

© Yee Ming Khaw, 2021

ASSESSMENT OF THE IMPACT OF EARLY LIFE TRAUMA, CHANGES IN ESTROGEN
SIGNALING, AND NEUROTOXIC MOLECULES IN A MOUSE MODEL OF
MULTIPLE SCLEROSIS

BY

YEE MING KHAW

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois Urbana-Champaign, 2021

Urbana, Illinois

Doctoral Committee:

Assistant Professor Makoto Inoue, Chair
Professor CheMyong Jay Ko
Associate Professor Andrew Steelman
Assistant Professor Daniel McKim

ABSTRACT

Multiple sclerosis (MS) is the most common neurological disease that affect young adults that causes the accumulation of disability over time due to irreversible neurodegeneration. Disease presentations vary significantly from person to person in terms of who gets the disease, who develops the more severe types of MS, the type of central nervous system injury that the disease inflicts, molecular signatures on disease driving immune cells, and one's sensitivity to prescribed treatment. While it is a deeply heterogenous disease, MS is widely accepted to be driven by an autoimmune response characterized by the infiltration of peripheral immune cells and activation of CNS-resident glial cells which results in demyelination, loss of oligodendrocytes, neuron atrophy. The etiology of MS is undetermined. Several genetic and non-genetic (a.k.a. environmental) factors have been identified to have significant impact on disease activity and its underlying mechanism. Importantly, evidence suggest that non-genetic environmental factors play a significant role in disease modification by acting on cellular drivers of the disease. Herein, using a MS model of experimental autoimmune encephalomyelitis (EAE) in four independent projects, I report that non-genetic environmental factors of early life stress, changes in estrogen level, immune cell expression of neurotoxic molecule contribute to disease heterogeneity. The studies also highlight the identity of membrane lymphotoxin-lymphotoxin receptor beta receptor signaling and CXCR2 signaling as potential biomarkers for neurodegenerative and interferon-beta resistant MS and EAE.

ACKNOWLEDGEMENTS

First and foremost, I want to thank my advisor, Dr. Makoto Inoue, for giving me the opportunity to pursue a Ph.D. in his lab. Under his mentorship, he taught me to be independent and helped me develop many skills that I will use for the rest of my career. The manuscripts I was fortunate enough to publish were only possible because of his relentless encouragement and guidance. I would also like to thank Dr. Mayandi Sivaguru, for setting me on a path to success by teaching me how to operate optical systems and perform stereological analysis. I want to thank my committee members Dr. CheMyong Jay Ko, for being so generous with sharing his lab resources and allowing me to learn multiple surgical procedures from his senior lab member to whom I also extend my gratitude: Patrick Po-Ching Lin; Dr. Andrew Steelman, for being the incredible teacher who ignited my interest in immunology and for collaborating with us for a project; last but not least, Dr. McKim, for teaching me the intricacies of literature reading and assessment which provided me with the confidence to discuss scientific knowledge. I would like to thank all the current and past members of the Inoue lab: Chris Oh, Danish Majid, Mary Clutter, Claire Cunningham, Abigail Tierney, Lazaro Pacheco, Dr. Ziyuan Song, Zhengzhong Tan, Eunjoo Kang, Ashley Otero, Dr. Tasuku Kawano, for all their help on my studies. I also want to thank Dr. Zigman for providing us with $\beta 1$ adrenergic receptor knockout mice. I want to thank the NSP program, especially Dr. Sam Beshers and Stephanie Pregent, without whom my graduate journey will not have been half as smooth sailing. I thank my family for their unwavering support and willingness to listen to me talk about my work, despite it not always being clear what I am researching. None of this would have been possible without the love and support from my better half, Daniel Sangiamo.

TABLE OF CONTENTS

ABBREVIATIONS	v
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: A CONFOCAL REFLECTION SUPER-RESOLUTION TECHNIQUE TO IMAGE GOLGI-COX STAINED NEURONS	36
CHAPTER 3: EARLY LIFE TRAUMA TRIGGERS INTERFERON BETA RESISTANCE AND NEURODEGENERATION VIA DOWNREGULATED β 1-ADRENERGIC SIGNALING	65
CHAPTER 4: LYMPH NODE-DERIVED ESTROGEN MODULATES DISEASE PHENOTYPE VIA ESTROGEN RECEPTOR ALPHA	112
CHAPTER 5: ASTROCYTES LURE CXCR2-EXPRESSING CD4 ⁺ T CELLS TO GRAY MATTER VIA TAK1-MEDIATED CHEMOKINE PRODUCTION	154
CHAPTER 6: NEUTROPHIL-SELECTIVE DELETION OF CXCR2 PROTECTS AGAINST CNS NEURODEGENERATION	184
CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS	215

ABBREVIATIONS

A-NP (albumin-coated nanoparticle)

APC (antigen presenting cell)

AR (adrenergic receptor)

ASC (Inflammasome adaptor protein Apoptosis-associated speck-like protein containing CARD)

BBB (blood brain barrier)

BMDC (bone marrow derived dendritic cell)

cAMP (cyclic-AMP)

CFA (complete Freund's adjuvant)

ChAT (choline acetyltransferase)

CORT (corticosterone)

CNS (Central nervous system)

CRSR (Confocal Reflection Super-Resolution)

CSF (cerebrospinal fluid)

CXCR2 (C-X-C Motif Chemokine Receptor 2)

DC (dendritic cell)

dGM (dorsal gray matter of lumbar spinal cord)

DMEM (Dulbecco's Modified Eagle Medium)

DPI (days post immunization)

E2 (17 β estradiol)

EAE (experimental autoimmune encephalomyelitis)

EBV (Epstein Barr Virus)

ELT (early life trauma)

ER (estrogen receptor)

ER α (estrogen receptor alpha)

EXE (exemestane)

GFAP (Glial fibrillary acidic protein)

GM (gray matter)

HEV (high endothelial venules)

HLA (human leukocyte antigen)

HPA (hippocampus pituitary axis)

i.p. (intraperitoneal)

i.th. (intrathecal)

Iba1/AIF-1 (Allograft inflammatory factor 1)

IFN β (interferon-beta)

Ig (immunoglobulin)

IHC (immunohistochemistry)

IL-1 β (interleukin 1 beta)

IRF3 (Interferon Regulatory Factor 3)

LN (lymph node)

LPS (lipopolysaccharide)

LT β R (lymphotoxin beta receptor)

MBP (myelin basic peptide)

MHC (major histocompatibility complex)

MPO (myeloperoxidase)

mLT (membrane lymphotoxin)

MOG (myelin oligodendrocyte glycoprotein)

MS (Multiple sclerosis)

Mtb (heat-killed mycobacterium tuberculosis)

N2a (Neuro2a neuroblastoma cell)

Ncf1 (neutrophil cytosolic factor 1)

NE (norepinephrine)

NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3)

OVX (ovariectomy)

PBMCs (peripheral blood mononuclear cells)

PBS (phosphate buffer saline)

PFA (paraformaldehyde)

PPMS (primary progressive multiple sclerosis)

PPT (4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)

PTX (pertussis toxin)

RFP (red fluorescence protein)

ROS (reactive oxygen species)

RRMS (relapse remitting multiple sclerosis)

s.c. (subcutaneous)

SNS (sympathetic nervous system)

SPMS (secondary progressive multiple sclerosis)

TAK1 (transforming growth factor β -activated kinase 1)

TGF- β (transforming growth factor beta)

Th1 (lineage of CD4⁺ effector T helper type 1)

Th17 (lineage of CD4⁺ effector T helper type 17)

Th2 (lineage of CD4⁺ effector T helper type 2)

TMEV (Theiler's murine encephalomyelitis virus)

T_{reg} (lineage of CD4⁺ effector T regulatory type)

Ub (ubiquitin)

VCAM-1 (Vascular Cell Adhesion Molecule 1)

vGM (ventral gray matter of lumbar spinal cord)

WM (white matter)

WT (wild type)

CHAPTER 1: INTRODUCTION

Multiple sclerosis (MS) is a chronic and debilitating inflammatory neurological disorder that currently affects 2.8 million people worldwide and ~ 500,000 individuals in the United States ¹. The disorder is estimated to cost the United States healthcare system \$28 billion per year, with an estimated lifetime cost per patient of more than \$4 million ². As the commonest non-traumatic disabling disease to affect young adults, MS is often diagnosed during young adulthood (18-30 years) ³. A prototypic person who receives the life-long diagnosis of the neuroinflammatory disorder is likely female (female: male, 2.5:1) ⁴, who recently detected bouts of limpness, altered sensations in her extremities, found vision to be blurry, or began to experience bladder dysfunction ⁵. Other common prodromal MS manifestations are depression, fatigue, and brain fog. Symptoms vary significantly between patients and change/worsen throughout a person's lifetime. Without appropriate treatment, within 10 years since diagnosis, 93% of persons with MS will require the assistance of a cane to walk or expect to be wheelchair bound due to irreversible central nervous system (CNS; i.e. brain and spinal cord) atrophy ⁶.

The current understanding of the pathogenesis of MS is that it is driven by an autoimmune response mainly due to the observation of T and B lymphocytes surrounding lesions in the tissues of the CNS such as the brain and spinal cord ("Is MS an autoimmune disease?" is discussed in section 1.1). Disease worsening is characterized by inflammatory and neurodegenerative events that occur in the CNS such as the infiltration of peripheral immune cells and activation of CNS-resident glial cells in the brain and spinal cord which results in demyelination, loss of oligodendrocytes, neuron atrophy ^{7,8} ("Disease pathogenesis" is discussed in section 1.2). Demyelination and failure to remyelinate is the hallmark MS pathology. There

is also evidence to support the neuron atrophy independent on demyelination may better account for disability accumulation ⁹ (“Disease pathology in the CNS” is discussed in section 1.3).

Since the 1990s, MS has been primarily differentiated into three disease subtypes based on synthesized assessment which takes into consideration pattern of disease trajectory (duration of remission and relapse), symptom presentation, and CNS pathology. 85% of persons with MS present with relapses or attacks – with symptoms referable to lesions in the CNS – followed by a complete or partial remission/return to normal, only to be followed by another relapse, thus presenting as relapsing and remitting MS (RRMS) ¹⁰. Within 25 years of disease onset, 90% of patients with RRMS will develop a progressive form of the disease that is without disease remission, termed secondary progressive MS (SPMS) ¹⁰. Approximately 15% of persons with MS present with a gradually progressive course since disease onset, without an initial well-defined attack. This is termed primary progressive MS (PPMS) ¹⁰. Unlike RRMS patients, most PPMS patients present with higher lesions burden in the spinal cord than in the brain ¹¹ and the most common presentation in PPMS is progressive paraparesis rather than visual or sensory disturbances ¹².

Adding to the heterogeneity of MS – though currently unaccounted for in differential diagnosis between disease subtypes – is the variation in patient responsiveness to current therapies. In fact, the first line treatment of interferon-beta (IFN β), approved since 1993, is only effective in 30-50% of patients¹³. Because MS is a CNS disorder and the CNS, unlike other parts of the body, has limited capacity at regeneration, a comprehensive understanding of disease pathogenesis is needed for better differential diagnosis and prescriptions of effective treatments to delay disease-mediated disability. There has been recent effort to identify elements of disease

pathogenesis that can act as biomarkers to differentiate between MS disease with varying underlying mechanisms. For example, persons with MS who have high serum endogenous IFN β levels are more likely to be resistant to exogenous treatment and may even deteriorate with IFN- β therapy. Additionally, serving as the inspiration of much of the current work, in 2016, a study reported by Inoue and colleagues showed that relative gene expression of *Ltbr* (which encodes for lymphotoxin beta receptor (LT β R)) and *Cxcr2* (which encodes for C-X-C Motif Chemokine Receptor 2 (CXCR2)) were enhanced in peripheral blood mononuclear cells (PBMCs) derived from IFN β non-responding MS patients¹⁴. These studies suggest the existence of peripherally derived biomarkers that can provide insight to drug sensitivity and disease pathogenesis of MS.

While what causes MS remains an incompletely solved puzzle, it is well known that genetic and non-genetic (a.k.a. environmental) factors contribute to disease onset and significantly informs disease pathogenesis. Genes within the human leukocyte antigen (HLA) gene complex are the strongest genetic risk factors for MS. In particular, the class II variant HLA-DRB1*15:01 has a striking association with a 30% increased risk of MS, whereas the class I variant HLA-A*02 is associated with protection from the disorder¹⁵⁻¹⁷. The discovery of the strong association between HLA class II and MS susceptibility, adding to the historical observation of CD4⁺ T lymphocytes around lesions in MS brain¹⁸, initiated a long line of research on the central role of T cells in MS disease. This is because HLA class II, which is the human version of major histocompatibility complex major histocompatibility complex (MHC) class II, are expressed on antigen-presenting cells (APCs) such as dendritic cell (DC), macrophages, and B cells for recognition and subsequent activation of CD4⁺ T lymphocytes. New genetic approaches such as genome-wide association studies along with genome project data and larger datasets have recently identified new MS susceptibility loci within and outside

the HLA region, such as interleukin-2 receptor α , interleukin-7 receptor α , CLEC16A, CD6, CD58, IRF8, IL12A, Olig3-TNFAIP3, PTGER4, RGS1 and TNFRSF1A genes ¹⁹.

Aside from genetic factors, environmental factors are key contributors to the risk of MS. Some established MS-associated non-genetic risk factors are endogenous estrogen levels ²⁰, smoking ²¹, vitamin D levels ²², and Epstein-Barr virus ²³. Initially proposed by Jean Charcot himself who named the disease “*la sclérose en plaques*” (MS in French), stress is another also long suspected risk factor associated with changes in disease presentation ^{24,25}. Early work on MS risk factors mostly focused on the impact of disease risk factors on disease activity (onset and progression); however more recent evidence shows that risk factors, in particular environmental risk factors, can significantly impact underlying mechanisms of disease, accounting for differences not only in disease activity but also in disease pathology, disease mechanism, and treatment sensitivity. For example, in addition to increasing the susceptibility to MS, smoking carries the risk of the development of neutralizing antibodies against biologics used in treatment of MS, including natalizumab ²⁶ and IFN β ²⁷. Additionally, viral infection increases endogenous IFN β synthesis which can render insensitivity to drug treatment ²⁷.

In Chapter 2, I present my work in the development of a new optical imaging approach to visualize Golgi-Cox-stained neurons at super-resolution with simultaneous visualization of fluorescence signals. This method is utilized in all following chapters as a key approach to examine neuron atrophy via quantitative morphological analysis. Using an MS animal model, experimental autoimmune encephalomyelitis (EAE) (MS animal models discussed in section 1.4), I explore the following concurrent independent theses. In Chapters 3 and 4, I present my work on understanding whether environmental factors of early life trauma (ELT) and estrogen

receptor (ER) signaling contribute to MS heterogeneity by altering mechanisms of disease pathogenesis defined within the parameters of disease trajectory, CNS pathology, disease mechanism, and treatment sensitivity. In Chapters 4 and 5, I present my work on understanding the cellular specific role of neurotoxic cytokine signaling in the context of neuron atrophy. CXCL1 is a chemokine ligand that are found to be in high levels in the plasma and cerebrospinal fluid (CSF) of persons with MS ²⁸. Evidence from human PBMCs suggest that enhanced CXCL1 signaling in peripheral immune cells and its activation of cognate receptor CXCR2 may play an important role in disease pathogenesis to the extent of informing drug sensitivity and CNS atrophy ¹⁴. As such, the second theses of this dissertation work explores whether CXCL1-CXCR2 signaling in neutrophils and T cells inform disease heterogeneity with a focus on its mechanism in mediating CNS atrophy. The rationale and specific aims of this dissertation are provided in section 1.5.

1.1 IS MS AN AUTOIMMUNE DISEASE?

From most references in the literature, MS is stated as an autoimmune disorder. However, the classification of MS as the result of autoimmunity is not without controversy. Direct proof that autoimmunity drives inflammation in MS requires the demonstration of two main criterion of (1) the detection of a precise autoantigen be present in all patients with the disease and that (2) the administration of autoantigen, autoantibodies, or T cells induces autoimmune disease in normal animals.

The search for specific pathogenic autoantibodies and their antigens has been notoriously challenging in MS. Most studies have suggested major myelin antigens as potential targets, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), as well as lipids. This long-standing hypothesis is supported by early evidence that demonstrates

significantly enhanced T- and B-cell responses to MOG in multiple sclerosis patients compared to healthy control ²⁹. Another study showed that plasma B cell derived serum anti-MOG-immunoglobulin (Ig) antibody is greater in MS patients when compared to patients with other non-inflammatory neurological diseases and patients with rheumatoid arthritis ³⁰. More recent studies confirmed that lymphocytes collected from MS patients have significantly altered functional responses in towards myelin protein when compared to control cohort, despite the comparable frequencies of these cells ³¹. Importantly, changes in the specific pattern of T cell response to myelin epitopes have been correlated with MS relapse ³². Furthermore, a trial of an altered peptide ligand of MBP showed increased disease activity linked to increased MBP reactivity ³³, fitting the narrative that MS is of autoimmune origin that is likely driven by antigen-specific responses towards myelin-related proteins. However, contradictory evidence and interpretation exists. The most damning of all is that despite considerable effort, analysis of oligoclonal bands in MS patient-derived CSF has generally failed to demonstrate evidence of antigen specificity. Autoantigens of non-myelin source has also been suggested to be important in MS pathogenesis. Thus, the first criteria used to determine whether MS is an autoimmune disease remains unsatisfied because there is still no conclusion as to the identity of a precise autoantigen be present in all MS patients.

In humans, the use of anti-rabies vaccine that had been proliferated and sub-optimally purified from brain tissue, resulted in neuromuscular symptoms in vaccine-treated persons that resemble MS relapse ³⁴. Some of the vaccine-induced MS symptoms can be directly attributed to the contamination of the specific vaccine with CNS tissue. Though the vaccine-induced neurological condition is not MS, because it usually exists as an acute episode and is not self-perpetuating, this observation provided valuable insight into the neurological effect that CNS-

related antigens have upon injection into a human's circulatory system. Despite these failures to determine the specificity of MS-inducing autoantigen, immunization with myelin peptides, specifically MOG, in human primates or marmosets was shown to induce demyelination reflective of human MS ³⁵, partially satisfying the autoimmune narrative. In mice, transfer of T cells, that had been programmed to exert autoimmunity towards myelin-peptides into recipient EAE animals without functional T cells is too sufficient to induce MS-like symptoms of demyelination and gray matter atrophy. However, manifestation of MS-like symptoms is also observed in animal models that do not employ the use of myelin antigen. For example, experimental infection of laboratory animals with various viruses induces demyelination in the CNS. In the viral MS of animal model Theiler's murine encephalomyelitis virus (TMEV), demyelination is in part the result of direct virus destruction of oligodendrocytes and the consequence of epitope spreading which generates CNS-targeted autoimmunity.

Regardless of whether MS is an autoimmune disease, this much is clear: The interplay between immunological and neurodegenerative processes of MS plays an important role for the manifestation of CNS atrophy. As such, the current work focuses on the pathogenesis of MS with focus on immune cell profiles and their potential interactions with resident cells of the CNS.

1.2 DISEASE PATHOGENESIS

The exact choreography of MS pathogenesis is unknown, though there is abundant insight into its potential mechanistic events observed in human cohorts and animal models of the disease. In particular, success in translational pharmacology of MS based on evidence gathered from EAE studies – as reflected in some of today's clinical applications such as glatiramer acetate (“proof of concept in EAE”=1971, FDA-approval = 1996), mitoxantrone (“proof of concept in

EAE”=1987, FDA-approval = 2000), VLA-4 ($\alpha_4\beta_1$ integrin)-VCAM-1 (drug name: natalizumab, “proof of concept in EAE”=1992, FDA-approval = 2006) – are hugely informative for disease pathogenesis³⁶. Briefly, glatiramer acetate is a synthetic peptide designed to simulate MBP. The mode of action of glatiramer acetate is by initial strong but promiscuous binding to MHC molecules and consequent competition with various myelin antigens for their presentation to T cells³⁷. A further aspect of its action is potent induction of T helper 2 (Th2) type that migrate to the CNS and lead to in situ bystander suppression. Mitoxantrone is a DNA intercalator with immunosuppressive effects. Among its many other functions, mitoxantrone reduces relapse rate by mainly enhancing T-cell suppressor function and inhibits B-cell function and antibody production³⁸. Natalizumab, a recombinant humanized antibody, binds to $\alpha_4\beta_1$ -integrin and blocks its interaction with Vascular Cell Adhesion Molecule 1 (VCAM-1)³⁹. As a result, immune cell infiltration into the CNS is inhibited, preventing the formation of lesions⁴⁰.

Together, while the initiating event remains unknown, these studies and others informed us that MS and EAE conditions are likely characterized by the following pathogenic events: **(1) T lymphocytes become activated by APCs in lymphoid organs such as spleen and lymph node, (2) T lymphocytes migrate from peripheral lymphoid organs and traverse across the BBB to induce CNS local inflammation, (3) Cytokines released by infiltrated peripheral immune cells and CNS resident cells (such as microglia, astrocyte, and endothelial cells) promote further invasion of peripheral immune cells into the CNS such as APCs which support reactivation of antigen specific T cells in the CNS, (3) Peripheral and central immune cells interact in orchestration to promote disease pathologies of demyelination, oligodendrocyte loss, and neuronal atrophy.** With regard to the disease’s phasic nature, the current view is that infiltration of lymphocyte subsets into the CNS signifies relapsing disease,

while CNS inflammation, as seen during disease progression, seems to be driven by tissue-resident populations ⁴¹.

Stromal cells such as endothelial cells and pericytes likely play a significant role in determination of disease pathogenesis and outcome as shown in recent reports ^{42,43}, but those studies are not discussed here as it is out of the current scope. Because the model of EAE is largely CD4⁺ T cell driven and is the choice of animal model in this work, the current studies focused on T cell effector function under the influence of APC interaction. Reflecting on key cell types studied within this dissertation work, I describe here the roles of CD4⁺ helper T (Th1, Th17, Treg) lymphocytes and APCs (DC, microglia, astrocyte) in MS pathogenesis:

T (Th1, Th17, Treg) lymphocytes in MS pathogenesis

Historically, MS is viewed as being driven by T cells, primarily because of the findings that the strongest genetic risk for MS is the MHC class II locus⁴⁴. Until recently, IFN- γ -secreting T helper type 1 (Th1) cells were thought to be the main effector T cells responsible for the autoimmune inflammation. This conclusion was based partly on the observation that mice lacking IL-12 gene were resistant to EAE, because IL-12 is required for differentiation of Th1 cells. Furthermore, treatment of MS patients with IFN- γ exacerbated disease⁴⁵. However, paradoxically, IFN- $\gamma^{-/-}$ mice that lack the IFN- γ cytokine strongly associated with a Th1 response were found to develop more EAE with greater severity ^{46,47}. Since then, the identity of T cell subpopulation that can account for neurodegeneration observed in MS shifted from Th1 cells to Th17 cells.

Since its first description in 2005, frequency of Th17 cells and its IL-17 mRNA – which encodes for key cytokine released, IL-17 – were detected in brain lesions of MS patients as

compared to healthy controls ^{16,48}. Moreover, correlations between disease activity and Th17 and IL-17 frequencies were found in MS lesions, leading to the suspicion that Th17 activity is neurotoxic. Later, a study using human-derived cells confirmed the relationship between Th17 and neurodegeneration. In the study, Th17 cells, but not other Th cell types, acquire an increased potential to disrupt the BBB and kill oligodendrocytes⁴⁹.

Unlike Th1 and Th17 which have suspected neurodegenerative effector functions in MS, T_{reg} cells is a T cell subpopulation that is thought to suppress autoimmunity. Supporting this notion, MOG-induced EAE transfer of T_{reg} reduced disease severity ⁵⁰. The action of T_{reg} in mitigating disease is mainly via the secretion of IL-10 and transforming growth factor beta (TGF- β) ⁵⁰. In addition to T_{reg}'s anti-inflammatory functions, these cells have been reported to facilitate remyelination and promote oligodendrocyte differentiation by secreting cellular communication network factor 3 (CCN3)⁵¹.

The mechanisms by which T cell exert impact upon cells in the CNS can be thought of in the terms of its function in BBB breakdown, glia activation, peripheral immune cell recruitment, and attack upon neurons and oligodendrocytes. Cytokines such as IL-17 (produced by Th17 cells) and IL-22 (produced by Th1, Th17, NK cells) can act on endothelial cells in the CNS to upregulate expression of inflammatory mediators such as chemokines CCL2, CCL5, and CXCL10 which further recruits monocytes and lymphocyte infiltration into the CNS ⁵². Specifically, IL-17 in combination with IL-6 (produced by monocytes) has been recently described to promote the breakdown of BBB in RRMS patients by altering the expression of adhesion molecules on endothelial cells and favoring the depolymerization of the cytoskeleton near the tight junctions⁵³. Secretion of IFN- γ by Th1 cells were shown to increase Fas receptor expression on CNS cells such as neurons and oligodendrocytes, thus mediating Fas-mediated cell

apoptosis⁵⁴. It was suggested that CD8⁺T cells may play a more significant role in inducing neuronal apoptosis by perforin-mediated mechanisms⁵⁵, however, contradictory evidence demonstrated that neurons are resistant to perforin-mediated lysis and instead mostly succumbed to Fas ligand-mediated apoptosis⁵⁶.

Cells that determine T cell effector function in MS pathogenesis

T cell activation and survival require two signals from APCs: (1) antigen presentation by the MHC to the T cell receptor and (2) signal provided by the interaction of co-stimulatory molecules such as CD80 and CD86 with CD28 on T cells. Here I discuss the role of APCs in MS pathogenesis with focus on peripheral DCs as well as centrally derived microglia and astrocytes. Though not described here, other cell types that have antigen-presenting capacity and are demonstrated to have a significant role in MS pathogenesis are B cells, macrophages, and neutrophils.

