined EAE severity or pathology in Mer-deficient models. By treating with the selective inhibitor LDC1267, which has been used to demonstrate the positive impact of TAM receptor signaling following cuprizone treatment, we inhibited TAM receptor signaling during EAE. The vast majority of LDC1267-treated mice displayed atypical, brain-targeted neurological deficits, while only few in the vehicle-treated group displayed this phenotype[220]. There was no observed hindbrain demyelination in either group, which is not surprising given the data was collected at an early timepoint of EAE. It is unclear why the mice did not display the more severe conventional EAE that occurs in *Axl*$^{-/-}$ and *Gas6*$^{-/-}$ mice, but differences in EAE induction between the two studies, incomplete Axl inhibition by LDC1267, or developmental effects of *Axl*-deficiency that impact inflammatory responses, are all possible explanations. Though previous studies have not reported any off-target effects, the effect of LDC1267 on neurological fitness of naïve mice was not assessed, and future studies should be mindful of drug side effects.

The development of atypical EAE was not associated with an increase in the number of cells measured by flow cytometry at the onset of disease or during peak inflammation, but instead was associated with a change in the distribution of the infiltrates, from the meningeal and perivascular spaces to deeper within the brainstem parenchyma. The majority of parenchymal-infiltrating CD45^{+}IBA1^{-} cells were neutrophils. Surprisingly, we observed no significant difference in the level of chemotactic molecules in the hindbrain between LDC1267-treated and

control mice. This data contradicts findings in other atypical models, where ataxia and neutrophil-rich hindbrain lesions are associated with elevated CXCL2[199,201,295]. More local differences in chemokine expression may exist that we could not detect with our approach. The enhanced parenchymal infiltration in LDC1267-treated mice could also be explained by alterations to chemokine receptor expression on $CD45^{hi}$ cells.

Mer has been identified as a genetic susceptibility locus for MS development[286,287,329]. However, the association of the risk factor with disease outcome is context dependent, as at least one polymorphism, which was linked to higher expression of Mer in monocytes, has a discordant association with MS development based on HLA-DRB1*15:01 status[287]. In *DR15* homozygous individuals, the minor allele confers protection, but in the absence of *DR15*, the minor allele confers MS risk. It is unclear the mechanism that drives this discordance. Some data indicate that a patient's *DR15* status can influence the location of lesions during MS[330–332], while other studies refute that finding[333].

Mechanistic patterns emerge when comparing our findings to the findings in other atypical EAE models. In the present study, we measured significant differences in hindbrain levels of G-CSF, IL-6, IL-1β, and TNF-α in mice treated with LDC1267 compared to controls. High levels of IL-1β and TNF-α are also detected in MOG_{35-55}-immunized LysMCre-$SOCS3^{fl/fl}$ mice, a model which has a high occurrence of atypical EAE[102]. In this same model, neutrophil depletion or CXCR2 blockade reduces the severity of atypical EAE, suggesting neutrophils are necessary for an atypical phenotype[102]. Higher levels of G-CSF and MPO in the hindbrain of LDC1267-treated mice suggest greater neutrophil activation, a hypothesis that would need to be further explored. Other studies have found that induced IL-6 expression in cerebellar astrocytes skews EAE towards an atypical presentation, implicating the elevated IL-6 levels in LDC1267-

treated mice in the development of atypical EAE[334]. The source of the elevated cytokines in the hindbrain following LDC1267 treatment are unknown. Activated microglia are capable of producing G-CSF, IL-6, IL-1β, and TNF-α[335–339]. Additionally, TAM receptors have been shown to directly regulate TNF-α, IL-1β, and IL-6 expression in macrophages[340]. In the absence of TAM signaling, microglia may acquire an activated phenotype and release these pro-inflammatory factors.

The use of an inhibitor that blocked signaling of all three TAM receptors controlled for any compensatory or overlapping functions, but limited our understanding of which receptor or receptors are critical for protection. The lack of an atypical phenotype in *Axl*$^{-/-}$, *Tyro3*$^{-/-}$, or *Gas6*$^{-/-}$ mice suggest that TAM-mediated suppression of hindbrain inflammation is mediated, at least in part, through Mer[221]. Previous literature supports diverging roles of Mer and Axl, the former maintaining homeostasis and the later responding to inflammation[341]. The hypothesis that Mer signaling in the CNS protects against inflammation would explain why Mer, rather than Axl or Tyro3, is a genetic susceptibility locus for MS development. While Gas6 is the most well-studied TAM ligand, it has a lower affinity for Mer than Axl[215]. Mer can also be activated by protein S (ProS), which does not bind Axl[215,342]. Gas6 and ProS have opposing roles neuronal stem cells, indicating diverging signaling pathways initiated by their receptors[343–345]. A critical role of ProS as a Mer ligand would explain the absence of an atypical phenotype in *Gas6*$^{-/-}$ mice[292].