DCs are thought to be a major population of APC that launch the activation pathogenic T cells in the periphery and in the CNS. In support, DCs are found at higher levels both in the blood and in CNS of MS patients and EAE animals^{57,58}. Additionally, DC differentiated in vitro from blood monocytes taken from patients with MS secrete higher levels of proinflammatory cytokines IL-6, TNF and/or IL-23 than the same cells taken from healthy individuals^{59,60}. DCs are located in peripheral tissues and remain in an immature state until they interact with the antigens. After cells activation, DCs initiate a maturation process in which mature DCs acquire stimulatory properties for the activation of naïve T-cells and the development of effector T-cells. DCs secrete cytokines such as IL-23, IL-12, and IL-6 to guide T cell differentiation. Aside from contributing to T cell activation and differentiation in an

antigen-specific manner, DCs also inform T cell properties via antigen-independent mechanisms. For example, DC expression of membrane lymphotoxin activates lymphotoxin beta receptor on T cells which we and others show to increase T cell neurotoxic profile (discussed in length in Chapter 4).

Microglia are resident brain macrophages that govern CNS homeostasis through multiple functions: immune surveillance, phagocytosis of debris, cytokine and chemokine secretion, complement activation and more. During MS and EAE, microglia are thought to exert both neuro -damaging and -protectant effects. In the case of the former, microglia secrete abundant chemokine signals in response to activation by antigen-specific T cells to promote further CNS invasion of peripheral immune cells. Like DCs, microglia also express MHC molecules and co-stimulatory factors which allows them to form bystander activation of infiltrating autoreactive T cells ^{61,62}. Some protective activities of microglia include phagocytosis of myelin debris, which if left accumulating in CNS microenvironment is likely to result in lipid-mediated inflammatory cascades. However, this act, and likely others, of microglia is a double-edge sword. Intracellular accumulation of phagocytosed myelin fragments in microglia can result in upregulated NADPH oxidase function which promote oxidative stress in the CNS microenvironmental to promote mitochondrial dysfunction ⁶³. On top of the dynamic function of microglia in disease pathogenesis, recent evidence using single-cell transcriptomic technology revealed the profound heterogeneity within microglia populations in the CNS. Briefly, four different clusters of disease-associated microglia were found in mice induced with EAE ⁶⁴. Thus, careful interpretation of the role of microglia in MS and EAE should take into the consideration of the function of disease-specific microglia phenotypes.

Astrocytes constitute the most abundant cell type and play diverse anatomical and functional roles in the CNS ⁶⁵. In MS and EAE, astrocytes play a role in immune cell recruitment through chemokine production. For example, astrocytes have been shown to produce CCL5, CCL2, CXCL1, CXCL2, CXCL8, CXCL10 during inflammation which promotes the infiltration of peripheral immune cells into the CNS ^{66,67}. Recently, we demonstrated the genetic ablation of TAK1 in mice prevented T cell migration in an EAE model (shown in Chapter 5). Like DCs and microglia, astrocytes also express MHC-II and up-regulate expression of the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) upon treatment with IFN- γ , suggesting its potential role in T cell reactivation in the CNS ⁶⁸⁻⁷⁰. Although some studies did not find CD80 or CD86 expression on astrocytes in EAE ^{71,72}, a more recent study found that astrocytes in chronic MS lesions do express CD80 and CD86 ⁷³. Together these results suggest that astrocytes, like microglia and dendritic cells, may play the role of antigen-presentation to reactivate pathogenic T cells.

1.3 CNS PATHOLOGY

In 1880, MS was pathologically conceptualized as a disseminated “plaque-like sclerosis” characterized by demyelination with a variable extent of axonal loss by Jean-Martin Charcot. MS lesions can be seen in the white matter and gray matter of multiple CNS regions, the most commonly affected regions being the brain stem, spinal cord, and optic nerve⁷⁴. A lesion is often characterized by a combination of pathogenic outcomes comprising of demyelination, neuronal atrophy, oligodendrocyte loss, gliosis, accumulation of peripherally derived immune cells, and BBB breakdown. For example, an active MS lesion is marked by the accumulation of T and B cells around the lesion rim, along with the abundance of reactive glia. A chronically active

lesion can be marked by glial scarring that is comprised of interwoven reactive astrocytes which serve as a physical barrier to isolate damaged CNS regions from “healthier” parts of the tissue⁷⁵.

Demyelination refers to the loss of myelin sheath surrounding the axons, which is a major contributor to impaired neurological function and disability through impaired saltatory conduction⁷⁶. In the white matter (WM), it is likely that demyelination occurs secondary to an inflammatory T-cell mediated insult to myelin and/or oligodendrocytes, although B-cell mediated effects, as well as oligodendrocyte apoptosis have also been implicated ⁷⁷.

Demyelination in the GM may also occur as the result of a similar immune pathology, although, interestingly, inflammatory changes and BBB breakdown tend to occur less often than in the white matter ⁷⁸.

The appearance of brain and spinal cord GM neurodegeneration is an important event in the pathogenesis of MS. As such, compared to WM atrophy or lesions, neurodegeneration in the GM is thought to reflect disease subtype and disability to a greater extent ⁷⁹, especially in patients with progressive forms of MS. In particular, spinal cord GM atrophy is a major source of clinical disability in MS and EAE. Initially, GM neurodegeneration during MS is thought to be the consequence of the neuron’s inability to support normal functioning as a result of demyelination. However, emerging evidence suggest that neurodegeneration can occur independent of demyelination and oligodendrocyte loss. Evidence from early studies corroborate the story: although myelin is completely lost, axons are preserved to a large extent with variability between different patients and even between lesions in the same patient ^{80,81}.

Furthermore, some studies demonstrated that signs of GM atrophy, to a greater extent than WM atrophy, was detected in patients with prodromal or early MS ^{82,83}. Thus, the GM demyelination

and axonal degeneration may be caused by an independent, primary disease process that arises in the GM or a secondary disease process caused by damage to WM.

1.4 MS ANIMAL MODELS

Due to the many possible etiologies of MS and profound heterogeneity in clinical presentation, a range of experimental paradigms have been employed in the history of MS research. Three main animal models are used to understand MS pathogenesis: EAE, TMEV, and cuprizone intoxication. Below I describe each animal MS model as well as their benefits and drawbacks.

EAE is the most commonly used paradigm to understand MS pathogenesis in the context of T cell mediated autoimmunity (see discussion on MS as an autoimmune disease in section below) ⁸⁴. Use of the model directly contributed to the successful development of 3 current MS therapeutics of glatiramer acetate, mitoxantrone, and natalizumab. The formation of the EAE model is based on a series of observations made between 1930s that showed that sensitization of humans with CNS elements (resultant of insufficiently purified anti-rabies vaccination that was propagated in brain tissues) can cause neuromyolytic complications and disseminated encephalitis as observed in MS patients ³⁴. Thus, initially, monkeys were immunized with emulsions of brain tissue dissolved in saline to produce MS-like symptoms, but the method only resulted inconsistent results. Since then strong adjuvants such as the complete Freund's adjuvant is used to provide a slow liberation of sensitizing antigen (such as MOG) and heat killed mycobacterium tuberculosis (Mtb) is used as the immune stimulant, resulting in today's use of the EAE model. First reported in 1960, another form of the EAE model is the passive EAE ⁸⁵. In passive EAE, myelin-specific T cells are isolated from EAE immunized animals then intravenously transferred into naïve animals to induce EAE symptoms.

EAE share many commonalities with MS with some distinct differences. Reflecting late stage MS or progressive MS, EAE results in motor paralysis that originate from inflammation-mediated CNS damage. Like MS, demyelination is present in models of EAE. However, clear discrepancies in the spatial localization and pathological manifestation of demyelination events are found between MS and various EAE models. In MS, demyelination lesions can be found in multiple brain regions and in the spinal cord. However, in EAE, demyelination is limited to the spinal cord, brain stem, and cerebellum⁸⁶. EAE model is biased towards CD4⁺ T-driven autoimmune responses. While CD4⁺ T cells are recognized to play a role in MS pathogenesis, recent evidence shows that cytotoxic CD8⁺ T cells and B cells may have comparable contribution to disease pathology. To illustrate, inflammatory peripheral immune cells that infiltrate the CNS and population lesion areas are more frequently identified to be CD8⁺ T cells rather than CD4⁺ T cells in MS post-mortem brain. In stark contrast, CD4⁺ T cells are the predominant CNS-infiltrating lymphocytes that accumulate in the CNS of EAE animals. B cells were recently shown to play important roles in MS pathogenesis^{87,88}. For example, a study published in 2020 demonstrated that, in addition to producing low levels of IL-10, B cells from patients with MS patients are defective in the suppression of Th1 cell effector functions. Additionally, B cell accumulation were found in accentuated abundance in a subgroup of patients who have extensive gray matter pathology and a rapidly progressive clinical disease course, suggesting that B cell accumulation causes or contributes to the worse clinical course^{89,90}. However, in EAE, B cell action in disease pathogenesis is likely underemphasized because the EAE condition is primarily driven by pathogenic T cell populations. In fact, some B cell-focused studies using the EAE model reported the protective effect of B cells during disease, though contradictory evidence exists due to differences in experimental paradigms.

So far, while a MS-specific virus infection has not been determined, ample evidence suggests that infection with Epstein Barr Virus (EBV) may play a role in its pathogenesis. A key finding associating EBV to MS is the higher titers of EBV antibodies in MS patients compared to age-matched controls ⁹¹. Thus, MS is also popularly modeled using viral pathogens. Developed by Theiler in 1934, the TMEV model, which uses virus from the Picornaviridae family, causes neurological disease and is used as a MS model because it causes MS pathology of CNS demyelination. A major criticism for the virus model is that while EBV correlates with MS susceptibility, EBV infection in mice cannot does not cause demyelination or encephalomyelitis symptoms. Instead, the two most widely used viral MS models are chronic encephalomyelitis induced by the Theiler's virus or MHV corona virus. This calls into question the human relevance of the TMEV model. Like EAE, the TMEV model results in demyelination which is more prevalent in the spinal cord than in the brain. Additionally, demyelination lesions are seen to present with progressive spinal cord atrophy and axonal loss with ensuing neurological deterioration. These evidences suggest that, like EAE, demyelination induced by the TMEV model is more likely secondary demyelination instead of primary demyelination which occurs in MS. Interestingly, TMEV infection in C57BL/6 mice can initiate spontaneous recurrent epileptic seizures in approximately half of all affected mice. While seizures are not classified as MS symptoms, people with MS do report a higher incidence of developing seizures. Thus, the TMEV model has been used to improve our understanding on study seizure activity that occurs in MS patients.

The most frequently used MS model of remyelination is induced by systemic exposure of animals to cuprizone. Cuprizone is a copper chelating drug, which triggers apoptosis in oligodendrocytes and induces demyelination through mechanisms of oxidative injury.

Demyelination lesions are predictably located in the corpus colosseum ⁹². Interestingly, replacing cuprizone supplemented chow with normal diet is sufficient to induce spontaneous remyelination. Additionally, a prolonged cuprizone feeding paradigm (12 week) was shown to resulting in the induction of chronically demyelinated lesions with limited remyelination capacity. Thus the cuprizone demyelinating model has been used to provide seminal insights into metabolic processes involved in demyelination and remyelination.

In the current work, I explored MS pathogenesis in the context of disease severity, drug sensitivity, and neuron atrophy status using the EAE model. The EAE model was chosen as the animal model to study MS heterogeneity based on practical and scientific rationale. In the former case, the EAE model is easy to induce and highly reproducible. Additionally, the lab has previously shown that disease heterogeneity can be elicited via manipulation of adjuvant concentration and injection schedule. In the latter case, like MS, the presentation of EAE is heterogeneous and was previously shown to inform disease pathogenesis with potential clinical relevance. In strong support, many of the drugs that are in current or imminent use in MS have been developed, tested, or validated on the basis of EAE studies.

1.5 RATIONALE AND SPECIFIC AIMS

MS is a chronic, often debilitating autoimmune disease that currently affects more than 500,000 individuals in the United States ^{93,94}. MS is characterized by demyelination and degeneration of neurons in the CNS with profound heterogeneity in disease presentation, trajectory, and treatment efficacy. Current treatment options slow disease progression but have limited efficacy in a portion of patients with non-relapsing disease and progressive CNS neurodegeneration. Further, even with efficacious medicine, persons with multiple sclerosis accumulate disabilities

over their lifetime. Several environmental factors such as early life trauma and changes in endogenous estrogen signaling have been identified as potential mediators of predictable changes in MS disease progression ⁹⁵. Therefore, there is an urgent need to identify critical molecular determinants of disease heterogeneity and to develop novel precision therapeutics for treating MS.

Exposure to stress has long been suspected to be a factor that can aggravate MS by altering the immune system ⁹⁶⁻⁹⁹. Specifically, early-life trauma (ELT) has been proposed to change immune cell properties in adults and is associated with an increased risk for chronic illnesses ¹⁰⁰⁻¹⁰⁴. Though no large epidemiological studies have been conducted, human case studies have shown that childhood trauma, such as emotional neglect and physical abuse, is a factor in increasing susceptibility, relapse rates, hospitalization likelihood, and an earlier onset in patients with MS ¹⁰⁵⁻¹⁰⁸. However, how childhood trauma increases MS disease susceptibility and sensitivity is still largely unknown.

High estrogen levels can alleviate MS symptoms ¹⁹. A seminal study reported that the rate of relapse of women with multiple sclerosis declines during pregnancy, especially in the third trimester when estrogen levels are highest ¹⁰⁹. Conversely, post-partum women with low endogenous estrogen levels are more prone to relapse, supporting an inverse relationship between endogenous estrogen and MS ^{110, 111}. Based on these observations, sex hormones have been tested as treatment options against MS. Treatment of patients with estriol, an endogenous form of estrogen, alone ^{112, 113} or in combination with an immune-modulatory drug produces favorable therapeutic outcomes such as reduced lesion load ¹¹³ and relapse rate ¹¹⁴. While the efficacy of systemic estrogen treatment against disease progression greatly is thought to greatly

depend on estrogen's effect on the activity of myelin antigen presenting dendritic cells and T lymphocytes^{115, 116}, little to known about its role in the context of disease phenotype.

In Chapter 2, I present a novel method to visualize Golgi-Cox staining which permits more accurate dendritic spine quantitation using the IMARIS automated software. The method is used to examine degree of neuron damage in the rest of the current dissertation work. Herein, in Aim 1 (Chapters 3 and 4), I explore the impact of environmental factors of early life trauma and changes in estrogen signaling on disease phenotype and pathogenesis using the EAE model to understand the impact of these factors in the context of heterogeneity. Through this work, I hope to encourage more epidemiological studies to be conducted to confirm the relationship between these environmental factors and MS heterogeneity characterized by disease trajectory, pattern and degree of CNS atrophy, and sensitivity to first-line treatment of IFN β .

CXCL1 is a chemokine ligand that are found to be in high levels in the plasma and cerebrospinal fluid (CSF) of persons with MS²⁸. A previous study has shown that a high level of CXCR2 is present in the chronic active lesions of MS patients, indicating that CXCR2 might be involved in MS pathogenesis¹¹⁷. While CXCR2 are known to be expressed by neutrophils, its expression on T cells was recently reported to be upregulated in an alternative EAE model with severe neurodegeneration (termed Type-B EAE, described in Chapters 5 and 6), suggesting the potential role of CXCR2 signaling as a neurotoxic molecule. Thus, in Aim 2, we sought to understand the role of neurotoxic molecule in the context of neuron damage by focusing on the role of CXCR2 activation in neutrophils and T cells using the Type-B EAE model with severe neurodegeneration.

1.6 REFERENCES

1. Walton C, King R, Rechtman L, et al. Rising prevalence of multiple sclerosis worldwide: Insights from the Atlas of MS, third edition. *Mult Scler*. Dec 2020;26(14):1816-1821. doi:10.1177/1352458520970841
2. Goodwin SJ. Multiple sclerosis: integration of modeling with biology, clinical and imaging measures to provide better monitoring of disease progression and prediction of outcome. *Neural Regen Res*. Dec 2016;11(12):1900-1903. doi:10.4103/1673-5374.195274
3. Kobelt G, Thompson A, Berg J, et al. New insights into the burden and costs of multiple sclerosis in Europe. *Mult Scler*. Jul 2017;23(8):1123-1136. doi:10.1177/1352458517694432
4. Harbo HF, Gold R, Tintore M. Sex and gender issues in multiple sclerosis. *Ther Adv Neurol Disord*. Jul 2013;6(4):237-48. doi:10.1177/1756285613488434
5. Compston A, Coles A. Multiple sclerosis. *Lancet*. Oct 25 2008;372(9648):1502-17. doi:10.1016/S0140-6736(08)61620-7
6. Coleman CI, Sidovar MF, Roberts MS, Kohn C. Impact of mobility impairment on indirect costs and health-related quality of life in multiple sclerosis. *PLoS One*. 2013;8(1):e54756. doi:10.1371/journal.pone.0054756
7. Karussis D. The diagnosis of multiple sclerosis and the various related demyelinating syndromes: a critical review. *J Autoimmun*. Feb-Mar 2014;48-49:134-42. doi:10.1016/j.jaut.2014.01.022
8. Lassmann H. Pathology and disease mechanisms in different stages of multiple sclerosis. *J Neurol Sci*. Oct 15 2013;333(1-2):1-4. doi:10.1016/j.jns.2013.05.010

9. Popescu V, Agosta F, Hulst HE, et al. Brain atrophy and lesion load predict long term disability in multiple sclerosis. *J Neurol Neurosurg Psychiatry*. Oct 2013;84(10):1082-91. doi:10.1136/jnnp-2012-304094
10. Ebers GC. Natural history of multiple sclerosis. *J Neurol Neurosurg Psychiatry*. Dec 2001;71 Suppl 2:ii16-9. doi:10.1136/jnnp.71.suppl_2.ii16
11. Tsagkas C, Magon S, Gaetano L, et al. Preferential spinal cord volume loss in primary progressive multiple sclerosis. *Mult Scler*. Jun 2019;25(7):947-957. doi:10.1177/1352458518775006
12. Thompson AJ, Polman CH, Miller DH, et al. Primary progressive multiple sclerosis. *Brain*. Jun 1997;120 (Pt 6):1085-96. doi:10.1093/brain/120.6.1085
13. Axtell RC, Raman C, Steinman L. Interferon-beta exacerbates Th17-mediated inflammatory disease. *Trends Immunol*. Jun 2011;32(6):272-7. doi:10.1016/j.it.2011.03.008
14. Inoue M, Chen PH, Siecinski S, et al. An interferon-beta-resistant and NLRP3 inflammasome-independent subtype of EAE with neuronal damage. *Nat Neurosci*. Dec 2016;19(12):1599-1609. doi:10.1038/nn.4421
15. Hollenbach JA, Oksenberg JR. The immunogenetics of multiple sclerosis: A comprehensive review. *J Autoimmun*. Nov 2015;64:13-25. doi:10.1016/j.jaut.2015.06.010
16. Lock C, Hermans G, Pedotti R, et al. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med*. May 2002;8(5):500-8. doi:10.1038/nm0502-500
17. Moutsianas L, Jostins L, Beecham AH, et al. Class II HLA interactions modulate genetic risk for multiple sclerosis. *Nat Genet*. Oct 2015;47(10):1107-1113. doi:10.1038/ng.3395

18. Traugott U, Reinherz EL, Raine CS. Multiple sclerosis. Distribution of T cells, T cell subsets and Ia-positive macrophages in lesions of different ages. *J Neuroimmunol*. Jun 1983;4(3):201-21. doi:10.1016/0165-5728(83)90036-x
19. Cotsapas C, Mitrovic M. Genome-wide association studies of multiple sclerosis. *Clinical & translational immunology*. 2018;7(6):e1018.
20. Miller DH, Fazekas F, Montalban X, Reingold SC, Trojano M. Pregnancy, sex and hormonal factors in multiple sclerosis. *Mult Scler*. Apr 2014;20(5):527-36. doi:10.1177/1352458513519840
21. Riise T, Nortvedt MW, Ascherio A. Smoking is a risk factor for multiple sclerosis. *Neurology*. Oct 28 2003;61(8):1122-4. doi:10.1212/01.wnl.0000081305.66687.d2
22. Sintzel MB, Rametta M, Reder AT. Vitamin D and Multiple Sclerosis: A Comprehensive Review. *Neurol Ther*. Jun 2018;7(1):59-85. doi:10.1007/s40120-017-0086-4
23. Houen G, Heiden J, Trier NH, et al. Antibodies to Epstein-Barr virus and neurotropic viruses in multiple sclerosis and optic neuritis. *J Neuroimmunol*. Jul 7 2020;346:577314. doi:10.1016/j.jneuroim.2020.577314
24. Karagkouni A, Alevizos M, Theoharides TC. Effect of stress on brain inflammation and multiple sclerosis. *Autoimmun Rev*. Aug 2013;12(10):947-53. doi:10.1016/j.autrev.2013.02.006
25. Nisipeanu P, Korczyn AD. Psychological stress as risk factor for exacerbations in multiple sclerosis. *Neurology*. Jul 1993;43(7):1311-2. doi:10.1212/wnl.43.7.1311
26. Hedstrom AK, Alfredsson L, Lundkvist Ryner M, Fogdell-Hahn A, Hillert J, Olsson T. Smokers run increased risk of developing anti-natalizumab antibodies. *Mult Scler*. Jul 2014;20(8):1081-5. doi:10.1177/1352458513515086

27. Hedstrom AK, Ryner M, Fink K, et al. Smoking and risk of treatment-induced neutralizing antibodies to interferon beta-1a. *Mult Scler*. Apr 2014;20(4):445-50. doi:10.1177/1352458513498635
28. Burman J, Svensson E, Fransson M, et al. The cerebrospinal fluid cytokine signature of multiple sclerosis: a homogenous response that does not conform to the Th1/Th2/Th17 convention. *J Neuroimmunol*. Dec 15 2014;277(1-2):153-9. doi:10.1016/j.jneuroim.2014.10.005
29. Lindert RB, Haase CG, Brehm U, Linington C, Wekerle H, Hohlfeld R. Multiple sclerosis: B- and T-cell responses to the extracellular domain of the myelin oligodendrocyte glycoprotein. *Brain*. Nov 1999;122 (Pt 11):2089-100. doi:10.1093/brain/122.11.2089
30. Reindl M, Linington C, Brehm U, et al. Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. *Brain*. Nov 1999;122 (Pt 11):2047-56. doi:10.1093/brain/122.11.2047
- 1990;346(6280):183-7. doi:10.1038/346183a0
31. Cao Y, Goods BA, Raddassi K, et al. Functional inflammatory profiles distinguish myelin-reactive T cells from patients with multiple sclerosis. *Sci Transl Med*. May 13 2015;7(287):287ra74. doi:10.1126/scitranslmed.aaa8038
32. Muraro PA, Wandinger KP, Bielekova B, et al. Molecular tracking of antigen-specific T cell clones in neurological immune-mediated disorders. *Brain*. Jan 2003;126(Pt 1):20-31. doi:10.1093/brain/awg021
33. Bielekova B, Goodwin B, Richert N, et al. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med*. Oct 2000;6(10):1167-75. doi:10.1038/80516

34. Rivers TM, Sprunt DH, Berry G. Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. *The Journal of experimental medicine*. 1933 Jun 30;58(1):39.
35. Genain CP, Nguyen MH, Letvin NL, et al. Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. *J Clin Invest*. Dec 1995;96(6):2966-74. doi:10.1172/JCI118368
36. Glatigny S, Bettelli E. Experimental Autoimmune Encephalomyelitis (EAE) as Animal Models of Multiple Sclerosis (MS). *Cold Spring Harb Perspect Med*. Nov 1 2018;8(11)doi:10.1101/cshperspect.a028977
37. Arnon R, Aharoni R. Mechanism of action of glatiramer acetate in multiple sclerosis and its potential for the development of new applications. *Proc Natl Acad Sci U S A*. Oct 5 2004;101 Suppl 2:14593-8. doi:10.1073/pnas.0404887101
38. Neuhaus O, Kieseier BC, Hartung HP. Mechanisms of mitoxantrone in multiple sclerosis--what is known? *J Neurol Sci*. Aug 15 2004;223(1):25-7. doi:10.1016/j.jns.2004.04.015
39. Gonzalez-Amaro R, Mittelbrunn M, Sanchez-Madrid F. Therapeutic anti-integrin (alpha4 and alphaL) monoclonal antibodies: two-edged swords? *Immunology*. Nov 2005;116(3):289-96. doi:10.1111/j.1365-2567.2005.02225.x
40. Rudick RA, Sandrock A. Natalizumab: alpha 4-integrin antagonist selective adhesion molecule inhibitors for MS. *Expert Rev Neurother*. Jul 2004;4(4):571-80. doi:10.1586/14737175.4.4.571
41. van Langelaar J, Rijvers L, Smolders J, van Luijn MM. B and T Cells Driving Multiple Sclerosis: Identity, Mechanisms and Potential Triggers. *Front Immunol*. 2020;11:760. doi:10.3389/fimmu.2020.00760
42. Derada Troletti C, Fontijn RD, Gowing E, et al. Inflammation-induced endothelial to mesenchymal transition promotes brain endothelial cell dysfunction and occurs during multiple

sclerosis pathophysiology. *Cell Death Dis.* Jan 18 2019;10(2):45. doi:10.1038/s41419-018-1294-

2

43. Torok O, Schreiner B, Schaffenrath J, et al. Pericytes regulate vascular immune homeostasis in the CNS. *Proc Natl Acad Sci U S A.* Mar 9

2021;118(10)doi:10.1073/pnas.2016587118

44. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KH. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol.* Oct 2010;162(1):1-11.

doi:10.1111/j.1365-2249.2010.04143.x

45. Panitch HS, Hirsch RL, Haley AS, Johnson KP. Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet.* Apr 18 1987;1(8538):893-5. doi:10.1016/s0140-

6736(87)92863-7

46. Ferber IA, Brocke S, Taylor-Edwards C, et al. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J*

Immunol. Jan 1 1996;156(1):5-7.

47. Bettelli E, Sullivan B, Szabo SJ, Sobel RA, Glimcher LH, Kuchroo VK. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp*

Med. Jul 5 2004;200(1):79-87. doi:10.1084/jem.20031819

48. Montes M, Zhang X, Berthelot L, et al. Oligoclonal myelin-reactive T-cell infiltrates derived from multiple sclerosis lesions are enriched in Th17 cells. *Clin Immunol.* Feb

2009;130(2):133-44. doi:10.1016/j.clim.2008.08.030

49. Kebir H, Kreymborg K, Ifergan I, et al. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med.* Oct 2007;13(10):1173-5.

doi:10.1038/nm1651

50. Kohm AP, Carpentier PA, Anger HA, Miller SD. Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol.* Nov 1 2002;169(9):4712-6. doi:10.4049/jimmunol.169.9.4712
51. Dombrowski Y, O'Hagan T, Dittmer M, et al. Regulatory T cells promote myelin regeneration in the central nervous system. *Nat Neurosci.* May 2017;20(5):674-680. doi:10.1038/nn.4528
52. Wilson EH, Weninger W, Hunter CA. Trafficking of immune cells in the central nervous system. *J Clin Invest.* May 2010;120(5):1368-79. doi:10.1172/JCI41911
53. Setiadi AF, Abbas AR, Jeet S, et al. IL-17A is associated with the breakdown of the blood-brain barrier in relapsing-remitting multiple sclerosis. *J Neuroimmunol.* Jul 15 2019;332:147-154. doi:10.1016/j.jneuroim.2019.04.011
54. Volpe E, Sambucci M, Battistini L, Borsellino G. Fas-Fas Ligand: Checkpoint of T Cell Functions in Multiple Sclerosis. *Front Immunol.* 2016;7:382. doi:10.3389/fimmu.2016.00382
55. Rensing-Ehl A, Malipiero U, Irmeler M, Tschopp J, Constam D, Fontana A. Neurons induced to express major histocompatibility complex class I antigen are killed via the perforin and not the Fas (APO-1/CD95) pathway. *Eur J Immunol.* Sep 1996;26(9):2271-4. doi:10.1002/eji.1830260945
56. Medana I, Li Z, Flugel A, Tschopp J, Wekerle H, Neumann H. Fas ligand (CD95L) protects neurons against perforin-mediated T lymphocyte cytotoxicity. *J Immunol.* Jul 15 2001;167(2):674-81. doi:10.4049/jimmunol.167.2.674

57. Lande R, Gafa V, Serafini B, et al. Plasmacytoid dendritic cells in multiple sclerosis: intracerebral recruitment and impaired maturation in response to interferon-beta. *J Neuropathol Exp Neurol*. May 2008;67(5):388-401. doi:10.1097/NEN.0b013e31816fc975
58. Serafini B, Rosicarelli B, Magliozzi R, et al. Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells. *J Neuropathol Exp Neurol*. Feb 2006;65(2):124-41. doi:10.1097/01.jnen.0000199572.96472.1c
59. Huang YM, Xiao BG, Ozenci V, et al. Multiple sclerosis is associated with high levels of circulating dendritic cells secreting pro-inflammatory cytokines. *J Neuroimmunol*. Sep 1 1999;99(1):82-90. doi:10.1016/s0165-5728(99)00106-x
60. Vaknin-Dembinsky A, Balashov K, Weiner HL. IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production. *J Immunol*. Jun 15 2006;176(12):7768-74. doi:10.4049/jimmunol.176.12.7768
61. Gerritse K, Laman JD, Noelle RJ, et al. CD40-CD40 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis. *Proc Natl Acad Sci U S A*. Mar 19 1996;93(6):2499-504. doi:10.1073/pnas.93.6.2499
62. Zrzavy T, Hametner S, Wimmer I, Butovsky O, Weiner HL, Lassmann H. Loss of 'homeostatic' microglia and patterns of their activation in active multiple sclerosis. *Brain*. Jul 1 2017;140(7):1900-1913. doi:10.1093/brain/awx113
63. Di Filippo M, de Iure A, Giampa C, et al. Persistent activation of microglia and NADPH oxidase [corrected] drive hippocampal dysfunction in experimental multiple sclerosis. *Sci Rep*. Feb 18 2016;6:20926. doi:10.1038/srep20926

64. Jordao MJC, Sankowski R, Brendecke SM, et al. Single-cell profiling identifies myeloid cell subsets with distinct fates during neuroinflammation. *Science*. Jan 25 2019;363(6425)doi:10.1126/science.aat7554
65. Miller SJ. Astrocyte Heterogeneity in the Adult Central Nervous System. *Front Cell Neurosci*. 2018;12:401. doi:10.3389/fncel.2018.00401
66. Chastain EM, Duncan DS, Rodgers JM, Miller SD. The role of antigen presenting cells in multiple sclerosis. *Biochim Biophys Acta*. Feb 2011;1812(2):265-74. doi:10.1016/j.bbadis.2010.07.008
67. Choi SS, Lee HJ, Lim I, Satoh J, Kim SU. Human astrocytes: secretome profiles of cytokines and chemokines. *PLoS One*. 2014;9(4):e92325. doi:10.1371/journal.pone.0092325
68. Wong GH, Bartlett PF, Clark-Lewis I, Battye F, Schrader JW. Inducible expression of H-2 and Ia antigens on brain cells. *Nature*. Aug 23-29 1984;310(5979):688-91. doi:10.1038/310688a0
69. Yong VW, Moudjian R, Yong FP, et al. Gamma-interferon promotes proliferation of adult human astrocytes in vitro and reactive gliosis in the adult mouse brain in vivo. *Proc Natl Acad Sci U S A*. Aug 15 1991;88(16):7016-20. doi:10.1073/pnas.88.16.7016
70. Cornet A, Bettelli E, Oukka M, et al. Role of astrocytes in antigen presentation and naive T-cell activation. *J Neuroimmunol*. Jul 1 2000;106(1-2):69-77. doi:10.1016/s0165-5728(99)00215-5
71. Cross AH, Ku G. Astrocytes and central nervous system endothelial cells do not express B7-1 (CD80) or B7-2 (CD86) immunoreactivity during experimental autoimmune encephalomyelitis. *J Neuroimmunol*. Oct 2 2000;110(1-2):76-82. doi:10.1016/s0165-5728(00)00327-1

72. Aloisi F, Ria F, Penna G, Adorini L. Microglia are more efficient than astrocytes in antigen processing and in Th1 but not Th2 cell activation. *J Immunol.* May 15 1998;160(10):4671-80.
73. Zeinstra E, Wilczak N, De Keyser J. Reactive astrocytes in chronic active lesions of multiple sclerosis express co-stimulatory molecules B7-1 and B7-2. *J Neuroimmunol.* Feb 2003;135(1-2):166-71. doi:10.1016/s0165-5728(02)00462-9
74. Brownlee WJ, Hardy TA, Fazekas F, Miller DH. Diagnosis of multiple sclerosis: progress and challenges. *Lancet.* Apr 1 2017;389(10076):1336-1346. doi:10.1016/S0140-6736(16)30959-X
75. Holley JE, Gveric D, Newcombe J, Cuzner ML, Gutowski NJ. Astrocyte characterization in the multiple sclerosis glial scar. *Neuropathol Appl Neurobiol.* Oct 2003;29(5):434-44. doi:10.1046/j.1365-2990.2003.00491.x
76. Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol.* Jun 2000;47(6):707-17. doi:10.1002/1531-8249(200006)47:6
77. Barnett MH, Prineas JW. Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann Neurol.* Apr 2004;55(4):458-68. doi:10.1002/ana.20016
78. Bo L, Vedeler CA, Nyland HI, Trapp BD, Mork SJ. Subpial demyelination in the cerebral cortex of multiple sclerosis patients. *J Neuropathol Exp Neurol.* Jul 2003;62(7):723-32. doi:10.1093/jnen/62.7.723
79. Fisniku LK, Chard DT, Jackson JS, et al. Gray matter atrophy is related to long-term disability in multiple sclerosis. *Ann Neurol.* Sep 2008;64(3):247-54. doi:10.1002/ana.21423

80. Ferguson B, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. *Brain*. Mar 1997;120 (Pt 3):393-9. doi:10.1093/brain/120.3.393
81. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med*. Jan 29 1998;338(5):278-85. doi:10.1056/NEJM199801293380502
82. Tiberio M, Chard DT, Altmann DR, et al. Gray and white matter volume changes in early RRMS: a 2-year longitudinal study. *Neurology*. Mar 22 2005;64(6):1001-7. doi:10.1212/01.WNL.0000154526.22878.30
83. Dalton CM, Chard DT, Davies GR, et al. Early development of multiple sclerosis is associated with progressive grey matter atrophy in patients presenting with clinically isolated syndromes. *Brain*. May 2004;127(Pt 5):1101-7. doi:10.1093/brain/awh126
84. Constantinescu CS, Farooqi N, O'Brien K, Gran B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol*. Oct 2011;164(4):1079-106. doi:10.1111/j.1476-5381.2011.01302.x
85. Paterson PY. Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *The Journal of experimental medicine*. 1960 Jan 1;111(1):119-36.
86. Lassmann H, Bradl M. Multiple sclerosis: experimental models and reality. *Acta Neuropathol*. Feb 2017;133(2):223-244. doi:10.1007/s00401-016-1631-4
87. Li R, Rezk A, Miyazaki Y, et al. Proinflammatory GM-CSF-producing B cells in multiple sclerosis and B cell depletion therapy. *Sci Transl Med*. Oct 21 2015;7(310):310ra166. doi:10.1126/scitranslmed.aab4176
88. Bar-Or A, Fawaz L, Fan B, et al. Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol*. Apr 2010;67(4):452-61. doi:10.1002/ana.21939

89. Bevan RJ, Evans R, Griffiths L, et al. Meningeal inflammation and cortical demyelination in acute multiple sclerosis. *Ann Neurol*. Dec 2018;84(6):829-842. doi:10.1002/ana.25365
90. Magliozzi R, Howell O, Vora A, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain*. Apr 2007;130(Pt 4):1089-104. doi:10.1093/brain/awm038
91. Maghzi AH, Marta M, Bosca I, et al. Viral pathophysiology of multiple sclerosis: A role for Epstein-Barr virus infection? *Pathophysiology*. Feb 2011;18(1):13-20. doi:10.1016/j.pathophys.2010.04.003
92. Steelman AJ, Thompson JP, Li J. Demyelination and remyelination in anatomically distinct regions of the corpus callosum following cuprizone intoxication. *Neurosci Res*. Jan 2012;72(1):32-42. doi:10.1016/j.neures.2011.10.002
93. Hunter SF. Overview and diagnosis of multiple sclerosis. *Am J Manag Care*. Jun 2016;22(6 Suppl):s141-50.
94. Nelson LM, Wallin MT, Marrie RA, et al. A new way to estimate neurologic disease prevalence in the United States: Illustrated with MS. *Neurology*. Mar 5 2019;92(10):469-480. doi:10.1212/WNL.0000000000007044
95. Disanto G, Berlanga AJ, Handel AE, et al. Heterogeneity in multiple sclerosis: scratching the surface of a complex disease. *Autoimmune Dis*. Dec 9 2010;2011:932351. doi:10.4061/2011/932351
96. Mohr DC, Hart SL, Julian L, Cox D, Pelletier D. Association between stressful life events and exacerbation in multiple sclerosis: a meta-analysis. *BMJ*. Mar 27 2004;328(7442):731. doi:10.1136/bmj.38041.724421.55

97. Artemiadis AK, Anagnostouli MC, Alexopoulos EC. Stress as a risk factor for multiple sclerosis onset or relapse: a systematic review. *Neuroepidemiology*. 2011;36(2):109-20. doi:10.1159/000323953
98. Kern S, Rohleder N, Eisenhofer G, Lange J, Ziemssen T. Time matters - acute stress response and glucocorticoid sensitivity in early multiple sclerosis. *Brain Behav Immun*. Oct 2014;41:82-9. doi:10.1016/j.bbi.2014.04.006
99. Briones-Buixassa L, Mila R, J MfA, Buñill E, Olaya B, Arrufat FX. Stress and multiple sclerosis: A systematic review considering potential moderating and mediating factors and methods of assessing stress. *Health Psychol Open*. Jul 2015;2(2):2055102915612271. doi:10.1177/2055102915612271
100. Gross C, Hen R. The developmental origins of anxiety. *Nat Rev Neurosci*. Jul 2004;5(7):545-52. doi:10.1038/nrn1429
101. Liu D, Diorio J, Day JC, Francis DD, Meaney MJ. Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nat Neurosci*. Aug 2000;3(8):799-806. doi:10.1038/77702
102. Szyf M. DNA methylation, the early-life social environment and behavioral disorders. *J Neurodev Disord*. Sep 2011;3(3):238-49. doi:10.1007/s11689-011-9079-2
103. Khulan B, Manning JR, Dunbar DR, et al. Epigenomic profiling of men exposed to early-life stress reveals DNA methylation differences in association with current mental state. *Transl Psychiatry*. Sep 23 2014;4:e448. doi:10.1038/tp.2014.94
104. Cattaneo A, Macchi F, Plazzotta G, et al. Inflammation and neuronal plasticity: a link between childhood trauma and depression pathogenesis. *Front Cell Neurosci*. 2015;9:40. doi:10.3389/fncel.2015.00040

105. Dube SR, Fairweather D, Pearson WS, Felitti VJ, Anda RF, Croft JB. Cumulative childhood stress and autoimmune diseases in adults. *Psychosom Med*. Feb 2009;71(2):243-50. doi:10.1097/PSY.0b013e3181907888
106. Goodwin RD, Stein MB. Association between childhood trauma and physical disorders among adults in the United States. *Psychol Med*. Apr 2004;34(3):509-20.
107. Spitzer C, Bouchain M, Winkler LY, et al. Childhood trauma in multiple sclerosis: a case-control study. *Psychosom Med*. Apr 2012;74(3):312-8. doi:10.1097/PSY.0b013e31824c2013
108. Shaw MT, Pawlak NO, Frontario A, Sherman K, Krupp LB, Charvet LE. Adverse Childhood Experiences Are Linked to Age of Onset and Reading Recognition in Multiple Sclerosis. *Front Neurol*. 2017;8:242. doi:10.3389/fneur.2017.00242
109. Confavreux C, Hutchinson M, Hours MM, Cortinovis-Tourniaire P, Moreau T. Rate of pregnancy-related relapse in multiple sclerosis. Pregnancy in Multiple Sclerosis Group. *N Engl J Med*. Jul 30 1998;339(5):285-91. doi:10.1056/NEJM199807303390501
110. Birk K, Ford C, Smeltzer S, Ryan D, Miller R, Rudick RA. The clinical course of multiple sclerosis during pregnancy and the puerperium. *Arch Neurol*. Jul 1990;47(7):738-42. doi:10.1001/archneur.1990.00530070026007
111. Damek DM, Shuster EA. Pregnancy and multiple sclerosis. *Mayo Clin Proc*. Oct 1997;72(10):977-89. doi:10.1016/S0025-6196(11)63371-5
112. MacKenzie-Graham A, Brook J, Kurth F, et al. Estriol-mediated neuroprotection in multiple sclerosis localized by voxel-based morphometry. *Brain Behav*. Sep 2018;8(9):e01086. doi:10.1002/brb3.1086

113. Sicotte NL, Liva SM, Klutch R, et al. Treatment of multiple sclerosis with the pregnancy hormone estriol. *Ann Neurol*. Oct 2002;52(4):421-8. doi:10.1002/ana.10301
114. Voskuhl RR, Wang H, Wu TC, et al. Estriol combined with glatiramer acetate for women with relapsing-remitting multiple sclerosis: a randomised, placebo-controlled, phase 2 trial. *Lancet Neurol*. Jan 2016;15(1):35-46. doi:10.1016/S1474-4422(15)00322-1
115. Matejuk A, Bakke AC, Hopke C, Dwyer J, Vandenbark AA, Offner H. Estrogen treatment induces a novel population of regulatory cells, which suppresses experimental autoimmune encephalomyelitis. *J Neurosci Res*. Jul 1 2004;77(1):119-26. doi:10.1002/jnr.20145
116. Liu HY, Buenafe AC, Matejuk A, et al. Estrogen inhibition of EAE involves effects on dendritic cell function. *J Neurosci Res*. Oct 15 2002;70(2):238-48. doi:10.1002/jnr.10409
117. Omari KM, John G, Lango R, Raine CS. Role for CXCR2 and CXCL1 on glia in multiple sclerosis. *Glia*. Jan 1 2006;53(1):24-31. doi:10.1002/glia.20246

CHAPTER 2: A CONFOCAL REFLECTION SUPER-RESOLUTION TECHNIQUE TO IMAGE GOLGI-COX STAINED NEURONS¹

2.1 INTRODUCTION

While diffraction limit is surpassed in fluorescence imaging ¹⁻³, resolution improvement in other conventional optical techniques such as reflection and other modalities are constantly attempted ⁴⁻¹⁸. Super-resolution techniques such as stimulated emission and depletion, structured illumination, Airyscan ^{1,19} and PALM/STORM ²⁰ have recently been commercialized to go beyond diffraction limit of resolution in biological samples labeled with fluorescent targets. However, it is important to note that fluorescent techniques are inherently photon limited ^{21,22}. One has to collect as much, if not all, photons to faithfully describe the sample at hand. In general, techniques that reject the photons from fluorescence targets from in focal plane or from out of focus focal planes usually result in poor resolution and low signal-to-noise (SNR) ratios ²¹⁻²⁵. While this photon-limitation holds true in fluorescence imaging, it does not apply to photon-unlimited imaging modalities such as confocal reflection microscopy involving metallic material samples or biological samples labeled with metallic targets (gold and silver) nanoparticles ^{4,13,26} such as Golgi staining.

Golgi staining has a long-held history in the field of neuroscience as an important tool to characterize neuron morphology. It remains a superior neuron staining method with unsurpassed clarity. Golgi staining is based on a metallic reaction which forms mercury- or silver-based aggregates in neurons and, more rarely, glia of the central nervous system. This central nervous system staining method was originally invented by the Italian physician and scientist Camillo

¹ This chapter is published in its entirety in Journal of Microscopy. This article is available from <https://onlinelibrary.wiley.com/doi/abs/10.1111/jmi.12821> and using DOI: 10.1111/jmi.12821

Golgi³¹ who named this procedure a “black reaction” to show the details of the nervous system. The original technique has been modified and given multiple names such as Golgi-Cox (GC), rapid Golgi, Golgi-Kopsch, and so on. The conventional method of visualizing Golgi staining is by using transmitted light, otherwise known as bright-field microscopy. Turner and Spiga et al.^{29,33,34} demonstrated that Golgi staining stain can be visualized using the confocal microscope under the reflection modality. Using confocal reflection microscopy instead of the transmitted light microscopy, GC-stained neurons can be visualized in a three-dimensional modality better than the extended depth of focus images with the ability to optically section (image z-Stacks) thick brain slices with a black background, which is necessary for better contrast and resolution. In addition, Spiga and others demonstrated that central nervous system tissue samples impregnated with Golgi staining can also be marked with protein-specific fluorescent tags to allow for the simultaneous visualization and acquisition of Golgi and antibody targeted fluorescence signals in additional channels. This discovery created a new avenue for the 3D-rendering of the biochemical and morphological details of neurons and glia. However, the resolution of the reported confocal reflection technique is limited by the diffraction limit of light.

Previously, we have demonstrated that using smaller pinholes combined with lowest wavelength of light (405 nm) in a confocal microscope, one could achieve theoretical resolutions in material samples⁴. In this manuscript, we have extended a similar approach of using smaller pinholes in confocal reflection mode to improve resolution. In combination with deconvolution, this technique, termed the Confocal Reflection Super-Resolution (CRSR) method, can improve resolutions up to two-fold over the diffraction limit in visualizing metal-based targets in biological samples with gold nanoparticles as well as GC in thick brain slices. This improved resolution is critical as it has enabled better characterizing of dendritic spines which can be

indicative of neuronal function. We hope the simple, yet robust CRSR technique may prove to be critical in investigations that concern changes in detailed neuron morphology under central nervous system disease conditions such as multiple sclerosis and Alzheimer's disease.

2.2 METHODS

Animals and Institutional Study Approval

Healthy male and female mice of the C57BL/6 background of 6-8-week-old were randomly selected and used in this study. C57BL/6 mice were purchased from Jackson Laboratories. All the mice were kept group-housed (3-5 mice per cage) in a specific pathogen free facility with 12h-12h light-dark cycle in the Veterinary Medicine Basic Sciences Building at University of Illinois at Urbana-Champaign. This study was approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (Protocol number 16114).

Experimental autoimmune encephalomyelitis induction

To visualize activated astrocytes in the brain, we induced 7-week old mice with an established rodent model of multiple sclerosis termed experimental autoimmune encephalitis (EAE) by one subcutaneous injection of myelin oligodendrocyte glycoprotein (MOG35-55) peptide (100 micro g/mouse) emulsified in complete Freund's adjuvant including heat-killed mycobacteria (BD Difco, 200 μ g/mouse) on day 0, and i.p. injection of pertussis toxin (200 ng/mouse) on days 0 and 2.

Golgi-Cox staining protocol

Mice were deeply anesthetized with isoflurane and perfused intracardially with phosphate buffered saline (PBS) (15 ml) followed by 4% paraformaldehyde (pH 7.4) (25 ml). Brain and spinal cord were removed and stained with Rapid Golgi according to kit protocol (FD Rapid Golgi-Stain Kit, FD Neuro-Technologies, INC), described as follows. Brain was placed in 5 ml of Solution A and B of ratio 1:1 for 24 hours and then immersed in an identical volume of fresh Solution A and B for an additional 14 days in dark. The samples were then transferred to solution C for 3 days, with a replacement of fresh solution after 24 hours. To attain cryoprotection, brains were then transferred in a 30% sucrose solution in PBS for 1-2 days. Afterward, samples were snap frozen in optimal cutting temperature compound (SakuraTek, Japan) on dry ice and kept frozen at -80°C to be cut into 100 µm thick coronal slices using Cryo-cut 1800 (Reichert Jung) at -14°C. Cut slices were stored in 24-well plates with 0.02% azide in PBS at 4°C. To develop GC staining, brain slices were washed in distilled water (2 x 4 minutes) and submerged in solution D and E according to kit protocol. Slices were rinsed in distilled water (2 x 4 minutes) again.

Immunofluorescence procedure

GC stained tissue slices were permeabilized with 0.5% Triton X-100 overnight at 4°C. To prevent unspecific binding, slices were washed with 0.05% Tween-20 in tris-buffered saline (TBS-T) (3 x 10 minutes) and then pre-incubated in 3% bovine serum albumin in TBS for 3 hours. Slices were then incubated in mouse monoclonal GFAP antibody, 5C10 (Novus Biologicals) at the dilution of (1:500) in TBS for 2 days and overnight at 4°C, respective to their thicknesses. All sections were washed (3 x 10 minutes) in TBS-T and incubated in Alexa Fluor

546 Goat Anti-Mouse (1:500) for 2 hours at RT. Rinsed slices were mounted on poly-lysine coated slides with Pro-Long Gold Antifade-Mounting media (Thermo Fisher Scientific) with 0.17 mm thick (#1.5) coverslips. Edges of coverslips were sealed with nail polish. Slides were stored at -20°C until confocal microscopy visualization.

Gold nanoparticle preparation

100 nm gold nanoparticles (GNP) were purchased from Nanocomposix under the catalog # AUCN100-25M and lot number of HKE0007. According to manufacturer's certificate of analysis, GNPs dimensions were measured at 103 ± 10 nm using transmission electron microscope and around 90% of particles are in the range of 100-120 nm. Gold nanoparticles were supplied in aqueous 2mM sodium citrate; an aliquot of 100 microliter of this solution was dispensed in the center of a Zeiss high performance cover glass (No. 1 1/2, Catalog # 22 x 22 mm-0107052) and kept in a fume hood and allowed to dry. A very small droplets of a quick dry translucent nail polish is applied on four corners and a microscopic slide is brought on the cover glass and left in the hood until completely dried. The sample is imaged through the cover glass slide for GNP reflection measurements.

Confocal reflection and fluorescence microscopy

Samples of GNP and GC stained brain slides were imaged under a Zeiss LSM 880 Laser Scanning Microscope set up on an AxioObserver stand (Carl Zeiss, Obercohen, Germany). A continuous wave laser of 405 nm was used to image GNP particles as well as GC metallic stain and a 561 nm laser was used to excite Alexa 568 labeled GFAP antibodies targeted to astrocytes as shown in the light path diagram in Figure 2.1. While the 400-410 nm bandwidth centering 405

reflection was collected with 405 laser source for CRSR, 565-615 nm bandwidth of emission signal collected for GFAP fluorescence from Alexa 568 was excited by the 561 nm laser. One could also use longer wavelengths for CRSR such as 488, 561 and 633 in the absence of 405 nm UV line in the system, but the resolution would be lower with increasing excitation wavelength (Table 2.1) according to the Abbe's diffraction limit $0.61 \cdot \lambda / \text{NA}$ principle³⁸. As indicated in Fig. 2.1, the guiding mirror was removed to allow light to be reflected on the main beam splitter (MBS) at a 90° angle to collect the maximum excitation light of the same wavelength that illuminated the samples. We used throughout a Plan- Apochromat 63x/ 1.40 NA Oil for CRSR modality and two other objectives for contextual visualization (larger fields of view with lower resolution) such as a Plan-Apochromat 10x/ 0.45 NA and a Plan-Apochromat 20x/0.8 NA. The MBS 690+ was used as a main beam splitter for CRSR modality to avoid laser suppression from MBS. For GFAP fluorescence, MBS 561 was used, to optimally reflect the 561 laser line. The guiding mirror was used to send the light at a 45° angle to avoid reflection from the excitation light in the emission path in fluorescence detection (Fig. 2.1). Digital zoom was set at 3.2x when imaging under 63x magnification. Master gain at the GaAsP photomultiplier was kept at 516 and 522 (lowest gain setting is 500) for 1 and 0.1 AU. Pinhole size for acquisition was set at 1AU (38 microns) and 0.1AU (4 microns). GFAP fluorescent signals were acquired under 1 AU pinhole size. These settings are very close to the lowest laser power and gain in the system and indicate that in the reflection mode obtaining an image without saturating the pixels is a challenge; the range indicator in the program was used to make sure the pixels are not saturated. A stack (40-50 slices) of optical sections were acquired at 0.10 μm (Z steps) increments with XY pixel dimensions of 0.04 x 0.04 microns were obtained to reveal the 3D structure of the GC neuron staining. For all acquisitions, images were integrated with a 1.03 μs pixel dwell time

with a raw data dimension of 42 x 42 x 4.9 microns from a 1024 x 1024 in XY and ~49 slices in Z (Voxel sizes of the images are 0.04 x 0.04 x 0.1 microns in X, Y and Z axes, respectively. The signals were integrated in 8 bit, line average= 4, digital gain= 1.00 with a digital offset set at 0.00.