Another limitation of our study is that we could not identify a specific cell type driving protection in the hindbrain, and multiple CNS-resident cell types express TAM receptors[346]. Of the infiltrating cells, the majority of Mer^{+} cells in the hindbrain at naïve and preclinical time points are microglia. This suggests the clinical effects of TAM receptor inhibition are mediated through microglia prior to the onset of EAE. Despite this, protective functions of TAM receptors

on non-microglial cells cannot be ruled out. A major role for TAM receptor signaling in the CNS is in myelination and neuronal survival. Gas6 and ProS act as trophic factors and protect against neuronal and endothelial damage and apoptosis[347–350]. In oligodendrocytes, TAM signaling promotes survival *in vitro*. Tyro3 signaling in astrocytes mediates supportive functions during myelination of the optic nerve in a mixed glial cell culture[303,351]. While microglia-expressed Mer and Axl mediate myelin debris clearance following demyelination, deficiencies in Tyro3 result in thinner myelin sheaths during remyelination without any changes to microglial activation[328,352]. We do not believe these roles of TAM receptors are important for the phenotype of our model because atypical EAE develops prior to extensive CNS damage. Additionally, astrocytes and neurons utilize TAM receptors when pruning neuronal synapses and during neural development, but we do not expect loss of these functions to impact EAE induced in adult mice[353]. Finally, Mer signaling within endothelial cells helps to maintain blood-brain barrier integrity in infectious models. However we see no evidence of enhanced extravasation in our model by flow cytometry, only a change in the distribution of the infiltrates[354]. It is also important to consider the difficulty in distinguishing microglia from BAM populations, discussed in Chapter 1. Our studies used microglial markers that would also be expressed in BAMs. Future studies utilizing cell-specific knockouts of Mer alone, or in tandem with Tyro3 and Axl, will clarify the mechanism of regulation.

Recent studies have begun to appreciate the diversity and plasticity of microglia in the CNS during homeostasis and disease[355–357]. Region-specific transcriptomic and functional differences in microglia have been established, leading to questions regarding how these microglia mediate the susceptibility to lesion development[358]. The high expression of Mer on hindbrain microglia together with the high levels of Gas6 suggest these molecules mediate

physiologically relevant functions. Our findings implicate regional glial differences may contribute to the propensity for lesion development in certain areas of the CNS during inflammatory demyelination. Our working hypothesis is that microglia-expressed Mer is critical for maintaining homeostasis in the hindbrain during adoptive transfer EAE. In progressive MS, microglia activation is abundant, and is associated with brain atrophy and neuronal damage[359,360]. Microglia in normal-appearing white matter also display an activated phenotype in progressive MS patients, yet lesions preferentially localize to certain CNS regions[58]. Understanding how regional differences in microglia protect against inflammation may lead to the development of novel therapeutics targeting microglia for patients with progressive MS.

Overarching Challenges and Limitations

EAE is a useful model for recapitulating certain aspects of autoimmune demyelinating disease. Immune cell infiltration and demyelination are hallmark pathogenic features in MS and EAE[361]. However, translating findings in EAE to MS is difficult. No animal model can accurately capture all features of disease, and murine models of progressive MS are lacking. There are substantial discrepancies in the pathogenesis and mechanisms regulating EAE and MS. The most notable of which is the lack of B cell involvement in most common EAE models, despite their presumed importance in the pathogenesis of RRMS[104,362]. Alternative models that require B cells for disability, such as EAE induced via immunization with human MOG protein/CFA, have been developed but are still less commonly used[104]. The necessity of B cells following immunization with whole protein, but not peptide, may be due to a unique ability of B cells to process and present relevant MOG epitopes[105]. Other commonly used EAE models fail to recapitulate pathogenic mechanisms such as a relapsing course or the clinical recovery.

Adoptive-transfer EAE induced in C57BL/6 mice presents as a monophasic inflammatory course and is not a chronic, progressive disease. It is thus difficult to know if findings in EAE can be appropriately applied to patients with MS.

These concerns must be considered when applying EAE findings to the development of MS therapeutics. Pathways that are protective in EAE may not be protective, or could be detrimental, in MS. In the late 20th century, two independent studies found that blocking IFN-γ in mice through the systemic administration of a neutralizing antibody exacerbated disease, suggesting IFN-γ was protective[363,364]. However, infusion of IFN-γ in a clinical trial for MS exacerbated neurological symptoms[365]. This example, and others, demonstrates the need for a cautious approach when translating animal model research to human disease.

Other challenges to translational models are technical in nature. Experimental options for relevant human tissue are limited by sample availability. Studies in RRMS often use blood samples for their analysis, which do not fully reflect the ongoing processes within the CNS. CSF samples are limited in quantity and in high demand. Analysis of post-mortem samples of CNS tissue can be biased, as they are largely collected from aged individuals with progressive disease.

Despite these challenges, many successes have come from studies in EAE and other animal models. Much of our understanding of pathogenesis is due to the ability to manipulate variables in a controlled manner. Understanding pathogenic mechanisms has aided the development of current DMTs. Our findings from chapters 2 demonstrate that TET2 is not involved in myeloid cell regulation in CNS autoimmunity. The results in chapter 3 suggest that TAM receptors on glial populations can protect against inflammation in a region-specific manner. These studies benefit from the ability to control for many variables, such as genetic and environmental inconsistencies within patient populations. Future studies in MS patient tissue will

complement our findings and are critical understanding the regulation of myeloid cells during MS.

Conclusions

The results presented in this dissertation demonstrate that myeloid cells are dynamically regulated during autoimmune demyelinating disease. The related discussion outlines the need for a deeper understanding of mechanisms driving the acquisition of protective myeloid cell phenotypes. This work was a small contribution to the paramount objective of developing more efficacious therapies for individuals affected by MS. It is my hope that these findings inform and direct future studies to achieve that goal.

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