3D Rendering and 2D and 3D Image Analysis

All images were processed for line intensity profile analysis in the programs Zen (LSM 880 confocal microscope manufacturer's software) or Autoquant (Deconvolution software). 3D image visualization of GC and quantification of dendrites morphometric parameters was done using IMARIS (Bitplane Inc., Zurich, Switzerland). Images of GNPs and GC speckles were deconvolved using the Autoquant blind deconvolution algorithm to test the impact of deconvolution in addition to the smaller pinholes. FWHM values of individual GC speckles or gold-nanoparticles in both XY and XZ dimensions were generated by conducting line profile analysis in Zeiss' Zen software and/or ImageJ (NIH open source software ³⁹). Image properties were calibrated to appropriate raw data voxel dimensions as described above. For statistical purposes, FWHM measurements were made on at least 4-6 distinct locations of each objects (GNP, GC speckle, GC dendrite) to confirm the statistical range of resolution improvement. The mean of means and standard error of means are presented. The images were finally composed in the program Adobe Photoshop with appropriate intensity adjustments in most cases min/max or when gamma is adjusted to 0.45. As mentioned above, 3D rendering and surfaces were created using the program IMARIS under the Surpass mode (Bitplane Inc., Zurich, Switzerland). 3D projections were generated from individual optical sections as described before ⁴¹. Using the filament tracer algorithm, a new filament was manually traced and created using the Autopath

mode ⁴². A single dendrite starting point was assigned at the edge of the ROI. Automatic thresholds were used for assigning dendrite end points and dendrite surface rendering. The minimum dendrite end diameter, maximum spine length, and minimum spine end diameter were measured in 2D slicer view and entered into the filament tracer algorithm. Automatic thresholds were also used for generating spine seed points and surface rendering. Filament statistics were exported into Microsoft Excel program and finally graphed in the program SigmaPlot 13 (Systat Inc., San Jose, CA). Representative images were presented after adjusting and optimizing the intensities in a similar way between 0.1 AU and 1AU in the program IMARIS as well as in Photoshop for visualization purposes without pixel saturation.

Statistical analysis

To determine the statistical significance of the data between 1 and 0.1 AU settings, GraphPad prism or Sigmaplot was used to conduct paired student t-tests.

2.3 RESULTS

Imaging GC under CRSR and conventional transmitted light at varying resolution

Figure 2.2 shows images of GC-stained murine hippocampal neurons acquired using the CRSR and transmitted light modalities under increasing magnifications, depth of field and resolution. Plan Apochromat objectives of 10x, 0.3 NA (Fig. 2.1 A-B), 20x, 0.8 NA (Fig. 2.2 C-D), and a 63x, 1.4 NA with a digital zoom of 3.2 (Fig. 2.2 E-F) were used to understand the quality of GC-stained neurons with increasing magnifications and numerical apertures. Fig. 2.2 B, D and F show the CRSR images while A, C and E show transmitted light images. Though using a 633 nm laser line has been shown to be better than 405 and 488 to avoid autofluorescence and reduce

scattering and absorption of a given sample ^{6,30}, we chose to use a 405 nm laser line to gain the maximum resolution (Table 2.1).

Evaluation of CRSR technique performance with gold nanoparticles and GC speckles

To understand the resolution improvement, a spherical object such as a fluorescent beads of sub-diffraction limit is routinely used to evaluate and test the optical system's point spread functions (PSF) under a given modality ^{23,44-46}. Here, we used 100 nm gold nanoparticles (GNPs) as such point objects. The metallic nature of GNP is ideal to test the resolution limit of reflection based CRSR technique. In addition, we also used a second fine point like target (unknown size) of GC metallic labels adjacent to neurons in the sample itself as point objects. Lateral and axial resolution of both targets were evaluated under CRSR technique in this study at pinhole settings of both 1 and 0.1 AU (Fig. 2.3). When comparing the central focal plane of the acquired Z stack in both cases, lateral resolution improved 32% and 26% in GNP and GC targets, respectively (Fig. 2.3 C and J). In addition, closing the pinhole clearly reduced the cover glass derived reflection fringes seen as ring patterns as evidenced from the obtained images in two settings (Fig. 2.3 A and B). To summarize, 0.1 AU produced relatively smaller PSFs and better band width compared to its 1 AU counterpart (Fig. 2.3 D, F, K, M). These improvements in smaller PSFs and better retrieved FT frequencies were more pronounced in GC data (Fig. 2.3 E, G, L and N), affirming that the CRSR technique effectively increases resolution of GC staining. In addition to the line profiles and full width at half-maximum (FWHM) shown in graphs in Fig. 2.3, we also provided a consolidated performance analysis of differences and % resolution improvement in CRSR technique in 1 AU and 0.1 AU settings of GNPs, GCs and GC dendritic spines of multiple locations presented with statistical analysis in Table 2.1.

Simultaneous visualization of CRSR-acquired Golgi-Cox stained neurons and fluorescently tagged astrocyte

After evaluating the resolution performance of CRSR using point objects with reflection properties, we compared the resolution of GC staining in actual brain slices under the same pinhole setting used for 1 AU and 0.1 AU (Fig. 2.4 A–D). In addition, we have also labeled the samples with fluorescently tagged astrocytes using a standard immunofluorescence staining method (pseudo-colored red) after completing GC staining in the same sample in a sequential manner. We imaged these astrocytes (pseudo-colored red) using routine confocal fluorescence microscopy technique together with GC-stained neurons (pseudo-colored green) using the CRSR technique, simultaneously in the same location. The two z-stacks were then merged to visualize the spatial distribution and localization of two signals from different modalities (Fig. 2.4 E–F’).

Precision of dendritic spine quantification enhanced by resolution and contrast improvement in CRSR technique

The logical next step was to examine quantitatively whether the resolution improvement from CRSR helped improve the accuracy of neuron-derived data from brain slices labeled with GC, such as changes in neuronal dendritic spine data. To answer this question, we present Figure 2.5, which shows data derived from five independent hippocampal neuron dendrite segments visualized using the CRSR 0.1 AU and 1 AU settings. To reconstruct the complete morphology of each dendrite segment, we used the Filament Tracer, an algorithm in the program IMARIS (as described above) that has been used and tested to quantify and characterize neuronal dendritic spines^{42,58}. Figure 2.5 A and B shows that spine density was overestimated by approximately 200% under the 1 AU setting in comparison to the CRSR 0.1 AU. As shown in Figure 2.5 C,

dendritic spines reconstructed from z-stacks acquired under the 1 AU setting has a statistically significant 2-fold increase in area size. Similarly, we found that the mean diameter of dendritic spines reconstructed from z-stacks under the 1 AU setting had a statistically significant 1.5-fold increase in mean diameter (Figure 2.5 D and E).

Using a post-acquisition deconvolution routine to further improve the CRSR resolution

As a final processing step, we applied blind deconvolution to GC images (0.1 AU) obtained from CRSR and compare them with 0.1 AU data without this step both laterally and axially (Fig. 2.6 and Table 2.1). We show that the added blind deconvolution routine to 0.1 AU setting data improved the resolution laterally and axially and conferred up to two-fold enhancement over the diffraction limit (Fig. 2.6) compared with a conventional confocal setting (220 nm). In addition to resolution improvement in CRSR that led to sub 100 nm resolution (Table 2.1 and Fig. 2.6 D and H) in FWHM of the deconvolved 0.1 AU datasets, the SNR in these data sets was also substantially improved (Fig. 2.6 C, D, G, and H). In Table 2.1, we summarize the results of 0.1 AU with and without deconvolution for the 405 nm laser used in this study and project how this will be slightly different when longer wavelengths are used. Overall, the data indicate an additional 25-30% improvement in resolution. These improvements are possible only if the images are acquired carefully without any saturation, which is one of the fundamental requirements for deconvolution^{46, 56, 66, 67} and as indicated in materials and methods, it is easy to saturate the reflection signal.

2.4 DISCUSSION

While comparing the two modalities, the presented data reveal that the optical sectioning ability of CRSR reduced out of focus blur, increased SNR, and improved resolution with increasing magnification, numerical aperture and smaller depth of fields. Since the resolution is improved while minimizing the pinhole at the cost of losing signal (see Introduction), higher laser power was required to visualize GNP and GC under the 0.1 AU pinhole setting in contrast to the 1 AU settings. Hence, laser power was adjusted (increased ~10 fold) while acquiring all 0.1 AU setting images irrespective of sample target. Resolution improvements in CRSR were also evident in maximum intensity projected multiple particles of GNPs, GC speckles and GC dendritic spots (Table 2.1). Results indicate that the CRSR technique at 0.1 AU setting consistently produced higher resolution anywhere from 21% improvement of GC dendrites to 37% improvement of GNPs resolution in terms of FWHM. The same target GC dendrites showed 31% improvement of 1 AU in axial resolution (XZ) and the GNPs yielded 36%, indicating that the extent of resolution improvement is better and higher in the Z dimension compared to XY, which is also supported by higher frequency retrieval in the FT images in axial dimension (Fig. 2.3 G and N; Table 2.2). These results are consistent with the in-focus optical transfer functions reported previously and the resolution improvement with smaller pinholes^{17, 18, 22, 24, 44, 47, 48}.

Technically, one can combine CRSR imaging with multiple fluorescent targets using 488, 561 and 633 nm laser excitations with the application of appropriate second antibodies with fluorophores excitable at those wavelengths. We present the data for one probe (GFAP) for demonstration purposes. From the presented results in figure 2.4, it is evident that the CRSR technique provided images with both better resolution and SNR in both XY and XZ dimensions,

closely following the resolution improvements recorded from GNPs and GC speckles (Fig. 2.3 and Table 2.1). Further, images generated using CRSR can be combined with fluorescence visualization to offer a powerful visualization technique to study cell-to-neuron interactions (Fig. 2.4 E-F').

Dendritic spines are neuronal projections that receive presynaptic signals from other neurons, providing feedforward excitatory input to the cell. GC staining is commonly used to extract these structural features of dendritic spine population and characterization which provide valuable clues regarding neuronal function⁵⁹⁻⁶¹. Spine area, size and diameter are key parameters in determining the functional characterization of dendritic spines⁶⁰⁻⁶³. In the hippocampus CA1 region, crucial relationships have been revealed between the dimensions of dendritic spines and its functional properties. As such, studies have shown that the area of active zones per synapse is proportional to spine volume⁶³⁻⁶⁵. Using the CRSR imaging modality to visualize Golgi-Cox stained dendrites, we show that spine density and morphology can be more accurately obtained via IMARIS automated quantitation.

Taken together, utilizing the optical sectioning ability of the confocal and the ability to keep the background dark to improve contrast, resolution and SNR, the presented CRSR technique is useful in providing 3D information of the metal-stained biological structures. By closing the pinhole size of the confocal microscope from 1AU to 0.1AU and adding a deconvolution routine, we achieved around two-fold improvement GC lateral and axial resolution using the CRSR technique. We hope that this improved resolution of GC will be useful in future investigations that concern changes in detailed neuron morphology that occur in the context of central nervous system diseases such as multiple sclerosis and Alzheimer's disease.

2.5 FIGURES AND TABLES

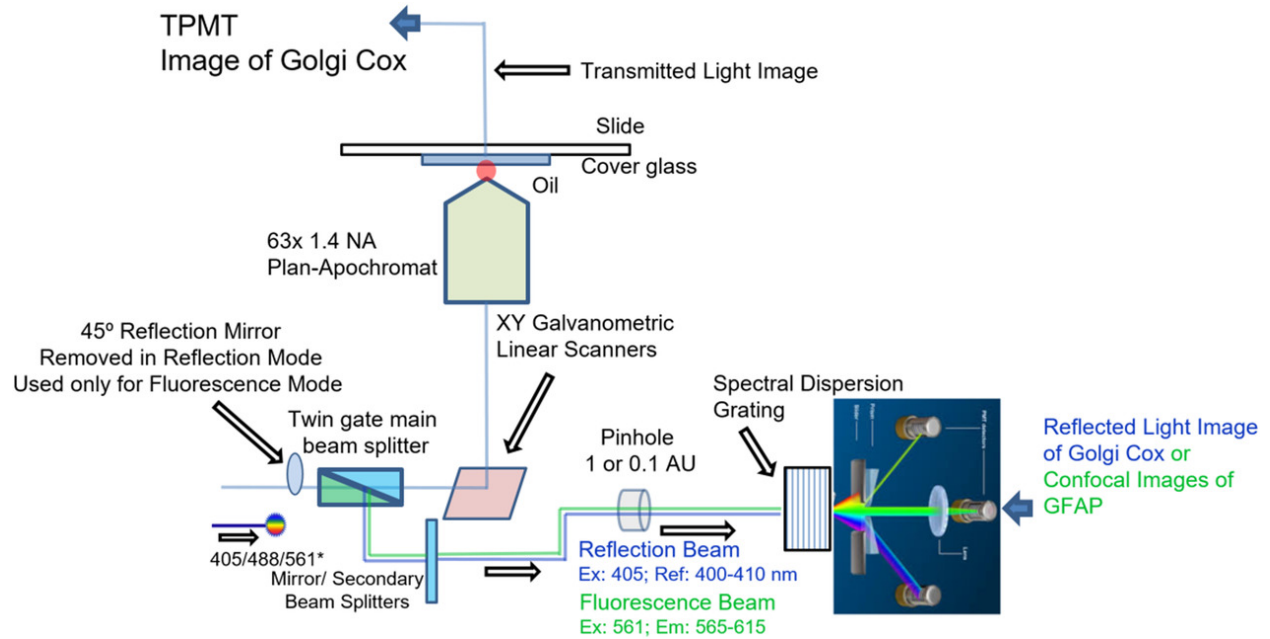


Fig 2.1. Light path diagram for CRSR and transmitted light. Schematic description of the light path and the optical components used in a confocal microscope LSM 880 for CRSR imaging. The 45 degree reflection mirror in front of the main beam splitter is the key for the CRSR technique. By removing this mirror, the excitation light hit the MBS at 90 degree angle, which allows the same wavelength to be detected using a different MBS which will not cut off 405nm. In fluorescence mode, this mirror will be used and an appropriate MBS 405 will be selected to suppress the excitation light in the emission path. In dual labeled samples, the same MBS for fluorescence (at 488, 561 and 633) could be used as well as for the 405 reflection beam sequentially without any hard ware movement.

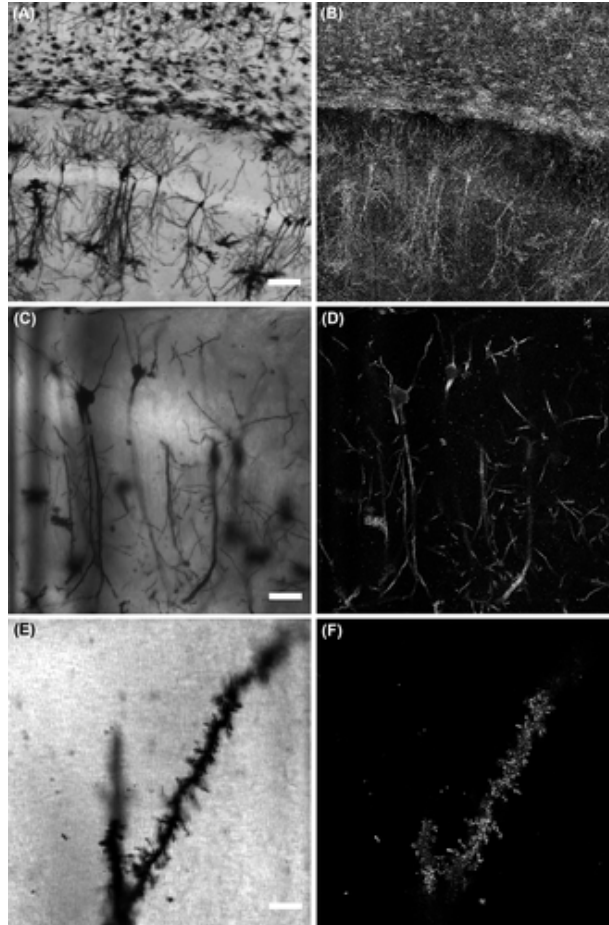


Fig 2.2. Imaging GC under conventional transmitted light and CRSR modalities at increasing magnifications. (A, C, E) Transmitted light field images of GC staining in murine hippocampal region under increasing magnifications (10x 0.3 NA, 20x 0.8 NA, and 63x 1.4 NA objective with 3.2x digital zoom). (B, D, F) CRSR images of the same focal plane. Note: out of focus light was eliminated with increasing numerical aperture and decreasing depth of field. For example, compare E and F, the dendrite at 90 degrees visible in E in the transmitted light but is not visible in F. In addition, having a dark background in CRSR images gives inherent increase in contrast. All images are acquired using 405 nm laser light. Scale bar is 100 μm for (A and B), 50 μm for (C and D) and 10 μm for (E and F).

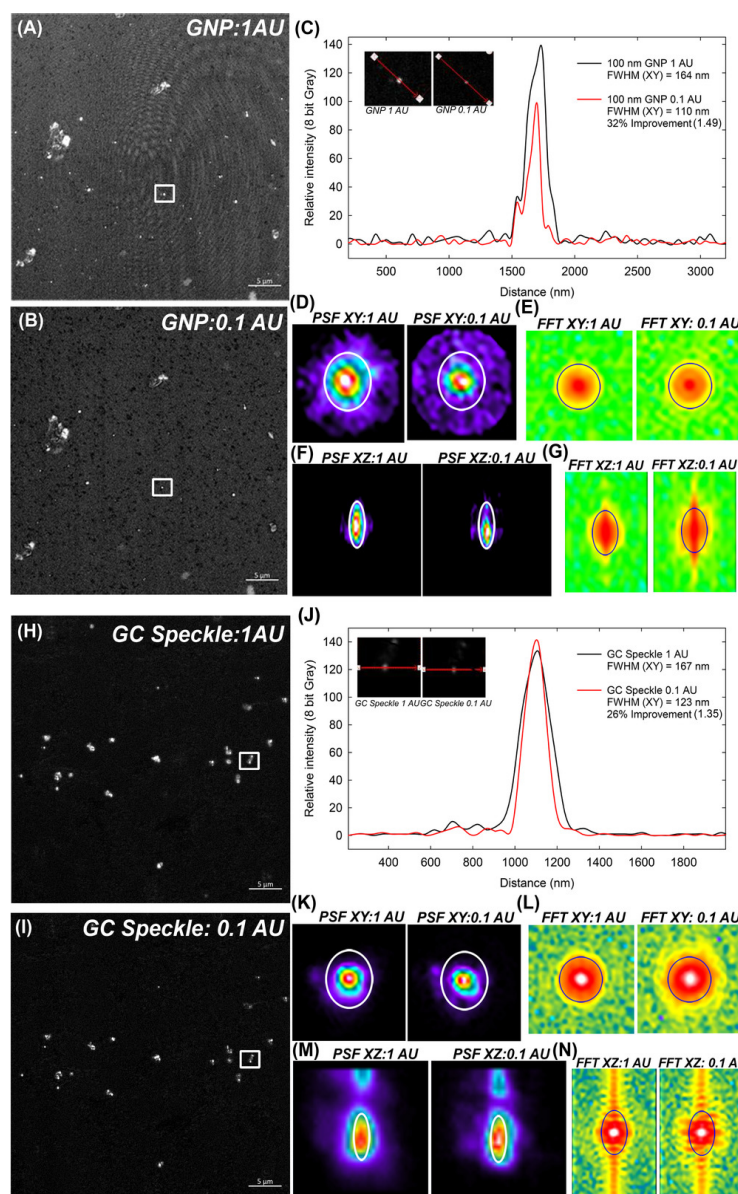


Fig 2.3. Evaluation of CRSR technique performance with PSFs of gold nanoparticles and GC Speckles. All images are acquired with 63x Plan Apochromat 1.4 NA objective with 3.2 times zoom. (A) Center focal plane (optical section) of a Z stack from GNP acquired under 1 AU setting. (B) Same field of view of GNPs at center focal plane of acquired Z Stack under 0.1 AU pinhole setting. (C) Line profile analysis of the GNP particle inside the white box in A and B also showed in insets and their indicated FWHMs. Experimental PSFs created from the same representative GNPs (white boxes in A and B) and their PSF shape in XY and XZ (D and F) and their respective Fourier transforms (FTs) (E and G). For comparison of PSF and FTs a circle (on XY images) or ellipse (on XZ images) are drawn covering the intensities above background (purple) on 1 AU PSF (circle) and its FTs (Ellipse-covering most frequencies in FTs). Then the same circle and ellipse from 1 AU PSF and FTs are copied over to all PSF and FT images from 0.1 AU. Similar analyses were done on the GC speckle images (H to N). Images A-B and H-I pairs are set at same min/max intensity levels. Scale bars A, B, H and I are 5 μm .

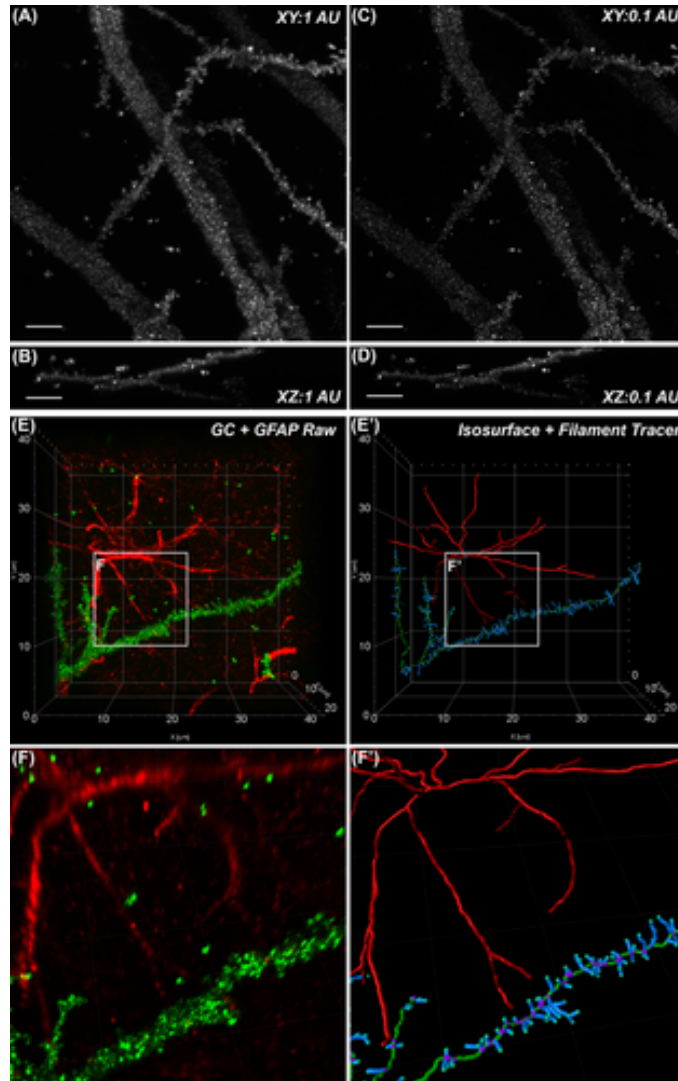


Fig 2.4. Resolution and SNR improvement in CRSR technique and simultaneous visualization of GC and GFAP. (A, C) XY and (B, D) XZ views of GC-stained hippocampal neuron dendrites visualized using the confocal reflection technique with 1 AU and 0.1 AU settings under high magnification of 63x with 3.2x digital zoom. Scale bars in A-D represents 10 μm . (E) Representative images of combination of GC (0.1 AU, pseudo-colored green) and the GFAP confocal fluorescence (1.0 AU, psuedocolored red) images of raw data where all optical sections are used to create a maximum intensity projected 3D image. (E') IMARIS-filament tracer rendered image of (E) with isosurface algorithm after thresholding. Noise and out of focus pieces neurons (in the bottom right corner of (E)) are filtered out for clarity. The white boxes indicate the images for F and F'. F is the raw data of the combined channel as in E and F' is the same as in E' but zoomed in to better visualize the spines and GFAP labeled neuron interaction and proximity. The image scale is shown in labeled grids in E and E'.

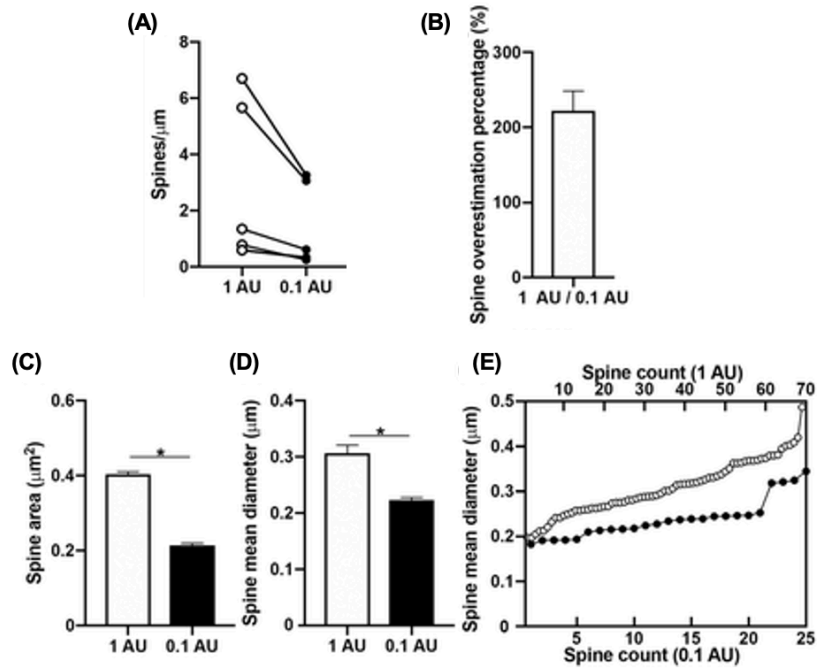


Fig 2.5. CRSR technique results in improved accuracy in dendritic spine quantification and characterization compared to conventional confocal setting of 1 AU. (A) Quantification of rendered dendritic spine density (spine count per μm dendrite length). (B) % overestimation of dendritic spine density in 1 AU while using 0.1 AU as baseline, N = 5. (C) Quantification of spine area between the 1 AU and 0.1 AU. (D) Quantification of spine mean diameter between the 1 AU and 0.1 AU. (E) Representative frequency plot of spine mean diameter Vs spine count of one dendrite showing 0.1 AU almost one third of spine count compared to 1 AU together with much lower spine diameter. Scale bars in A-D represents 0.4μm. Error bars represent means ± SEM. *, P < 0.05; Student's t test.

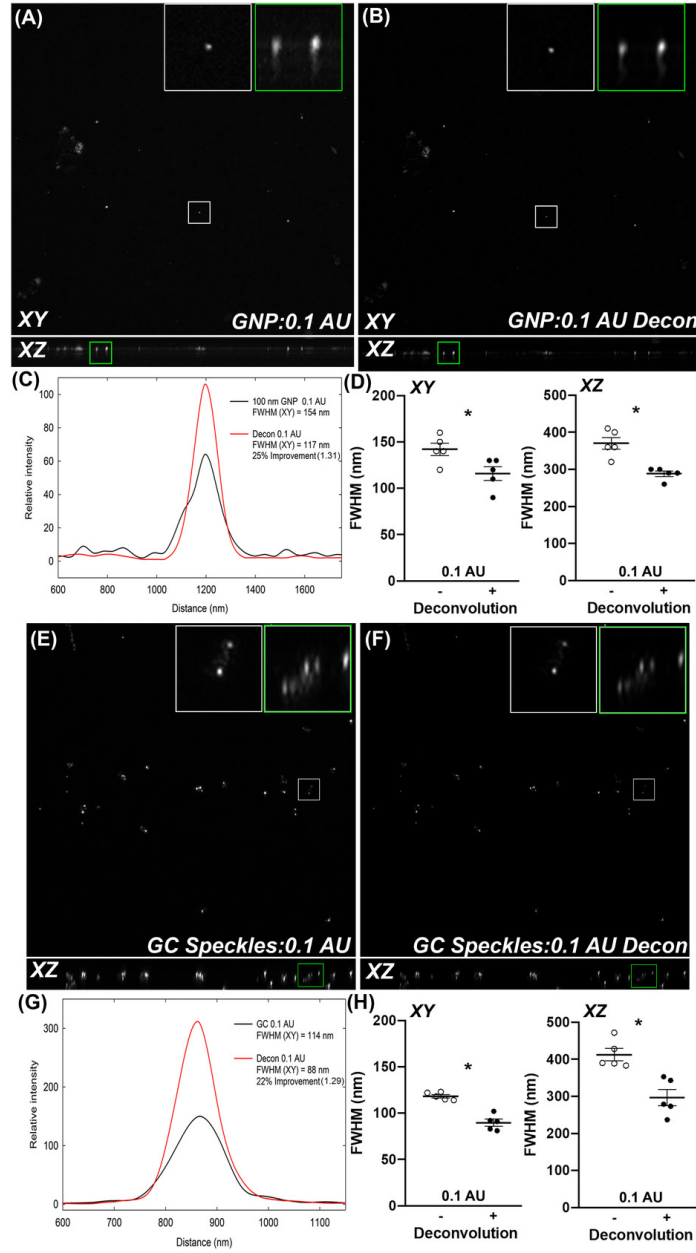


Fig 2.6. Impact of a deconvolution routine on resolution and SNR of 0.1 AU GNP and GC Speckles data sets. For better comparison, the Z stacks from the same data sets from Fig. 2.3 B (GNPs 0.1 AU) and I (GC speckles 0.1 AU) were used for the deconvolution using a blind deconvolution routine. (A-D) images are from GNPs and (E-H) are from GC speckles at indicated XY, XZ dimensions. The line profiles in C and G indicate the FWHM calculated from particles highlighted in white lines across a specific set of particles in A and E. (D and H) showing statistical analysis of over 4-5 particles quantified for assessing the improvement in FWHM in both lateral and axial directions before and after deconvolution. Representative FWHM values are represented in Table 2.1 as well for 405 nm and compared with the influence of longer wavelength on resolution. Scale bar in A is 5 μm . Error bars represent means $\pm$ SEM. *, $P < 0.05$; Student's t test.

Wavelength (nm)	Abbe's resolution limit (nm) (% reduction, factor)	0.1 AU GNP (nm)	0.1 AU GNP + deconvolution (nm)	0.1 AU GC speckles (nm)	0.1 AU GC speckles + deconvolution (nm)
405 [*]	176	142 [*]	116 [*]	118 [*]	90 [*]
488	212 (20%, 0.83)	170	139	142	108
561	244 (38%, 0.72)	196	160	163	124
633	275 (56%, 0.64)	222	181	184	140

Table 2.1. Potential impact of using longer wavelengths on CRSR resolution based on Abbe's resolution limit. The extent of influence is also compared with and without deconvolution in final resolution of CRSR is also calculated*. See also Fig. 2.6.

Dimension	Target	1 AU Resolution (FWHM in nm)	0.1 AU Resolution (FWHM in nm)	Resolution (FWHM) improvement% (factor)	<i>p</i> Value (paired <i>t</i> - test)
XY – single optical section	GNP	192 ± 17 (<i>N</i> = 6)	121 ± 5 (<i>N</i> = 6)	37% (1.58)	0.001*
XY – MIP	GNP	180 ± 10 (<i>N</i> = 5)	140 ± 9 (<i>N</i> = 5)	22% (1.28)	0.003*
XZ – MIP	GNP	550 ± 82 (<i>N</i> = 5)	350 ± 28 (<i>N</i> = 5)	36% (1.51)	0.045*
XY – single optical section	GC speckle	191 ± 9 (<i>N</i> = 5)	129 ± 9 (<i>N</i> = 5)	32% (1.48)	0.002*
XY – MIP	GC speckle	150 ± 9 (<i>N</i> = 5)	110 ± 7 (<i>N</i> = 5)	27% (1.40)	0.007**
XZ – MIP	GC speckle	600 ± 45 (<i>N</i> = 4)	450 ± 24 (<i>N</i> = 4)	25% (1.33)	0.050*
XY – MIP	GC dendrite	205 ± 15 (<i>N</i> = 6)	161 ± 12 (<i>N</i> = 6)	21% (1.27)	0.042*
XZ – MIP	GC dendrite	728 ± 44 (<i>N</i> = 5)	503 ± 32 (<i>N</i> = 5)	31% (1.44)	0.004**

Table 2.2. Comprehensive statistical analysis of resolution improvement and performance of CRSR technique on GNP and GC targets of interest.

2.6 REFERENCES

1. C. J. Sheppard, Super-resolution in confocal imaging. *Optik* 80, 53-54 (1988).
2. S. W. Hell, J. Wichmann, Breaking the Diffraction Resolution Limit by Stimulated-Emission - Stimulated-Emission-Depletion Fluorescence Microscopy. *Optics Letters* 19, 780-782 (1994).
3. M. G. Gustafsson, Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J Microsc* 198, 82-87 (2000).
4. B. P. Azeredo et al., Direct Imprinting of Porous Silicon via Metal-Assisted Chemical Etching. *Adv Funct Mater* 26, 2929-2939 (2016).
5. M. Sivaguru et al., Application of an advanced maximum likelihood estimation restoration method for enhanced-resolution and contrast in second-harmonic generation microscopy. *J Microsc* 267, 397-408 (2017).
6. M. Sivaguru et al., Geobiology reveals how human kidney stones dissolve in vivo. *Sci Rep-Uk* 8, (2018).
7. J. S. Ploem, Ed., Reflection-contrast microscopy as a tool for investigation of the attachment of living cells to glass surface, (Blackwell, Oxford, 1975), pp. 405-421.
8. I. Cornelesetenvelde, J. Wiegant, H. J. Tanke, J. S. Ploem, Improved Detection and Quantification of the (Immuno) Peroxidase Product Using Reflection Contrast Microscopy. *Histochemistry* 92, 153-160 (1989).
9. H. K. Koerten, J. S. Ploem, W. T. Daems, Ingestion of Latex Beads by Filopodia of Adherent Mouse Peritoneal-Macrophages - a Scanning Electron Microscopical and Reflection Contrast Microscopical Study. *Exp Cell Res* 128, 470-475 (1980).

10. H. K. Koerten, J. S. Ploem, W. T. Daems, Sem and Reflection Contrast Study of the Filopodia of Adherent Mouse Resident Peritoneal Macrophages. *Ultramicroscopy* 4, 150-150 (1979).
11. J. S. Ploem, F. A. Prins, Reflection-CONtrast Microscopy-Review. *Infocus* September, 38-56 (2017).
12. F. A. Prins, I. C. Velde, E. de Heer, Reflection contrast microscopy: The bridge between light and electron microscopy. *Methods Mol Biol* 319, 363-401 (2006).
13. F. A. Prins, R. Vandiemensteenvoorde, J. Bonnet, I. Cornelesetenvelde, Reflection Contrast Microscopy of Ultrathin Sections in Immunocytochemical Localization Studies - a Versatile Technique Bridging Electron-Microscopy with Light-Microscopy. *Histochemistry* 99, 417-425 (1993).
14. G. J. Brakenhoff, P. Blom, P. J. Barends, C. Bakker, High-Resolution Confocal Scanning Light-Microscopy. *Ultramicroscopy* 4, 115-115 (1979).
15. G. J. Brakenhoff, H. T. M. Vandervoort, N. Nanninga, High-Resolution Confocal Scanning Light-Microscopy in Biology. *Anal Chim Acta* 163, 231-236 (1984).
16. G. J. Brakenhoff, M. Muller, A new approach towards improved fluorescence imaging beyond confocal resolution. *P Soc Photo-Opt Ins* 2655, 271-279 (1996).
17. I. J. Cox, C. J. Sheppard, T. Wilson, Improvement in resolution by nearly confocal microscopy. *Appl Opt* 21, 778-781 (1982).
18. I. J. Cox, C. J. R. Sheppard, T. Wilson, Super-Resolution by Confocal Fluorescent Microscopy. *Optik* 60, 391-396 (1982).
19. J. Huff, The Airyscan detector from ZEISS: confocal imaging with improved signal-to-noise ratio and super-resolution. *Nature Methods* 12, 1205 (2015).

20. E. Betzig et al., Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313, 1642-1645 (2006).
21. J. B. Pawley, *Handbook of biological Confocal Microscopy*. (Springer Science, New York, NY, 2006).
22. T. Wilson, in *Handbook of Biological Confocal Microscopy*, J. B. Pawley, Ed. (Plenum Press, New York, 1995), pp. 167-182.
23. M. Sivaguru, L. Mander, G. Fried, S. W. Punyasena, Capturing the Surface Texture and Shape of Pollen: A Comparison of Microscopy Techniques. *Plos One* 7, (2012).
24. J. B. Pawley, in *Handbook of Biological Confocal Microscopy*, J. B. Pawley, Ed. (Springer, New York, NY, 2006), chap. 2, pp. 20-41.
25. G. J. Brakenhoff, H. T. M. Vandervoort, K. Visscher, *Confocal Microscopy for the Biological and Material Sciences - Principle, Applications, Limitations*. *P Soc Photo-Opt Ins* 1439, 121-127 (1992).
26. S. Y. Kim, M. Sivaguru, G. Stacey, Extracellular ATP in plants. Visualization, localization, and analysis of physiological significance in growth and signaling. *Plant Physiol* 142, 984-992 (2006).
27. I. Cornelesetenvelde, F. A. Prins, New Sensitive Light Microscopical Detection of Colloidal Gold on Ultrathin Sections by Reflection Contrast Microscopy - Combination of Reflection Contrast and Electron-Microscopy in Postembedding Immunogold Histochemistry. *Histochemistry* 94, 61-71 (1990).
28. E. C. M. Hoefsmit, C. Korn, N. Blijleven, J. S. Ploem, Light Microscopic Detection of Single 5 and 20 Nm Gold Particles Used for Immunolabeling of Plasma-Membrane Antigens

with Silver Enhancement and Reflection Contrast. *Journal of Microscopy-Oxford* 143, 161-169 (1986).

29. J. N. Turner, D. H. Szarowski, J. S. Deitch, K. L. Smith, J. W. Swann, in XIIth International Congress on Electron Microscopy, P. L. D., D. B. Williams, Eds. (1990), vol. 3, pp. 152-153.

30. D. H. Szarowski et al., Optimized Reflection Imaging in Laser Confocal Microscopy and Its Application to Neurobiology - Modifications to the Biorad Mrc-500. *Scanning* 14, 104-111 (1992).

31. C. Golgi, Sulla struttura della sostanza grigia dell cervello. *Gazz. Med. Lombarda* 33, 244-246 (1873).

32. M. Ai et al., Fluorescence imaging of dendritic spines of Golgi-Cox-stained neurons using brightening background. *Journal of Biomedical Optics* 20, (2015).

33. S. Spiga et al., Simultaneous Golgi-Cox and immunofluorescence using confocal microscopy. *Brain Struct Funct* 216, 171-182 (2011).

34. J. J. Mancuso, Y. Chen, X. Li, Z. Xue, S. T. C. Wong, Methods of Dendritic Spine Detection: From Golgi to High-Resolution Optical Imaging. *Neuroscience* 251, 129-140 (2013).

35. V. Centonze, J. B. Pawley, in *Handbook of biological confocal microscopy*, J. B. Pawley, Ed. (Springer, New York, 2006), chap. 35, pp. 627-648.

36. M. Sivaguru, Special Issue: Intact Organs: Super Resolution Multimodal Optical 4D Imaging Preface. *Microsc Res Techniq* 81, 99-100 (2018).

37. V. L. Kolossov et al., Airyscan super-resolution microscopy of mitochondrial morphology and dynamics in living tumor cells. *Microsc Res Techniq* 81, 115-128 (2018).

38. E. Abbe, Beitrage zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung. . Arkiv. Mikroskop. Anat. 9, 413-468 (1873).
39. C. T. Rueden et al., ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics 18, 529 (2017).
40. D. W. Crome, Avoiding Twisted Pixels: Ethical Guidelines for the Appropriate Use and Manipulation of Scientific Digital Images. Sci Eng Ethics 16, 639-667 (2010).
41. A. Goldraj et al., Compartmentalization of S-RNase and HT-B degradation in self-incompatible Nicotiana. Nature 439, 805-810 (2006).
42. S. A. Swanger, X. Yao, C. Gross, G. J. Bassell, Automated 4D analysis of dendritic spine morphology: applications to stimulus-induced spine remodeling and pharmacological rescue in a disease model. Mol Brain 4, 38 (2011).
43. J. S. Deitch, K. L. Smith, C. L. Lee, J. W. Swann, J. N. Turner, Confocal Scanning Laser Microscope Images of Hippocampal-Neurons Intracellularly Labeled with Biocytin. J Neurosci Meth 33, 61-76 (1990).
44. R. Juskaitis, in Handbook of Biological CONfocal Microscopy, J. B. Pawley, Ed. (Springer, New York, NY, 2006), chap. 11, pp. 239-250.
45. M. Sivaguru et al., Comparative performance of airyscan and structured illumination superresolution microscopy in the study of the surface texture and 3D shape of pollen. Microsc Res Techniq 81, 101-114 (2018).
46. H. T. M. Vandervoort, K. C. Strasters, Restoration of Confocal Images for Quantitative Image-Analysis. Journal of Microscopy-Oxford 178, 165-181 (1995).
47. T. Wilson, The Role of the Pinhole in Confocal Imaging-Systems. Handbook of Biological Confocal Microscopy, Revised Edition, 113-126 (1989).

48. T. Wilson, Resolution and optical sectioning in the confocal microscope. *J Microsc-Oxford* 244, 113-121 (2011).
49. H. Nishida, S. Okabe, Direct astrocytic contacts regulate local maturation of dendritic spines. *J Neurosci* 27, 331-340 (2007).
50. R. Nitsch et al., Direct impact of T cells on neurons revealed by two-photon microscopy in living brain tissue. *J Neurosci* 24, 2458-2464 (2004).
51. K. Vints et al., Modernization of Golgi staining techniques for high-resolution, 3-dimensional imaging of individual neurons. *Sci Rep-Uk* 9, (2019).
52. M. S. Kassem, S. Y. Y. Fok, K. L. Smith, M. Kuligowski, B. W. Balleine, A novel, modernized Golgi-Cox stain optimized for CLARITY cleared tissue. *J Neurosci Meth* 294, 102-110 (2018).
53. A. Ranjan, B. N. Mallick, A modified method for consistent and reliable Golgi-cox staining in significantly reduced time. *Front Neurol* 1, 157 (2010).
54. S. Zaqout, A. M. Kaindl, Golgi-Cox Staining Step by Step. *Front Neuroanat* 10, 38 (2016).
55. M. Sivaguru et al., Enhancing Resolution and Contrast in Second-Harmonic Generation Microscopy Using an Advanced Maximum Likelihood Estimation Restoration Method. *Proc Spie* 10069, (2017).
56. D. S. C. Biggs, Deconvolution of Fluorescence Microscope Imagery. *Microsc Microanal* 15, 1528-1529 (2009).
57. D. S. C. Biggs, M. Andrews, Conjugate-Gradient Acceleration of Maximum-Likelihood Image-Restoration. *Electron Lett* 31, 1985-1986 (1995).

58. D. Dumitriu, A. Rodriguez, J. H. Morrison, High-throughput, detailed, cell-specific neuroanatomy of dendritic spines using microinjection and confocal microscopy. *Nat Protoc* 6, 1391-1411 (2011).
59. C. Sala et al., Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* 31, 115-130 (2001).
60. K. M. Harris, S. B. Kater, Dendritic Spines - Cellular Specializations Imparting Both Stability and Flexibility to Synaptic Function. *Annu Rev Neurosci* 17, 341-371 (1994).
61. Y. Hayashi, A. K. Majewska, Dendritic spine geometry: Functional implication and regulation. *Neuron* 46, 529-532 (2005).
62. W. C. Risher, T. Ustunkaya, J. Singh Alvarado, C. Eroglu, Rapid Golgi analysis method for efficient and unbiased classification of dendritic spines. *PLoS One* 9, e107591 (2014).
63. J. Vangindertael et al., Super-resolution mapping of glutamate receptors in *C. elegans* by confocal correlated PALM (vol 5, 13532, 2016). *Sci Rep-Uk* 6, (2016).
64. V. Ramirez-Leon, J. K. Utvik, Y. Takumi, E. Rinvik, O. P. Ottersen, Modes of glutamate receptor expression in postsynaptic specializations. *Biom Hlth R* 45, 11-19 (2001).
65. C. Racca, F. A. Stephenson, P. Streit, J. D. B. Roberts, P. Somogyi, NMDA receptor content of synapses in stratum radiatum of the hippocampal CA1 area. *Journal of Neuroscience* 20, 2512-2522 (2000).
66. D. S. C. Biggs, M. Andrews, Acceleration of iterative image restoration algorithms. *Appl Optics* 36, 1766-1775 (1997).
67. D. S. C. Biggs, M. Andrews, Iterative blind deconvolution of extended objects. *International Conference on Image Processing - Proceedings, Vol Ii*, 454-457 (1997).

68. J. Vangindertael et al., An introduction to optical super-resolution microscopy for the adventurous biologist. *Methods Appl Fluores* 6, (2018).

CHAPTER 3: EARLY LIFE TRAUMA TRIGGERS INTERFERON BETA RESISTANCE AND NEURODEGENERATION VIA DOWNREGULATED β 1 ADRENERGIC SIGNALING²

3.1 INTRODUCTION

Multiple sclerosis (MS) is characterized by demyelination of neurons in the central nervous system (CNS), and an estimated 2.5 million individuals currently have MS worldwide ¹. MS is thought to be a disease of diverse subtypes characterized by distinct clinical presentations, immune signatures, and reactivity and/or resistance to pharmaceutical treatments ^{2,3}. Patients with MS experience various neurological symptoms that differ in type and severity ^{3,4}. While its etiology remains debated, MS is recognized as a multifactorial heterogeneous disease with both genetic and environmental causes. To illustrate, environmental factors like Epstein-Barr virus ⁵, vitamin D exposure levels ⁶, cigarette smoking^{7,8}, obesity⁹, and stress ¹⁰ are thought to converge with genetic determinants to influence the manifestation of MS progression.

Childhood trauma or early-life trauma (ELT), characterized by events such as emotional neglect or physical abuse, increases MS susceptibility later in life along with MS relapse rates and likelihood of MS-related hospitalization ^{11,12}. Animal studies using multiple models of MS in rats and mice also indicate an association between ELT and disease severity ^{13,14,15,16}. ELT is proposed to alter immune cell properties in adults and is associated with increased risk of chronic illnesses ^{17,18,19}. However, the mechanism through which ELT alters MS disease progression

² This chapter is published in its entirety in Nature Communications. This article is available from <https://www.nature.com/articles/s41467-020-20302-0> and using DOI: <https://doi.org/10.1038/s41467-020-20302-0>

remains elusive. Importantly, existing studies did not investigate changes in disease phenotype characterized by immune cell properties that influence drug efficacy and disease symptoms.

Here we used C57BL/6J mice and the experimental autoimmune encephalitis (EAE) model to demonstrate that ELT induces not only increased severity and susceptibility in EAE but also a phenotype shift characterized by interferon- β insensitivity, spinal cord neuronal damage, in addition to membrane bound lymphotoxin and CXCR2 involvement in both male and female mice. We also observed downregulated β 1 adrenergic receptor (AR) in immune cells of ELT-EAE mice. Rescue of β 1 receptor function restored interferon- β sensitivity and protected against neuronal damage. Therefore, our results indicate that phenotype changes in mice subjected to ELT can be explained by downregulated adrenergic signaling in immune cells.

3.2 METHODS

Animals

Healthy C57BL/6J mice aged 6–8 weeks were used in this study. In most experiments, male mice were used. C57BL/6J mice and TCR2D2 mice (Jackson Laboratory, #6912) were purchased from Jackson Laboratories. *Adrb1^{fl/fl}* mice were a gift from Dr. Zigman (The University of Texas Southwestern Medical Center) under material transfer agreement (MTA)⁸³. DC-specific and T cell-specific *Adrb1^{-/-}* mice were generated by crossing *Adrb1^{fl/fl}* mice with *Itgax-Cre* (Jackson Laboratory 008068) or *Lckicre* mice (Jackson Laboratory 012837) mice, respectively. *Asc^{-/-}* mice were a gift Genentech under MTA. All mice were kept in group housing (3–5 mice per cage) in a specific pathogen-free facility with a 12-h light/dark cycle and temperature control at 20–22°C at the Veterinary Medicine Basic Sciences Building at the University of Illinois at Urbana-Champaign. Breeders (one male with one female) were housed

in solid-bottom caging with standard bedding. Rodent diet (Teklad) and water were provided *ad libitum*. Mouse litter size and sex ratio varies between breeders and pregnancies. Male mice were used for experiments unless otherwise mentioned. The study was approved by the University of Illinois Institutional Animal Care and Use Committee (protocol number 19171).

ELT model

Mouse pups were separated daily from their mother and father (3–6 months old) for 3 h (3–6 pm) during postnatal days 2–14. During maternal separation, pups were kept together in a dark covered cage with supplemental heat to avoid pup abandonment due to low body temperature. Intraperitoneal injections of 40 μ L PBS were administered to pups with a 27G needle during postnatal days 4–10 while they were separated from the dam as an additional stressor. Normal parental care-taking behavior was observed after reunification despite the intervention (data not shown). Pups were weaned from their mothers on postnatal day 28. Newborn mice share their cage with parents until wean time. Neonates subjected to ELT or no neonatal treatment were derived from different litters.

Induction of active EAE

In 6–8-week-old mice, EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ peptide (100 μ g/mouse) emulsified in complete Freund's adjuvant including heat-killed *Mycobacteria* (Mtb, BD Difco, DF3114-33-8, 200 μ g/mouse) on day 0, and intraperitoneal injection of pertussis toxin (200 ng/mouse) on days 0 and 2. For 30 days, EAE clinical score was assessed as such: 1: tail limpness; 1.5: reversible impaired righting reflex; 2: impaired righting reflex; 2.5: one hind limb paralysis; 3: both hind limb paralysis; 3.5: both hind

and one fore limb paralysis; 4: hind and fore limb paralysis; 5: death. Water gel and powder feed were provided for mice that scored ≥ 2 to avoid body weight reduction as a result of being unable to reach standard food and water. Disease score analysis was performed in the middle of the day during the light cycle. To optimize EAE dosage to study susceptibility to mild induction, EAE was induced in 6-8-week-old mice using Mtb (50 or 100 $\mu\text{g}/\text{mouse}$) under same protocol as mentioned above. Mice that achieved EAE score of ≥ 1 were considered to be successfully induced.

Pharmaceutical treatment of mice

All treatments were conducted through i.p. injections. Day 0 represents day of EAE induction. Interferon β (3×10^4 unit/mouse) was i.p. injected into mouse every other day or three days from days 0 to 9 as previously performed^{27, 29}. CXCR2 inhibitor (SB225002, 0.1 mg/kg mouse) was i.p. injected into mouse every day from day 0 to day 20²⁷. $\alpha 1$ - selective antagonist (Prazosin, 3 mg/kg)³⁸, $\alpha 2$ - selective antagonist (Atipamezole, 3 mg/kg)³⁹, $\beta 1$ -selective antagonist (Metoprolol, 3 mg/kg)⁴⁰, and $\beta 2$ -selective antagonist (ICI 118,551, 0.5 mg/kg)⁴¹, β -adrenergic receptor non-selective antagonist (Propranolol, 5 mg/kg), and $\beta 1$ -agonist (Xamoterol, 3 mg/kg)⁴² were i.p. injected every other day from day 0 to day 10. PBS was treated as vehicle control.

Immunohistochemistry and analyses

Spinal cords were harvested from PBS-perfused and 4% paraformaldehyde-fixed mice at 30 dpi. Spinal cords were post-fixed in 4% paraformaldehyde overnight and cryoprotected by immersion in 30% sucrose solution for 24 h. Samples were frozen in OCT compound and stored at -80°C until cryostat sectioning. Transverse sections (30- μm) of spinal cords were

mounted on poly-L-lysine-coated glass slides. To visualize microglia and cholinergic neurons, mounted samples were permeabilized with 0.05% Triton-X for 15 min at room temperature, blocked with 2% BSA for 2 h at room temperature, incubated overnight at 4°C with goat polyclonal AIF-1/Iba1 primary antibody or cholinergic acetyltransferase antibody diluted in PBS, and incubated with chicken anti-goat Alexa 647 secondary antibody or chicken anti-goat Alexa 488 secondary antibody for 2 h. Labeled samples were dried, covered with mounting media (Prolong Gold Antifade Mountant, Invitrogen, Cat# P36930), and sealed with a coverslip. For Iba1 analysis, tissue sections (at least 3 images of ventral roots from individual L4–L6 lumbar spinal cord sections per animal) were visualized using a Nikon A1 confocal scanning microscope at 20x magnification. A total of 614 Iba1⁺ cells (in 3 animals per condition) were included in our analyses of soma size and roundness using the ImageJ morpholibj plugin⁸⁴. Briefly, a region of interest (ROI) was manually placed over spinal cord areas. Grey scale attribute filtering was applied with Operation set to “Opening”, Attribute set to “Area”, Minimum value set to “25 pixels”, and Connectivity set to “8”. Morphological filter was subsequently applied with Operations set to “Opening”, Element set to “Octagon”, and Radius (in pixels) set at “1”. A standardized threshold was applied across all ROIs. Measurements of “soma area” and “roundness” were obtained using “analyze particles” with measurements set to include “area, shape description, fit eclipse”. For ChAT analysis, tissue sections (at least 3 images of ventral roots from individual L4–L6 lumbar spinal cord sections per animal) were visualized using a Nikon A1 confocal scanning microscope at 20x magnification. Neurons labeled with ChAT were counted manually by a single blinded experimenter.

Luxol fast blue staining and analysis

Transverse sections (30 μm) of frozen spinal cord from mice at 30 dpi mounted onto poly-L-lysine coated slides and left to dry overnight. Sections were hydrated with 95% ethyl alcohol, then placed in 1:1 ethanol/chloroform for 5 minutes. Sections were rehydrated with 95% ethyl alcohol then incubated in 0.1% Luxol fast blue solution for 12 hours. After 12 hours, 95% ethanol was used to wash away excess dye solution, followed by rinsing step with distilled water. 0.05% Li_2CO_3 solution was applied to sections for 30 seconds, followed by a wash step with 70% ethanol until a sharp contrast between gray and white matter becomes apparent. Sections were immersed in 90%, 95%, and 100% ethanol gradient dehydration washes, followed by the application of a coverslip. For image analysis, grey scale images were opened in ImageJ and an automatic threshold was applied. Region of interest was manually drawn to restrict analysis to the area of white matter regions. Analyses were performed on at least 5 serial sections. Quantitative analyses measured the area of Luxol fast blue staining and normalized this area to the total analysis area.

Fluor Jade C staining

Transverse sections (30 μm) of frozen spinal cord from mice at 30 dpi mounted onto poly-L-lysine coated slides and left to dry overnight. Sections were immersed in 1% NaOH/80% EtOH for 5 minutes followed by one 2-minute rinse with 70% EtOH and one 2-minute rinse with distilled water. Sections were incubated in 0.06% KMgO_4 solution for 10 minutes and rinsed with distilled water for 2 minutes. Sections were then immersed in 0.0001% FluoroJade solution for 10 minutes. After 10 minutes, sections were rinsed with distilled water for 1 minute, repeated 3 times. Slides were dried at 50°C for 5 minutes followed by immersion in xylene for 1

minute. Promount and coverslip were applied to dried slides for extended storage. For image analysis, ventral gray matter region of lumbar spinal cord sections were visualized using a Nikon A1 confocal scanning microscope at 20x magnification. The percent of stained area was averaged across serial sections per animal. Positively stained neurons from at least 5 images of ventral roots from individual L4–L6 lumbar spinal cord sections per animal were counted manually by a single blinded experimenter. Count value was normalized by image area.

Golgi-Cox staining

At 30dpi, mice were deeply anesthetized with isoflurane and perfused intracardially with PBS followed by 4% paraformaldehyde. Spinal cord was removed and transferred into solutions designation by the FD Rapid Golgi-Stain Kit (FD Neuro-Technologies, INC). The protocol is described as follows: organs were placed in 10 ml of Solution A and B of ratio 1:1 for 24 hours and then immersed in an identical volume of fresh Solution A and B for an additional 14 days in dark. The spinal cord and brain were then transferred to solution C for 3 days, with a replacement of fresh solution after 24 hours. To attain cryoprotection, spinal cords were then transferred in a 30% sucrose solution overnight or until organs sunken to bottom. Spinal cord was individually snap frozen in OCT on dry ice and kept frozen at -80°C to be cut into 50µm thick coronal slices using Cryo-cut 1800 (Reichert Jung) at -14°C. Slices were then incubated in solution D and E before being mounted on a glass slide, dehydrated, and sealed by prolong gold mountant. Samples were visualized using Leica DM4000 bright field modality equipped with 20x objective. 5 fields of spinal cord ventral gray matter were analyzed per animal to obtain average value per animal. For image analysis, grey scale images were opened in ImageJ and an automatic threshold was applied. Wand tool was used to distinguish white from gray matter to

identify ventral gray matter region as of interest. Percentage stained with Golgi-Cox was determined as “Area of thresholded positive staining/Total ROI area”.

Confocal reflection super-resolution acquisition and analysis

Samples of Golgi-Cox-stained spinal cords were imaged using a Nikon A1 confocal scanning microscope under the confocal modality and CRSR modality (with minimized pinhole at 0.3 AU) using 100x/1.49 NA oil objective. A 405-nm continuous wave laser was used, and the reflectance mirror (BS 20/80) was applied. For dendritic spine analysis, z-stacks of at least 100 intervals were acquired. Pixel dimensions are: x: 0.0628 μm ; y: 0.0628 μm ; z: 0.075 μm . 4-8 z-stacks of spinal cord ventral roots from 4-8 individual 50 μm -thick spinal cord sections per animal were visualized. 20 dendrites per animal were included in our analyses using the filament tracer autopath function (IMARIS)²⁴. Briefly, dendritic regions that was 40 -60 μm in length and void of dendritic branch points and crossing neurites were selected. Gaussian filter and background subtraction were applied to z-stacks of cropped individual dendrites prior to tracing filaments.

Flow cytometry

Spleen, lymph node (axillary and inguinal), brain, and spinal cord were removed, then minced with a sterile razor blade. Minced spleen and lymph nodes were passed through a mesh filter. Brain and spinal cord were digested in collagenase for 30 min at 37 °C, and then passed through a 70- μm filter to remove debris. CNS-infiltrating lymphocytes were isolated by percoll gradient centrifugation. After washing, the cells were suspended in ice-cold staining buffer (sterile PBS containing 2% FBS). Cells were blocked with anti-CD16/32 on ice for 10 min. After blocking,

1×10^6 cells were suspended in 0.1 mL of ice-cold staining antibody buffer and stained on ice in the dark for 20 min. After washing twice in staining buffer, the samples were acquired on a Cytex Aurora flow cytometer. Gates were determined using unstained and single-stained samples obtained from the same tissue of origin. Results were analyzed using FCS Express 6 flow cytometry software (De Novo Software).

Dendritic cell and T cell co-culture

DCs (CD11c⁺) were isolated from inguinal and axillary lymph nodes of EAE mice with or without ELT condition (both 6-week-old) or EAE mice with vehicle or β 1 AR antagonist treatment by beads positive selection (EasySep Mouse Biotin Positive Selection kit) with Biotinylated CD11c antibody. CD4⁺ T cells were also isolated from inguinal and axillary lymph nodes of naïve TCR2D2 mice, a myelin oligodendrocyte glycoprotein (MOG)-specific transgenic T cell mouse, EAE mice with or without ELT condition, or EAE mice with vehicle or β 1 AR antagonist treatment by beads negative selection (EasySep Mouse CD4⁺ T cell isolation kit). CD4⁺ T cells were labeled by CellTrace Violet Cell proliferation kit or CFSE Cell Proliferation kit according to manufacturer's protocol. Cells derived from inguinal and axillary lymph nodes of mice with or without ELT condition were also labeled by CFSE Cell Proliferation kit. After washing, these CD4⁺ T cells were co-cultured with DCs (each 10^5 cells) and treated with MOG35-55 peptide (5 μ g/ml) in complete RPMI 1640 medium for 72 hours in 96 well round plates. Lymph node-derived cells were also treated with MOG35-55 peptide (5 μ g/ml) in complete RPMI 1640 medium for 72 hours. 72 hours after treatment, CD4⁺ T cell proliferation was evaluated by flow cytometry (Cytex Aurora, UIUC).

ELISA

Blood samples were taken at approximately 9 am from all mice to ensure consistency in sample quality. Serum or plasma were isolated and stored at 80°C until ELISA analysis. ELISA was conducted according to manufacturer's protocol. Triplicate wells were made for each sample derived from one mouse. Sample diluent alone was used as a negative control.

RNA and cDNA preparation for standard qPCR analyses

For standard qPCR analysis, DCs (CD11c⁺) were isolated from inguinal and axillary lymph nodes by beads positive selection (EasySep Mouse Biotin Positive Selection kit) with Biotinylated CD11c antibody. Macrophages were isolated from lymph nodes of ELT-EAE and control EAE mice at EAE 10 dpi by beads separation (EasySep Mouse CD11b Positive Selection Kit II, STEMCELL) after removing neutrophil population by beads selection (STEMCELL). Total RNA was extracted from cells with RNeasy Kit (Qiagen). cDNA synthesis was performed with qScript cDNA SuperMix (Quanta). qPCR analysis was performed with KAPA-SYBR-FAST (KAPA Bio Systems) with an initial denaturing step at 95°C for 3 min, followed by 35 cycles of a denaturation step at 94°C for 3 s and an annealing and extension step at 60°C for 30 s. The relative amounts of qPCR products were determined with the $\Delta\Delta C_t$ method to compare the relative expression of target genes and beta actin housekeeping genes. The expression of the gene encoding β -actin was used as an internal control.

DNA methylation assay

DCs (CD11c⁺) were isolated from inguinal and axillary lymph nodes by beads positive selection (EasySep Mouse Biotin Positive Selection kit) with Biotinylated CD11c antibody from spleen of

control-EAE and ELT-EAE mice at EAE at 9-dpi. Genomic DNA was obtained with the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma)²⁷. Bisulfite conversion was performed with the MethylDetector kit (Active Motif), and the Lta promoter (between –363-nt and +65-nt from the transcription start site) was cloned into the pCR4-TOPO-TA vector (Life Technologies) and transformed into *E. coli*. Methylation status was evaluated by sequencing 17-22 clones per group. Percentages of unmethylated CpG in all the tested CpG sites were calculated.

In vitro stimulation of bone marrow-derived DC

Bone marrow was isolated from naïve C57BL/6J mice. Isolated cells were treated with recombinant GM-CSF (200 ng/ml) for 6 days to generate bone marrow-derived DCs (BMDC). For qPCR experiment, BMDC (10^6 cells) were treated with AR agonists (10 µg/ml) for 18 hours at 37°C according to a previous report, then treated with Mtb (100 µg/ml) or lipopolysaccharides (1 µg/ml) for 3 hours at 37°C. As control, BMDC were treated with vehicle (PBS) instead of AR agonists, and vehicle instead of Mtb or LPS. For Western blotting experiments, BMDC (5×10^6 cells) were used under same treatment condition, respectively. To detect TRAF3 and actin, we analyzed BMDC lysates by Western blotting with antibody against TRAF3 (1:1000) and actin (1:1000). To normalize TRAF3 signals by beta-actin in same blotting membrane, membrane was cut and stained with specific antibodies. Horseradish peroxidase (HRP)–conjugated secondary antibody (1:1000) was used to detect signals, which were visualized by enhanced chemiluminescence (Peace). Images were taken by FluorChem Systems (Biotech). Quantification of image was performed by Image J.

Statistical analysis

Statistical analysis in all results was evaluated with two-tailed unpaired Student's t tests and t-values. The criterion of significance was set as $P < 0.05$. All behavior experiments were performed in a blinded and randomized fashion. No animals or data points were excluded. Quantifications were performed from at least two experimental groups in a blinded fashion. Measurements were taken from distinct samples for each experiment. For each test, the experimental unit was an individual animal. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. Data distribution was assumed to be normal, but this was not formally tested.

3.3 RESULTS

ELT impacts EAE susceptibility, severity, and duration in male and female mice

Early life trauma (ELT) was modeled by neonatal maternal separation and sterile phosphate buffered saline (PBS) injections to induce emotional and physical distress (see Methods). EAE scores of ELT mice were compared against mice that did not receive any handling (control mice) (Fig. 3.1A). ELT-EAE male mice showed slightly early induction of EAE disease with prolonged severe paralysis in comparison to control EAE mice (Fig. 3.1B). Quantification of cumulative disease severity from 0- to 30-day post-EAE induction (dpi) revealed that male ELT-EAE mice had significantly exacerbated disease in comparison to EAE mice (Fig. 3.1B). Similarly, female ELT mice also developed EAE at an earlier time and prolonged severe disease, when compared with non-ELT control mice (Fig. 3.1C). Because EAE is a demyelinating disease of the CNS, Luxol-fast blue myelin staining was conducted using lumbar spinal cord samples to reveal that ELT-EAE mice had significantly more severe demyelination in the spinal

cord compared to control EAE mice at 20 dpi (Fig. 3.1D). To test the hypothesis that ELT increases EAE susceptibility, we titrated the amounts of heat-killed *Mycobacterium tuberculosis* (Mtb) (adjuvant) used in complete Freund's adjuvant to identify a weak disease induction dose in control mice. While only 17% of control mice displayed an EAE score ≥ 1 over the course of 30 days using low-dose (50 $\mu\text{g}/\text{mouse}$) of Mtb for EAE induction, all ELT mice developed scores ≥ 1 (Fig. 3.1E). Taken together, these results suggest that ELT increases EAE severity with no sex specificity and increases mice susceptibility to EAE with low-dose Mtb.

ELT alters peripheral and CNS immune cell profiling

ELT affects immune system development and alter an organism's response to future immune challenges^{20,21}. In young adult mice without EAE induction, there was no difference of immune cell population was observed in lymph nodes of control and ELT mice (Fig. 3.2A). However, upon EAE induction, ELT mice had significantly increased numbers of CD4⁺ T cells, CD8⁺ T cells, and macrophage in axillary and inguinal lymph nodes at 10 dpi compared to non-ELT controls (Fig. 3.2B). To understand the mechanism of T cell upregulation, we first examined co-stimulatory molecule expression on antigen-presenting cells (APCs) and secondly evaluated T cell proliferative abilities. 4-1BBL and CD80 were significantly increased on DCs from lymph nodes of ELT-EAE mice at 10 dpi compared to controls (Fig. 3.2 C). In vitro co-culture experiments showed that CD4⁺ T cells produce significantly a greater abundant of cytokines, namely, IL-17, IFN γ , and GM-CSF, but not IL-2, in the presence of DCs upon incubation with MOG35-55 peptide, whereas the absence of DC yielded undetected amounts of cytokine from culture supernatant (Fig. 3.2D). These results suggest that the heightened T cell immune

response to EAE generated in ELT mice can be attributed amplified antigen-dependent interactions between innate and adaptive immune cells.

Neuroinflammation characterized by glia activation and peripheral immune cell infiltration to the CNS is important for EAE and MS pathology^{22,23}. ELT-EAE mice had significantly more spinal cord-infiltrated CD4⁺ T cells at 10 dpi (disease onset), 18 dpi (disease peak), and 25 dpi (disease chronic phase) when compared to control EAE mice (Fig. 3.2 E). T cell subtypes were also characterized based on cytokine producing profiles. Significant upregulation of IL-17- and/or IFN γ -producing CD4⁺ T cells was observed in the spinal cord of ELT-EAE mice (Fig. 3.2 F). Additionally, there was a significant increase in microglia cellularity in the spinal cord of ELT-EAE mice (Fig. 3.2 G). These findings demonstrate that the increased severity and early induction of disease in ELT-EAE mice may be attributed to heightened immune cells in the periphery and CNS which likely results from immune cell property changes observed in peripheral lymphoid organs.

Severe neuron damage in ELT-EAE mice

To test the impact of ELT on EAE-induced neuronal damage, we examined morphological abnormalities in motor neurons using Golgi-Cox staining. We compared the percentage of area stained by Golgi-Cox in the ventral horn of lumbar 3-6 spinal cord slices at 30 dpi (Fig. 3.3 A). While Golgi-Cox staining did not differ between control and ELT mice before EAE induction, staining was significantly reduced in ELT-EAE mice (Fig. 3.3 B). We then sought to determine dendritic spine density using the recently reported Golgi-visualizing super-resolution confocal reflection method (CRSR)²⁴. Reduced spine density was found in the dendrites of neurons that reside in the lumbar spinal cord ventral horn in control EAE mice and ELT-EAE mice at 30 dpi,

compared to naïve and ELT mice without EAE (Fig. 3.3D, 3.3D). Notably, spine density loss in ELT-EAE mice was significantly worse than in control EAE mice (Fig. 3.3 C, 3.3. D). To further evaluate the functional status of spinal cord neurons, we conducted FluoroJade C staining to detect degenerating neurons ²⁵. Neurodegenerative signals were significantly more prominent in the spinal cord lumbar region of ELT-EAE mice compared to control EAE mice (Fig. 3.3 E). Further, we quantitated motor neurons in the lumbar spinal cord using immunohistochemistry to label choline acetyltransferase (ChAT). The number of ChAT-positive motor neurons was significantly reduced in ELT-EAE mice, compared with controls (naïve mice, naïve ELT mice, and control-EAE mice) (Fig. 3.3.F). Next, we assessed activation status of microglia in the lumbar spinal cord region by examining soma area (Fig. 3.3G). A significant increase in Iba1⁺ cells of large soma areas, indicative of microglial activation, was observed in ELT-EAE mice when compared with control EAE mice (Fig. 3.3H). Collectively, these results demonstrate that ELT alters CNS neuron and microglia properties upon EAE induction.

Different drug sensitivities and distinct immune markers in ELT-EAE mice

IFN β sensitivity is used here as a proxy for an altered EAE phenotype that is neurodegenerative ^{26, 27}. While IFN β suppressed EAE development in control mice, the same treatment did not suppress EAE development in male and female mice subjected to ELT (Fig. 3.4 A, B).

Previously, we showed that while IFN β -sensitive EAE development is dependent on NLRP3-inflammasome-mediated IL-1 β ^{28, 29}, whereas IFN β -resistant EAE is driven by membrane-bound lymphotoxin (mLT) and lymphotoxin β receptor (LT β R) function, not inflammasome-mediated IL-1 β ²⁷. To examine if the IFN β resistant profile of ELT-EAE disease is mediated by NLRP3-inflammasome independent pathway, we monitored EAE symptoms in Asc^{-/-} mice with and

without ELT. Inflammasome adaptor protein Apoptosis-associated speck-like protein containing CARD (ASC) is an adaptor molecule that plays a pivotal role in inflammasome assembly mainly implicated in innate immune cells. ASC depletion did not impact the disease development of ELT-EAE mice whereas ASC is necessary in control EAE disease progression (Fig. 3.5A), suggesting that the development of ELT-EAE was indeed NLRP3-inflammasome independent. Additionally, while similar amounts of IL-1 β was found in the serum of ELT and control mice without EAE induction, IL-1 β was significantly reduced in ELT mice with EAE when compared to control mice with EAE at 10 dpi (Fig. 3.5B). This suggests the lack of function of IL-1 β in promoting ELT-EAE disease development. These results demonstrate that ELT-EAE mice were resistant to IFN β in a NLRP3-inflammasome independent pathway.

Next, we asked if markers of NLRP3-inflammasome independent IFN β resistance share the same critical cellular markers as observed previously in animal model and in IFN β resistant clinical cohort²⁷. Namely, our previously reported IFN β resistant EAE model was characterized by heightened membrane lymphotoxin (mLT: a heterotrimer containing LT α and LT β ³⁰) expression in APCs and upregulated chemokine receptor CXCR2 expression in T cells. Consistent with experimental evidence, peripheral blood mononuclear cells isolated from IFN β non-responder patients with MS highly express LT β R and CXCR2 compared to IFN β responder patients²⁷. In comparison to control EAE mice, ELT-EAE mice higher expression of mLT protein on DCs and macrophages (Fig. 3.5 C). Transcription of *lta* and *ltbr* are also highly upregulated in DCs derived from ELT-EAE mice when compared with control EAE mice (Fig. 3.5 D). Additionally, *Lta* promoter region in DCs isolated from ELT-EAE mice had a significantly increased un-methylated status compared to control EAE mice (Fig. 3.5 E). Further, we found increased number of CXCR2-expressing CD4⁺T cells in the lymph nodes of

ELT-EAE mice at 10 dpi (Fig. 3.5 F) and higher amounts of CXCL1, a ligand for CXCR2, in serum of ELT-EAE mice compared to control EAE mice (Fig. 3.5 G). While SB225002, a CXCR2 antagonist, did not impact EAE disease progression in control mice, as previously reported²⁷, it significantly ameliorated EAE in ELT mice (Fig. 3.5 H). Taken together, these data suggest that altered disease phenotype in ELT-EAE mice is mediated by an alternative inflammatory mechanism that involves mLT and CXCR2 signaling.

ELT downregulates adrenergic signaling in immune cells

ELT causes short-term or enduring changes in the systemic sympathetic nervous system (SNS) and the hypothalamic–pituitary–adrenal axis (HPA)³¹ which are shown to have major implications on immune cell property and response to stimuli^{33,34}. Levels of norepinephrine, a SNS neurotransmitter, in blood plasma of 2 to 6-week-old ELT mice were increased compared to controls (Fig. 3.6 A). Additionally, norepinephrine levels in the inguinal and axillary lymph nodes of 4-week-old ELT mice were significantly heightened compared to controls (Fig. 3.6 B). SNS hyperactivity results in downregulation of AR signaling and expression levels in immune cells^{35,36,37}. Therefore, we evaluated AR expression in DCs and CD4⁺T cells from lymph nodes of ELT mice. Gene expression of all classes of ARs except β 3-AR (i.e., α 1, α 2, β 1, and β 2) were detected in both DCs and CD4⁺T cells isolated from lymph nodes of control mice. α 1, α 2, β 1, and β 2 AR expression were significantly downregulated on DCs (Fig. 3.6 C) and CD4⁺T cells (Fig. 3.6 D) in the lymph nodes of ELT mice compared to control mice. To examine if reduced level of AR expression affects AR signaling in immune cells, DC and CD4⁺T cell isolated from control and ELT mice were treated with β 1-AR agonist (Xamoterol) and were examined for the amount of downstream signaling molecule cyclic-AMP (cAMP). While

treatment of β 1-AR agonist increased cAMP levels in DC and CD4⁺T cell of control mice, the treatment failed to upregulate cAMP levels in both DC and CD4⁺T cell of ELT mice (Fig. 3.6 E, F). These results suggest that ELT-mediated sustained SNS activation induces desensitization and downregulation of ARs in DCs and CD4⁺T cells.

β 1-AR antagonism is sufficient to reproduce the ELT-induced EAE phenotypes.

To determine the role of reduced AR signaling in mediating key phenotypes in ELT-EAE mice, we systemically treated control mice with AR antagonists against α 1 (prazosin)³⁸, α 2 (atipamezole)³⁹, or β 1/ β 2 (propranolol) within the first 10 days of EAE induction (disease induction phase). Injections of α 1- and α 2-AR antagonists to control EAE mice did not increase EAE severity, when compared with vehicle treated groups (Fig. 3.7 A). In contrast, β -AR antagonist treatment to control mice was sufficient to induce severe and prolonged EAE (Fig. 3.7 A). In addition, control EAE mice treated with β -AR antagonist were resistant to interferon- β (Fig. 3.7 B). Specifically, interferon- β -resistant EAE was observed in control EAE mice treated with metoprolol tartrate, a β 1-AR antagonist⁴⁰, but not with ICI 118,551, a β 2-AR antagonist⁴¹ (Fig. 3.7 B). Furthermore, we observed significant neuron damage reflected by reduction of Golgi-Cox staining in the ventral horn region of lumbar spinal cord sections in β -AR antagonist and β 1-AR antagonist-treated ELT mice (Fig. 3.7 C), suggesting that the inhibition of β 1-AR signaling in disease induction phase was sufficient to produce interferon- β -resistant phenotype with severe neuron damage in disease chronic phase.

Co-culture supernatant of DC and CD4⁺T cells isolated from β 1-AR antagonist-treated control mice had significantly increased production of IL-17, IFN γ , and GM-CSF upon incubation with MOG35-55 peptide, compared with vehicle-treated group (Fig. 3.7 D).

Furthermore, β 1-AR and β -AR antagonist treatment in control EAE mice was sufficient to induce mLT upregulation on DCs and CXCR2 upregulation on CD4⁺T cells (Fig. 3.7 E), which are mechanistic signatures of interferon- β -resistant EAE phenotype. Further, higher amounts of CXCL1 were detected in EAE mice with β 1-AR antagonist treatment when compared to EAE mice that did not receive treatment (Fig. 3.7 F). These results suggest that disease phenotypes of ELT-EAE are a result of dysfunctional β 1-AR signaling.

β 1-AR agonist treatment is sufficient to rescue ELT-EAE subtype

We attempted to rescue β 1 adrenergic signaling in ELT-EAE mice by administering β 1-AR agonist before EAE disease onset in ELT mice. β 1-AR agonist (Xamoterol)⁴² treatment was sufficient to revert ELT-mediated IFN β insensitivity back to an IFN β sensitive phenotype (Fig. 3.8 A). Additionally, β 1-AR agonist treatment in ELT EAE mice was sufficient to inhibit mLT upregulation on DCs and CXCR2 upregulation on CD4⁺T cells (Fig. 3.8 B). Furthermore, we observed significant rescue of neuron damage reflected by increase of Golgi-Cox staining in the ventral horn region of lumbar spinal cord sections in β 1-AR agonist treated ELT EAE mice when compared with ELT EAE (Fig. 3.8 C). To determine if the rescue function of β 1-AR agonist for EAE phenotype change in ELT mice is dependent β 1-AR on DC or T cells, DC- or T- cell specific β 1-AR knockout mice were subjected to ELT, EAE, and β 1-AR agonist treatment. While control β 1-AR floxed ELT mice with β 1-AR agonist treatment remains sensitive to IFN β , DC-specific and T cell-specific β 1-AR knockout ELT mice canceled the β 1-AR agonist-induced rescue function on IFN β sensitivity (Fig. 3.8 D). Additionally, both DC-specific and T cell-specific β 1-AR knockout ELT mice also canceled the β 1-AR agonist-induced rescue function on mLT upregulation on DCs and CXCR2 upregulation on CD4⁺T cells (Fig. 3.8

E). Interestingly, β 1-AR agonist-induced rescue function on neuron damage in the ventral horn region of lumbar spinal cord sections was canceled in T cell-specific β 1-AR knockout ELT EAE mice, but not in DC-specific conditional β 1-AR knockout ELT EAE mice (Fig. 3.8 F). These results demonstrate that reduction of β 1-AR signal in immune cells of ELT-EAE mice induces EAE phenotype shift.

β 1-AR agonist treatment prevents upregulation of Lta in BMDC in vitro

DCs in ELT mice showed upregulation of mLT in DCs, and downregulation of β 1-AR in DCs. We sought to determine whether β 1-AR signaling suppresses expression of mLT in DCs in vitro. Strikingly, pretreatment of β 1-AR agonist (Xamoterol), but not β 2- (Procaterol), α 1- (Cirazoline), or α 2-AR agonist (Medetomidine), suppressed both Mtb- and LPS-induced upregulation of Lta mRNA (encodes mLT) in bone-marrow-derived DCs (BMDCs) (Fig. 3.9 A). We previously found that Lta expression requires TRAF3 signaling under toll-like receptor using TRAF3 shRNA (Khaw et al., submitted). Therefore, we tested if β 1-AR signal affects TRAF3 expression and found that TRAF3 expression was significantly reduced in BMDCs after β 1-AR agonist treatment compared to vehicle control (Fig. 3.9 B). These results suggest that the β 1-AR signaling directly suppresses mLT in DCs, and reduction of β 1-AR-mediated suppressive function may trigger mLT upregulation in ELT-EAE mice.

3.4 DISCUSSION

MS clinical pathology and immunological phenotype show a high degree of variation between individuals. Notably, no single pathway, reliable biomarker, or specific treatment has yet been identified for all patients with MS. At present, the response to currently approved therapeutic

agents such as IFN β ⁴³, glatiramer acetate⁴⁴, and natalizumab⁴⁵ largely varies across the MS population. Mounting evidence suggests that environmental factors play a central role not only in triggering disease onset but also in modifying the course of disease by influencing individual susceptibility and immunological responses, which likely lead to the diverse heterogeneity observed in patients with MS.

Exposure to severe stress and trauma in early life can disrupt various regulatory processes such as the HPA axis⁴⁶, autonomic nervous system⁴⁷, immune system^{19,48,49}, and gut microbiome^{48,50} across the life span in both animals and humans⁵¹. A human case study published in 1982 demonstrates that ELT increases MS susceptibility among female patients⁵². More recently, a 2012 study found that patients with MS with histories of physical and/or sexual abuse had significantly higher relapse rates than patients without early-life stress¹¹. Here we performed side-by-side comparison of the EAE disease phenotype in adult mice subjected to ELT.

Multiple models of ELT have been proposed, such as maternal separation⁵³, neonatal handling⁵⁴, neonatal saline intraperitoneal injections⁵⁵, or a combination of those models^{56,57,58}. Because emotional neglect and physical abuse affect MS disease¹¹, we selected an ELT model that involves aspects of both psychological and physical abuse through maternal separation and daily injections, respectively⁵⁷. Differences in observations regarding the effect of ELT on EAE—such as our present finding of the effect of ELT on EAE severity in female mice, contrary to previous results in rats¹⁴—may be attributed to differences in experimental design or animal species. Further, human case studies demonstrate that ELT increases MS susceptibility among female patients⁵², so our mouse model may be to be more analogous to the human condition. In this study, we determined that EAE induced in mice exposed to ELT has significantly more

lasting and rampant inflammatory responses peripherally and in the CNS, which correspond to prolonged severity in observed hindlimb paralysis and severe neuron damage and demyelination pathologies.

Elevated invasion of immune cells to the CNS and increased number of microglia suggest the ability to sustain local CNS inflammation, mediate neuron damage, and support lymphocyte survival^{59,60}. We also found changes in membrane surface expression of co-stimulatory molecules CD80 and 4-1BBL on APCs. APC-T cell interaction through the 4-1BB: 4-1BBL pathway plays a crucial role in CD4⁺T and CD8⁺T cell clonal expansions⁶¹. CD80 is a potent co-stimulator of T cell proliferation and generation of cytotoxic lymphocytes⁶². Thus, upregulation of these co-stimulatory molecules on APCs may account for increased lymph node and splenic CD4⁺ and CD8⁺ T cells observed in ELT-EAE mice.

Strikingly, we discovered that ELT altered responsiveness to drug treatment during EAE when EAE was induced in adulthood. IFN β sensitivity is a useful marker in distinguishing EAE phenotype and mechanism. For instance, treatment of IFN β given prior to disease onset^{27, 63, 64} suppresses disease severity in naïve C57BL/6J mice induced with inflammasome-dependent EAE but not in mice induced with inflammasome-independent EAE²⁷. While EAE controls remained sensitive to IFN β , ELT-EAE mice had prolonged IFN β resistance. Consistent with our previous finding²¹, IFN β resistant ELT-EAE is independent of inflammasome activity and is largely driven by the mLT-LT β R axis. A possible reason for the IFN β -resistant inflammasome-independent feature of ELT-EAE may be attributed to upregulated lymphotoxin signaling. Previously we showed that treatment of recombinant lymphotoxin to control EAE animals were sufficient to render them non-responsive to IFN β treatment and induce severe EAE symptoms in inflammasome knockout (Nlpr3^{-/-}) animals, suggesting that lymphotoxin may be sufficient to

“convert” EAE phenotype from being inflammasome-dependent to being inflammasome-independent.

ELT-EAE mice present altered CNS neuron morphology, as shown in IFN β -resistant EAE disease^{27,64}. The observation of neuron damage was further fortified by reduced Golgi-Cox staining in the spinal cord ventral horn region as well as significant dendritic spine loss in the same spinal cord region. Dendritic spine loss has been shown to be associated with motor dysfunction in MS and EAE^{66,67}. Further, we found significantly less ChAT⁺ cholinergic motor neurons in ELT-EAE mouse spinal cords, implying impaired activity of neuron acetylcholine production and reduced neurotransmission⁶⁸. Reflective of damaged neuron status, the combination of ELT and EAE increased microglial proliferation and activation in the spinal cord. Microglia are readily activated by proinflammatory cytokines IFN- γ and IL-17 under EAE and Alzheimer’s disease conditions^{69,70,71}. Because ELT-EAE mice have highly heightened IFN- γ -secreting Th1 cells and IL-17-secreting Th17 cells, it is likely that these proinflammatory cytokines contribute to microglial activation under the ELT-EAE condition, leading to CNS neuronal damage. In addition, because mLT-LT β R signal is reported to equip CD4⁺T cells with neurotoxic property changes²⁷, a large amount of infiltrated CD4⁺T cells in the CNS should be also considered to contribute to CNS neuronal damage in ELT-EAE mice. Further studies are needed to clarify and deconvolute the exact mechanism of neurodegeneration in ELT-EAE.

The altered EAE disease phenotype induced by ELT shares symptomatic and mechanistic similarities with a previously reported EAE subtype induced by severe activation of innate immunity using high dosages of adjuvant (Mtb)²⁷. This implies that ELT causes lasting changes to innate immune cell properties, which result in immune response hyperactivation upon EAE induction and change the disease phenotype. Because ELT causes enduring changes in HPA³²

and SNS³¹ activity, both of which regulate immune function, we examined the role of these pathways in the observed disease phenotype alteration. Chronic stress induces HPA activation, as reflected by heightened corticosterone. However, we found no significant differences between corticosterone levels in control EAE and ELT-EAE mice at adulthood, suggesting that corticosterone may not be an immune-modulating factor. On the other hand, norepinephrine was significantly upregulated in both plasma and lymph nodes, indicative of chronic SNS hyperactivity. This is similar to a previous study demonstrating that chronic stress results in prolonged SNS hyperactivity⁷². Notably, heightened norepinephrine levels were sustained in ELT mice after EAE induction.

Chronic SNS hyperactivity causes downregulation of AR expression levels in immune cells^{35,37}. Accordingly, we found significantly reduced DC and CD4⁺T cells that express AR subtypes (α_1 , α_2 , β_1 , β_2) in ELT-EAE mice. Furthermore, cells derived from ELT mice showed lower amount of β_1 -AR agonist-mediated cAMP production which suggest that cells derived from ELT mice express a reduced amount or function of β_1 -AR. Human and animal studies of MS show evidence of the recovery role of immune cell AR activation^{73,74}, consistent with anti-inflammatory effects for immune cell ARs. Therefore, the altered EAE phenotype of ELT-EAE mice may be mediated by a hyperactive immune system as a consequence of a reduced regulatory role of the SNS. Importantly, control mice treated with β_1 -AR antagonist, but not α_1 -, α_2 -, or β_2 -AR antagonist, mimic disease phenotypes of ELT-EAE mice. The finding that β -AR antagonist treatment successfully reproduced severe and prolonged EAE symptoms was unsurprising due to the established immunosuppressive role of β -AR activation on immune cells and a recent article reporting similar results using the EAE model⁷⁵. In contrast, our findings that β_1 -AR antagonist treatment resulted in interferon- β insensitivity and severe neuron

damage are surprising and novel. Further, β 1-AR agonist treatment to ELT-EAE mice was sufficient to restore IFN β sensitivity and lumbar spinal cord neuronal status. This suggests that downregulated β 1-AR signaling in immune cells may involve phenotype change upon EAE induction in mice exposed to ELT.

As evidence of dysfunctional immune cell specific β 1-AR being the critical factor for EAE phenotype change, both DC and T cell specific β 1-AR knockout ELT mice did not recover from their interferon- β resistant status in spite of β 1-AR agonist treatment. Furthermore, β 1-AR agonist treatment to these specific knockout ELT-EAE animals did not inhibit production of mLT⁺ DCs and CXCR2⁺ CD4⁺T cells suggesting that β 1-AR dysfunction in neither DC nor CD4⁺T is sufficient to induce these cellular phenotypes. Our present data suggest that β 1-AR signaling inhibits mLT transcription by downregulating upstream TRAF3 expression and downregulated mLT expression had previously been associated with reduced CXCR2 expression on CD4⁺T cells, thus immunity of DC-specific β 1-AR knockout ELT-EAE mice to β 1-AR functional rescue is unsurprising. In contrast, immunity of T cell-specific β 1-AR knockout ELT-EAE mice to β 1-AR functional rescue was unexpected and suggests that β 1-AR signaling in T cells has a regulatory role in mLT expression on DCs via secreted molecule. Additionally, β 1-AR agonist treatment still rescues neuron damage in DC-specific β 1-AR knockout ELT-EAE mice, but not in T cell-specific β 1-AR knockout animals. Thus, it is plausible that T cell β 1-AR signaling is crucial in regulating T cell neurotoxic effector functions that may be independent of CXCR2. For example, β -AR signaling in T cells have been reported to inhibit production of multiple inflammatory cytokines such as IL-1 and TNF- α which had been shown to perpetuate EAE disease^{76,77}. Identification of critical signals for regulating mLT expression and T cell neurotoxic effector are next critical topics.

We have observed significant change in microglia/macrophage population and morphology in the CNS in ELT-EAE mice. Because microglia/macrophages are known to express β -ARs which regulate cell proliferation and cytokine secretion^{33, 78, 79}, altered NE levels in ELT mice may also affect β -AR expression levels on microglia/macrophage that may contribute to microglia/macrophage phenotype change and severe disease development as observed in ELT-EAE mice. Previous studies revealed that CNS resident microglia and monocyte-derived macrophages each have distinct roles during EAE development. Specifically, Ransohoff and team reported that monocyte-derived macrophages associate with nodes of Ranvier and initiate demyelination, whereas microglia appear to clear debris⁸⁰. The function of microglia/macrophage and involvement of β -AR signaling for their function in the manifestation of the ELT-EAE disease phenotype will be identified as next topic.

It is known that MS and EAE progression can be determined by DC phenotype and functional properties. In humans, DC properties were shown to vary significantly between patients who suffer from relapse-remitting MS versus progressive MS⁸¹. In animals, DC-targeting treatments have been shown to dramatically influence EAE outcomes⁸². Our experiment revealed that activation of DC β 1-AR signal was sufficient to downregulate expression of mLT which is an identifying molecule for EAE phenotype alteration in ELT-EAE mice. Because mLT-LT β R signaling is sufficient to induce CXCR2 expression²⁷, the upregulation of CXCR2, which is another identifying molecule for EAE phenotype alteration observed in ELT-EAE mice, may be mediated by such signaling in ELT-EAE mice. Importantly, we found β 1-AR signal in DCs suppresses expression of TRAF3, which is required for Lta transcription in DCs (Khaw et al., submitted). Further study to identify the mechanism by which β 1-AR signal suppresses TRAF3 expression is our next subject of interest in the effort

to identify reliable biomarkers and therapeutic targets for patients with MS who experienced ELT.

3.5 FIGURES

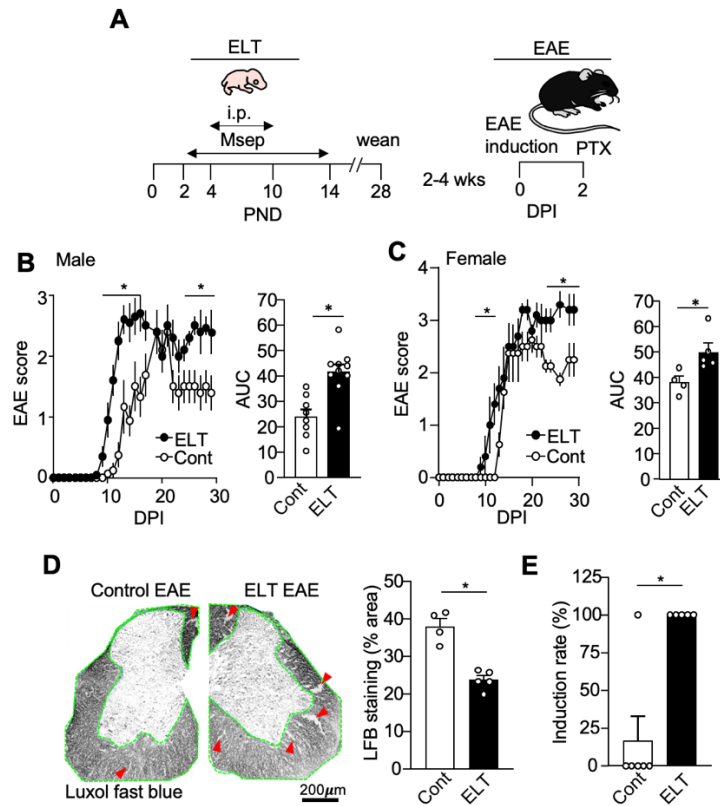


Fig. 3.1. ELT impacts EAE susceptibility, severity, and duration in both male and females. (A) Schematic representation of experimental procedure. PND and DPI indicate postnatal day and day post EAE induction, respectively. (B) Mean clinical EAE scores and cumulative scores of male mice (Cont EAE: n=8, ELT-EAE: n=10). (C) Mean clinical EAE scores and cumulative scores of female mice (Cont EAE: n=4, ELT-EAE: n=5). (D) Spinal cord ventral root sections of EAE mice stained for myelin using Luxol fast blue at 20 dpi (Cont EAE: n=4, ELT-EAE: n=5) with quantitative analysis of stained area. Green outline indicates analyzed white matter region of interest. Red arrows indicate demyelination regions. (E) EAE induction rate among mice treated with low-dose Mtb (50 ug/mouse, Cont EAE: n=6, ELT-EAE: n=5). Results are representative of at least two independent experiments. Each dot represents averaged data per animal. Data is represented as mean $\pm$ SEM. Two-tailed student's t-test, * $P < 0.05$.

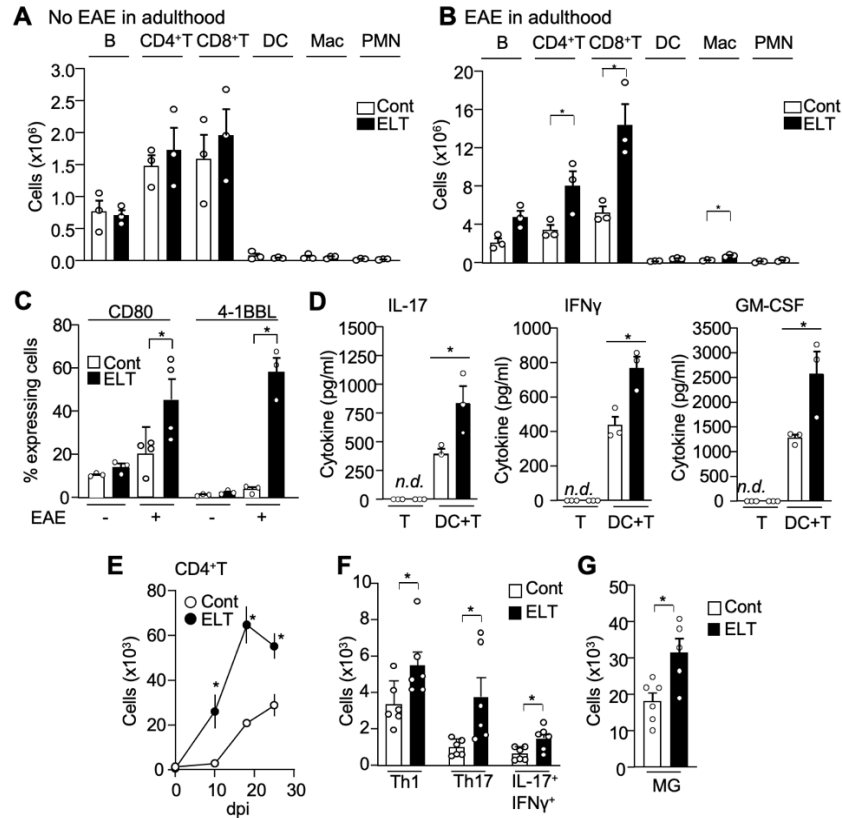


Fig. 3.2. ELT alters peripheral and CNS immune cell profiling. (A, B) Absolute immune cell numbers (B cell: CD19⁺, CD4⁺T cell: CD3⁺CD4⁺, CD8⁺T: CD3⁺CD8⁺, Dendritic cell: CD11b⁺CD11c⁺, Macrophage: CD11b⁺CD11c⁺, Neutrophil: CD11b⁺Ly6G^{high}) in control and ELT mice without (A) and with (B) EAE. Cells were isolated from draining lymph nodes were isolated from EAE mice at 10 dpi (n=3 per group). (C) Percentage of co-stimulatory molecule (CD80 and 4-1BBL) expressing dendritic cells (CD80: n=4 per group: 4-1BBL: n=3 per group). (D) Amount of cytokines IL-17, interferon γ (IFN- γ), and GM-CSF in supernatant derived from culture conditions 72 hours post culture initiation. CD4⁺T cells were isolated from draining lymph nodes of ELT-EAE or control-EAE mice on 10 dpi to be supplemented with MOG (10 ug/ml) and cultured alone or with isolated dendritic cell isolated from ELT-EAE or control-EAE mice (n=3 per group). (E) Absolute numbers of CD4⁺T (CD45⁺CD3⁺CD4⁺) cells in spinal cord isolated from EAE mice on 0, 10, 18, and 25 dpi (n=5 per group). (F) Absolute numbers of IL-17⁺ secreting CD4⁺T (Th17), interferon γ ⁺ secreting CD4⁺T cells (Th1), and IL-17⁺ interferon γ ⁺ CD4⁺T cells in the spinal cord of EAE mice at 25 dpi (n=6 per group). (G) Absolute numbers of microglia (MG: CD45^{low}CD11b⁺) in the spinal cord at 25 dpi (n=5 per group). Each dot represents averaged data per animal. Data is represented as mean $\pm$ SEM. Two-tailed student's t-test, *P < 0.05.

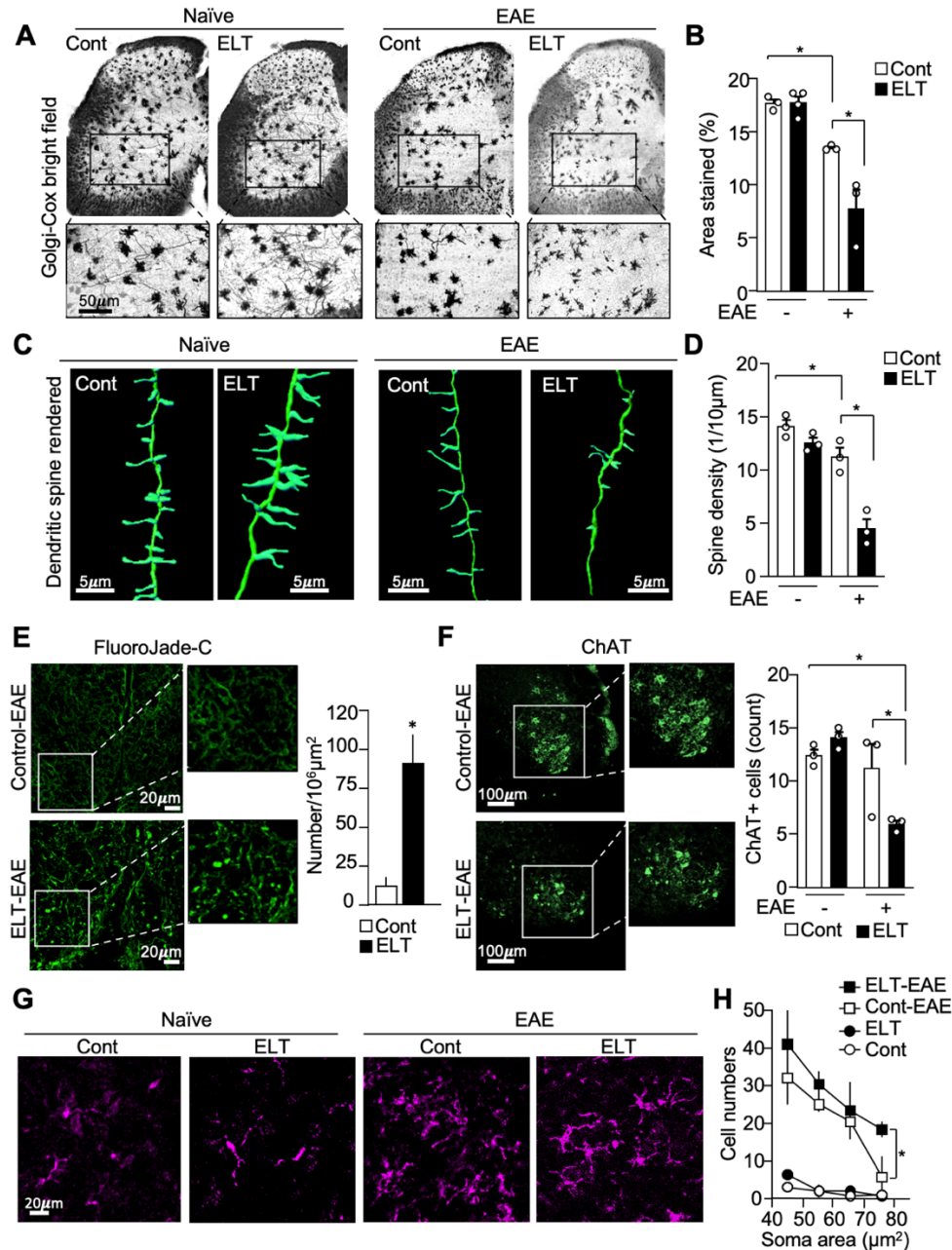


Fig. 3.3. Severe neuron damage in ELT-EAE mice. (A, B) Golgi-Cox stained lumbar spinal cord sections of EAE mice isolated at 30 dpi (A) with quantification of area stained/per field (b) (Cont naïve: n=3, ELT naïve: n=4, Cont EAE: n=3, ELT-EAE: n=3). (C, D) CRSR imaging of Golgi-Cox stained dendrites in lumbar spinal cord ventral horn region (c) with quantification of dendritic spine density at 30 dpi (D) (n=3 animals/group, 60 dendrites/group) (E) FluoroJade-C stained lumbar spinal cord sections of EAE mice isolated at 20 dpi with quantification of area stained/per field (Cont EAE: n=7, ELT-EAE: n=4). (F) ChAT⁺ neuron stained lumbar spinal cord sections of EAE mice isolated at 30 dpi (n=3 per group). (G) Representative image of Iba1⁺ cells in spinal cord ventral horn at 30 dpi. Representative image was randomly selected from pooled images from two independent staining experiments. (H) Iba1⁺ cell count categorized by soma size in the lumbar spinal cord ventral horn (L5-6) at 30 dpi (n=3 per group). Each dot represents averaged data per animal. Data is represented as mean $\pm$ SEM. Two-tailed student's t-test, * $P < 0.05$.

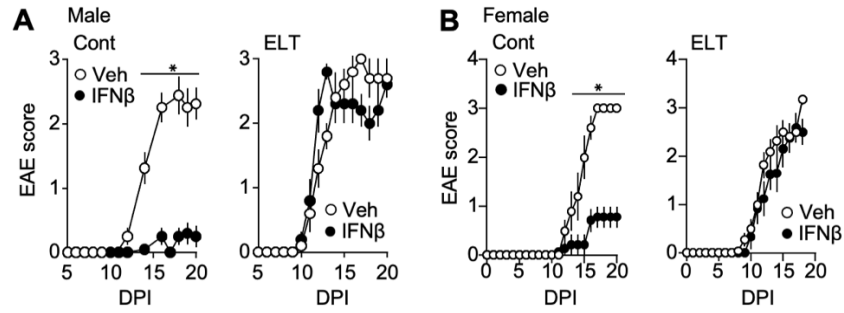


Fig. 3.4. ELT-EAE mice are IFN β resistant. (A, B) Mean clinical EAE scores of control-EAE and ELT-EAE male (A) and female (B) mice with or without interferon β (IFN β) treatment (Male Cont-Vehicle: n=8, Male-Cont-IFN β : n=10, Male ELT-Vehicle: n=5, Male-ELT-IFN β : n=5, Female Cont-Vehicle: n=5, Female-Cont-IFN β : n=7, Female ELT-Vehicle: n=5, Female-ELT-IFN β : n=5). IFN β was treated from 0 dpi to 10 dpi every other day. All data represent mean $\pm$ SEM. Two-tailed student's t-test, *P < 0.05.

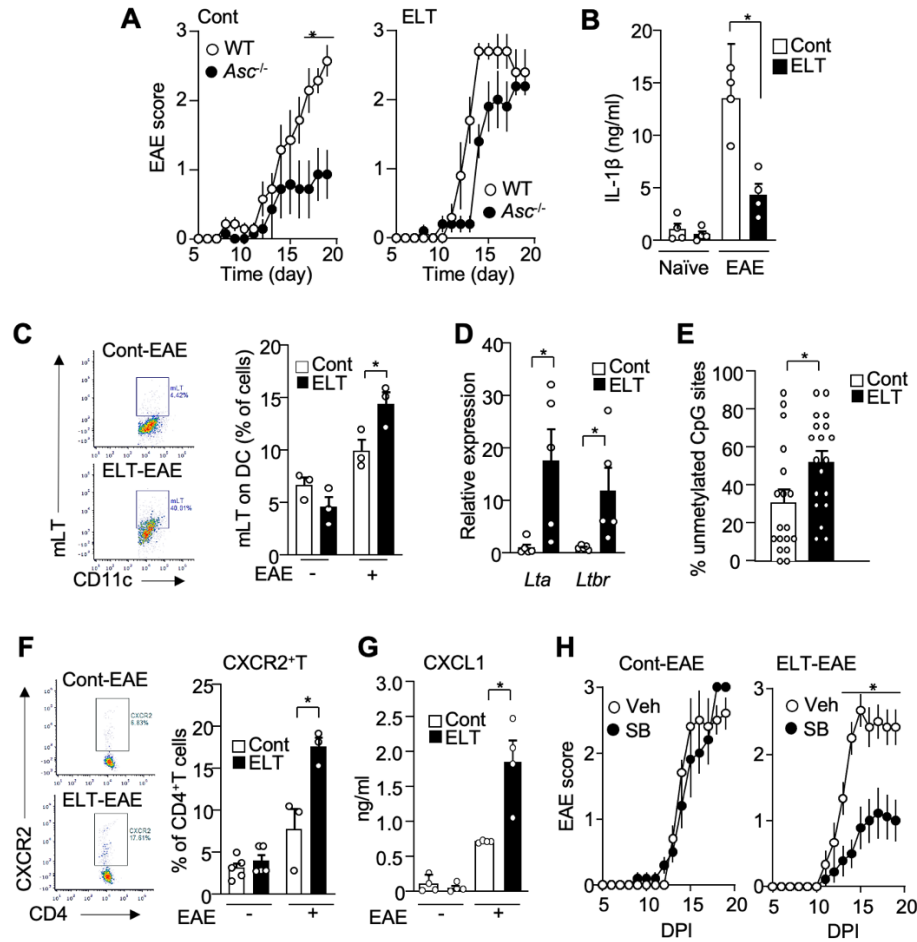


Fig. 3.5. Distinct immune markers in ELT-EAE mice. (A) EAE scores after EAE induction in control- and ELT-mice of wild type and *Asc*^{-/-} mice (Cont-WT: n=4, Cont-*Asc*^{-/-}: n=5, ELT-WT: n=6, ELT-*Asc*^{-/-}: n=5). (B) Serum levels of IL-1 β at 10 dpi (Cont naïve: n=4, ELT naïve: n=4, Cont EAE: n=3, ELT-EAE: n=3). (C) Percentage of mLT-expressing dendritic cells (DC) at 10 dpi (n=3 animals/group). (D) Expression of *Lta* and *Ltbr* mRNA in DC obtained from lymph nodes of control and EAE mice at 10 dpi (n=5 animals/group). (E) Percentage of unmethylated CpG sites in the promoter region of LT gene in dendritic cells isolated from lymph nodes of control and ELT mice without EAE at 10 dpi (n=18 colonies). (F) Percentage of CXCR2-expressing CD4⁺T cells at 10 dpi (Cont naïve: n=5, ELT naïve: n=5, Cont EAE: n=3, ELT-EAE: n=3). (G) Serum levels of CXCL1 at 10 dpi (n=4 animals/group). (H) EAE score of mice treated with CXCR2 inhibitor SB225002 (SB) or saline (Cont-Vehicle: n=5, Cont-SB: n=5, ELT-Vehicle: n=6, ELT-SB: n=9). Male mice were used for experiments. Each dot represents averaged data per animal. Data is represented as mean $\pm$ SEM. Two-tailed student's t-test, *P < 0.05.

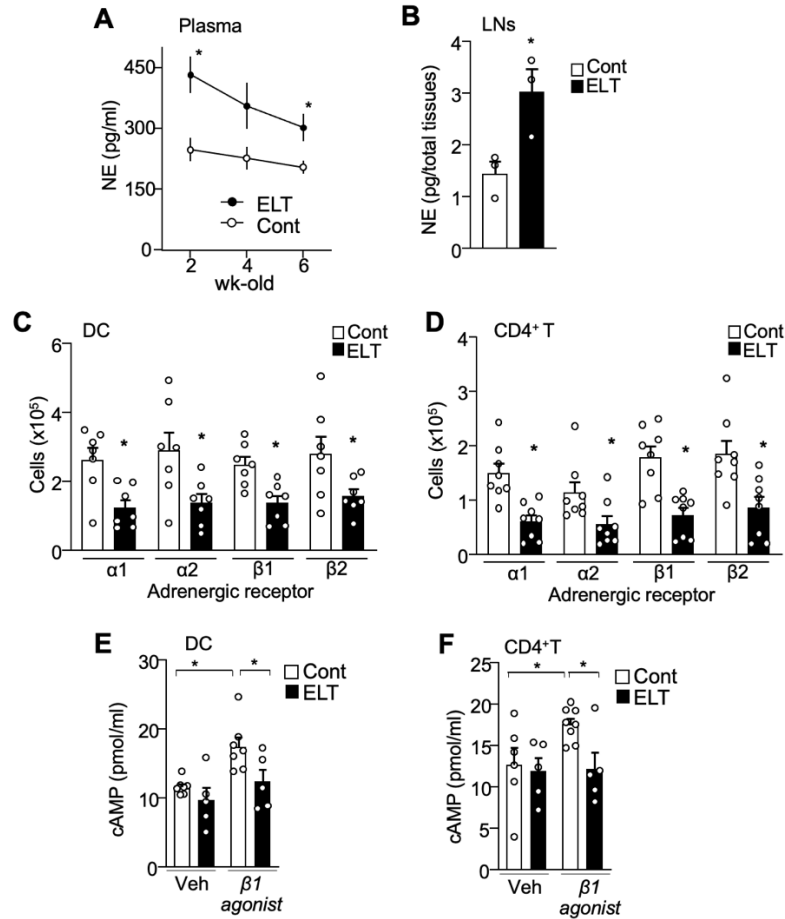


Fig. 3.6. ELT downregulates adrenergic signaling in immune cells. (A) Plasma levels of norepinephrine (NE) in 2-, 4-, and 6-week-old control and ELT mice (Cont-2wk-old: $n=5$, Cont-4wk-old: $n=7$, Cont-6wk-old: $n=8$, ELT-2wk-old: $n=4$, ELT-4wk-old: $n=4$, ELT-6wk-old: $n=9$). (B) Inguinal and axillary lymph node levels of norepinephrine (NE) in 4-week-old control and ELT mice ($n=3$ animals/group). (C, D) Number of (C) dendritic cells (DC, $n=7$ animals/group) and (D) CD4⁺T cells ($n=8$ animals/group) expressing adrenergic receptors: $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$ in lymph nodes of 3–4-week-old control and ELT mice. (E, F) Amount of cAMP derived from DC and CD4⁺T cells treated with vehicle or $\beta 1$ AR agonist (Cont-DC-Veh: $n=7$, ELT-DC-Veh: $n=5$, Cont-DC- $\beta 1$ agonist: $n=7$, ELT-DC- $\beta 1$ agonist: $n=5$, Cont-CD4⁺T-Veh: $n=6$, ELT-CD4⁺T-Veh: $n=5$, Cont-CD4⁺T- $\beta 1$ agonist: $n=8$, ELT-CD4⁺T- $\beta 1$ agonist: $n=5$). Immune cells were isolated from control and ELT mice without EAE. Male mice were used for experiments. Each dot represents averaged data per animal. Data is represented as mean $\pm$ SEM. Student's t-test, * $P < 0.05$.

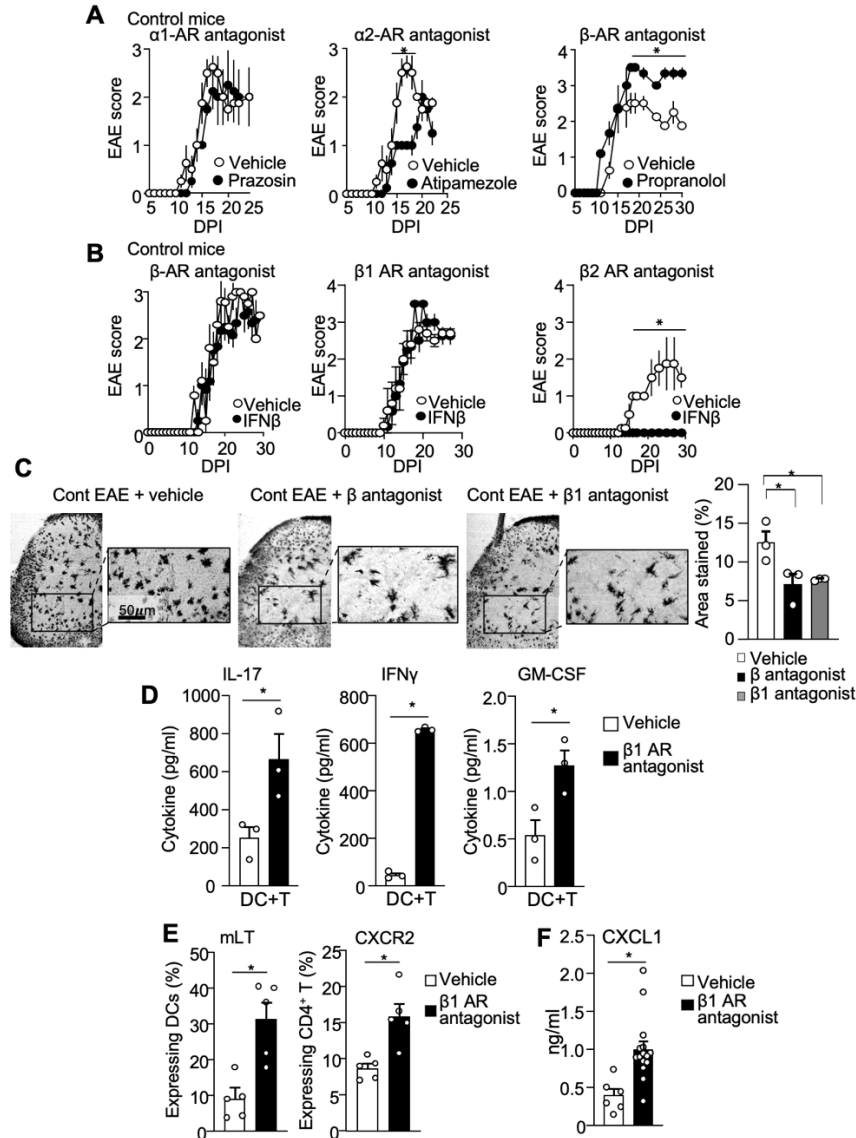


Fig. 3.7. β 1 adrenergic receptor antagonism is sufficient to reproduce the ELT-induced EAE phenotypes. (A) EAE scores of control mice with intraperitoneal injections of α 1-AR antagonist (prazosin, 5 mg/kg), α 2-AR antagonist (atipamezole, 5 mg/kg), or β -AR antagonist (propranolol, 5 mg/kg) with vehicle control (n=4 animals/group). All drugs were treated from 0 dpi to 10 dpi every other day. (B) EAE scores of IFN β -treated control EAE mice subjected to β -AR antagonist (propranolol, 5 mg/kg), β 1-AR antagonist (metoprolol tartrate, 5 mg/kg), β 2-AR antagonist (ICI 118,55, 1 mg/kg), or vehicle treatment (β -AR antagonist-Veh: n=5, β -AR antagonist - IFN β : n=5, β 1-AR antagonist-Veh: n=5, β 1-AR antagonist-IFN β : n=6, β 2-AR antagonist-Veh: n=4, β 2-AR antagonist-IFN β : n=4). (C) Representative images and quantification of Golgi-Cox staining in the ventral lumbar spinal cord region of vehicle-treated and β 1-AR antagonist-treated control EAE mice at 30 dpi. Quantification of area stained by Golgi-Cox (n=3 animals/group). (D) Amount of cytokines IL-17, interferon γ (IFN- γ), and GM-CSF in supernatant derived from culture conditions 72 hours post culture initiation. CD4 $^{+}$ T cells were isolated from draining lymph nodes of control EAE mice and mice subjected to β 1-AR antagonist (n=3 animals/group). (E) Percentages of mLT expressing lymph node DCs and CXCR2 expressing CD4 $^{+}$ T cells from EAE mice with β 1-AR antagonist or vehicle treatment (n=5 animals/group). (F) Serum levels of CXCL1 at 10 dpi (n=4 animals/group). Male mice were used for experiments. Each dot represents averaged data per animal. Data is represented as mean $\pm$ SEM. Student's t-test, *P < 0.05.

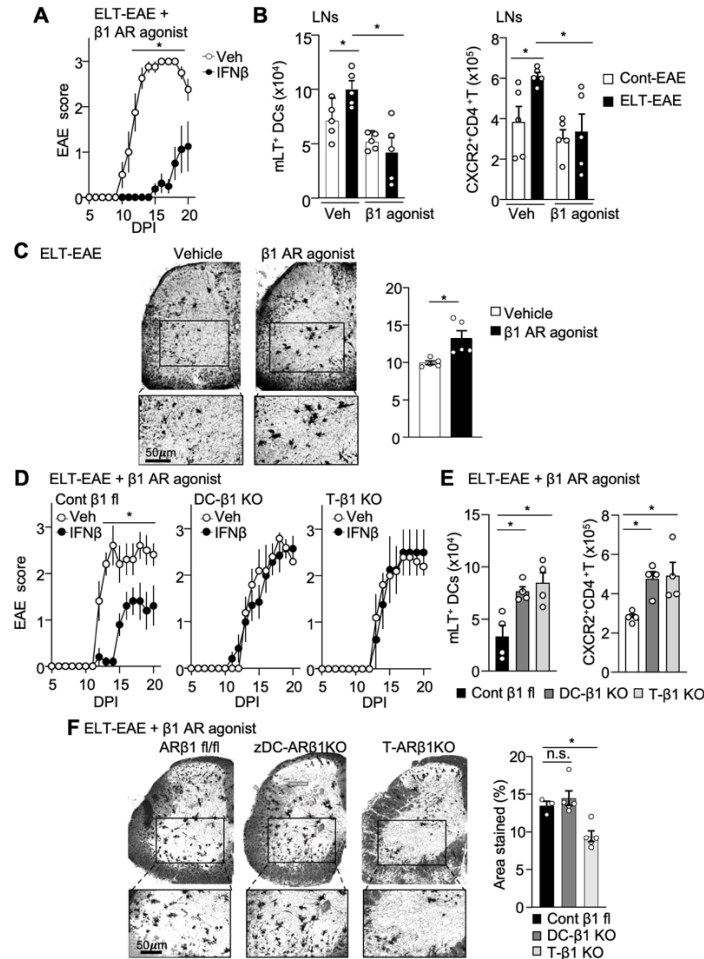


Fig. 3.8. β 1-AR agonist treatment is sufficient to rescue ELT-EAE subtype. (A) EAE scores of IFN β -treated ELT-EAE mice subjected to β 1-AR agonist (Xamoterol, 3 mg/kg) treatment (ELT- β 1-AR agonist-Veh: n=4, ELT- β 1-AR agonist-IFN β : n=8). IFN β was treated from 0 dpi to 9 dpi once every 3 days. Xamoterol was treated from 0 dpi to 10 dpi every other day. (B) Absolute number of mLT expressing lymph node DCs and CXCR2 expressing CD4⁺T cells from EAE mice with β 1-AR agonist or vehicle treatment (n=5 animals/group). (D) Golgi-Cox stained lumbar spinal cord sections of ELT EAE mice treated either with vehicle or β 1-AR agonist isolated at 30 dpi with quantification of area stained/per field. (n=5 animals/group). (D) EAE scores of IFN β -treated control β 1-AR fl ELT-EAE mice, dendritic cell (DC)-specific β 1-AR knockout ELT-EAE mice, and T cell-specific β 1-AR knockout ELT-EAE mice subjected to β 1-AR agonist (Xamoterol, 3 mg/kg) treatment (ELT- β 1-AR agonist-Cont-Veh: n=5, ELT- β 1-AR agonist-Cont-IFN β : n=5, ELT- β 1-AR agonist-DC- β 1KO-Veh: n=5, ELT- β 1-AR agonist-DC- β 1KO-IFN β : n=7, ELT- β 1-AR agonist-T- β 1KO-Veh: n=5, ELT- β 1-AR agonist-T- β 1KO-IFN β : n=5). Interferon β was treated from 0 dpi to 9 dpi once every 3 days. Xamoterol was treated from 0 dpi to 10 dpi every other day. (E) Absolute number of mLT expressing lymph node DCs and CXCR2 expressing CD4⁺T cells from control β 1-AR fl ELT-EAE mice, dendritic cell (DC)-specific β 1-AR knockout ELT-EAE mice, and T cell-specific β 1-AR knockout ELT-EAE mice subjected to β 1-AR agonist (Xamoterol, 3 mg/kg) treatment (n=4 animals/group). (F) Golgi-Cox stained lumbar spinal cord sections of ELT EAE mice treated with β 1-AR agonist of different transgene knockout conditions isolated at 30 dpi with quantification of area stained/per field. (ELT- β 1-AR agonist-Cont: n=3, ELT- β 1-AR agonist-DC- β 1KO: n=5, ELT- β 1-AR agonist-T- β 1KO: n=5). Male mice were used for experiments. Each dot represents averaged data per animal. Data is represented as mean $\pm$ SEM. Student's t-test, *P < 0.05.

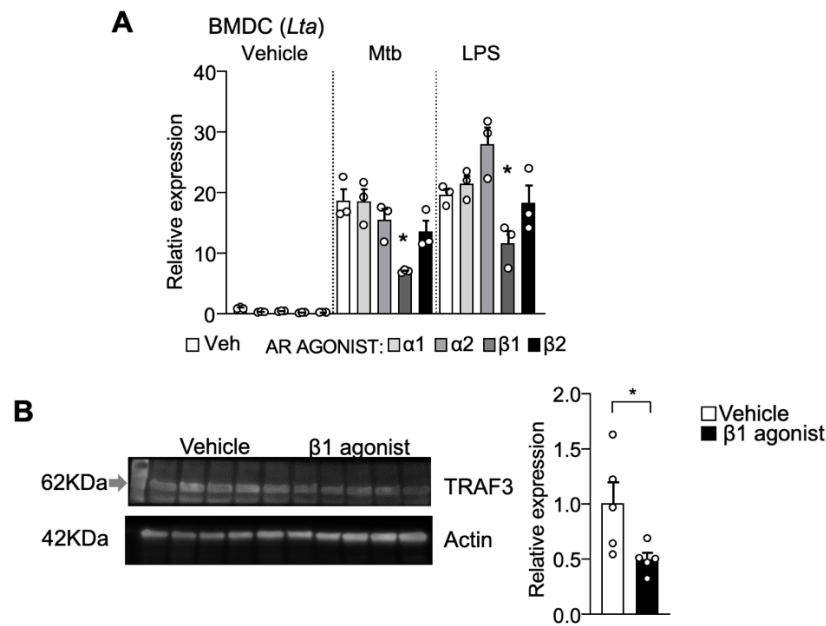


Fig. 3.9. $\beta 1$ -AR agonist treatment prevents upregulation of mLT in BMDC. (A) Relative expression of *Lta* mRNA in BMDC treated with adrenergic agonists and/or LPS and Mtb in vitro ($n=3$ per group). Vehicle or AR agonists ($100 \mu\text{M}$) were pretreated to BMDC for 18 h. Then LPS ($1 \mu\text{g/ml}$) or Mtb ($100 \mu\text{g/ml}$) was treated for 3h. (B) Representative western blot and quantification for TRAF3 in BMDC 18 h after vehicle or $\beta 1$ -AR agonist ($100 \mu\text{M}$). $n=5$ replicates/group. Each dot represents averaged data per animal. Data is represented as mean $\pm$ SEM. Student's t-test, $*P < 0.05$.

3.6 REFERENCES

1. Hunter SF. Overview and diagnosis of multiple sclerosis. *Am J Manag Care* 22, s141-150 (2016).
2. Quintana FJ, et al. Antigen microarrays identify unique serum autoantibody signatures in clinical and pathologic subtypes of multiple sclerosis. *Proc Natl Acad Sci U S A* 105, 18889-18894 (2008).
3. Lassmann H, Bruck W, Lucchinetti C. Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. *Trends Mol Med* 7, 115-121 (2001).
4. Disanto G, et al. Heterogeneity in multiple sclerosis: scratching the surface of a complex disease. *Autoimmune Dis* 2011, 932351 (2010).
5. Cepok S, et al. Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis. *J Clin Invest* 115, 1352-1360 (2005).
6. Ascherio A, Munger KL, Simon KC. Vitamin D and multiple sclerosis. *Lancet Neurol* 9, 599-612 (2010).
7. Hedstrom AK, Baarnhielm M, Olsson T, Alfredsson L. Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis. *Neurology* 73, 696-701 (2009).
8. Ghadirian P, Dadgostar B, Azani R, Maisonneuve P. A case-control study of the association between socio-demographic, lifestyle and medical history factors and multiple sclerosis. *Can J Public Health* 92, 281-285 (2001).
9. Ghadirian P, Jain M, Ducic S, Shatenstein B, Morisset R. Nutritional factors in the aetiology of multiple sclerosis: a case-control study in Montreal, Canada. *Int J Epidemiol* 27, 845-852 (1998).

10. Nisipeanu P, Korczyn AD. Psychological stress as risk factor for exacerbations in multiple sclerosis. *Neurology* 43, 1311-1312 (1993).
11. Spitzer C, et al. Childhood trauma in multiple sclerosis: a case-control study. *Psychosom Med* 74, 312-318 (2012).
12. Dube SR, Fairweather D, Pearson WS, Felitti VJ, Anda RF, Croft JB. Cumulative childhood stress and autoimmune diseases in adults. *Psychosom Med* 71, 243-250 (2009).
13. Manni L, Micera A, Pistillo L, Aloe L. Neonatal handling in EAE-susceptible rats alters NGF levels and mast cell distribution in the brain. *Int J Dev Neurosci* 16, 1-8 (1998).
14. Meagher MW, et al. Neonatal maternal separation alters immune, endocrine, and behavioral responses to acute Theiler's virus infection in adult mice. *Behav Genet* 40, 233-249 (2010).
15. Stephan M, Straub RH, Breivik T, Pabst R, von Horsten S. Postnatal maternal deprivation aggravates experimental autoimmune encephalomyelitis in adult Lewis rats: reversal by chronic imipramine treatment. *Int J Dev Neurosci* 20, 125-132 (2002).
16. Columba-Cabezas S, Iaffaldano G, Chiarotti F, Alleva E, Cirulli F. Early handling increases susceptibility to experimental autoimmune encephalomyelitis (EAE) in C57BL/6 male mice. *J Neuroimmunol* 212, 10-16 (2009).
17. Cattaneo A, et al. Inflammation and neuronal plasticity: a link between childhood trauma and depression pathogenesis. *Front Cell Neurosci* 9, 40 (2015).

18. Khulan B, et al. Epigenomic profiling of men exposed to early-life stress reveals DNA methylation differences in association with current mental state. *Transl Psychiatry* 4, e448 (2014).
19. Huang QQ, et al. Neonatal genetics of gene expression reveal potential origins of autoimmune and allergic disease risk. *Nat Commun* 11, 3761 (2020).
20. Danese A, S JL. Psychoneuroimmunology of Early-Life Stress: The Hidden Wounds of Childhood Trauma? *Neuropsychopharmacology* 42, 99-114 (2017).
21. Agorastos A, Pervanidou P, Chrousos GP, Baker DG. Developmental Trajectories of Early Life Stress and Trauma: A Narrative Review on Neurobiological Aspects Beyond Stress System Dysregulation. *Front Psychiatry* 10, 118 (2019).
22. Quan N, Whiteside M, Herkenham M. Time course and localization patterns of interleukin-1beta messenger RNA expression in brain and pituitary after peripheral administration of lipopolysaccharide. *Neuroscience* 83, 281-293 (1998).
23. Vitkovic L, Konsman JP, Bockaert J, Dantzer R, Homburger V, Jacque C. Cytokine signals propagate through the brain. *Mol Psychiatry* 5, 604-615 (2000).
24. Sivaguru M, Khaw YM, Inoue M. A Confocal Reflection Super-Resolution Technique to Image Golgi-Cox Stained Neurons. *J Microsc* 275, 115-130 (2019).
25. Ehara A, Ueda S. Application of Fluoro-Jade C in acute and chronic neurodegeneration models: utilities and staining differences. *Acta Histochem Cytochem* 42, 171-179 (2009).
26. Sellebjerg F, et al. Identification of new sensitive biomarkers for the in vivo response to interferon-beta treatment in multiple sclerosis using DNA-array evaluation. *Eur J Neurol* 16, 1291-1298 (2009).

27. Inoue M, et al. An interferon-beta-resistant and NLRP3 inflammasome-independent subtype of EAE with neuronal damage. *Nat Neurosci* 19, 1599-1609 (2016).
28. Inoue M, Williams KL, Gunn MD, Shinohara ML. NLRP3 inflammasome induces chemotactic immune cell migration to the CNS in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 109, 10480-10485 (2012).
29. Inoue M, et al. Interferon-beta therapy against EAE is effective only when development of the disease depends on the NLRP3 inflammasome. *Sci Signal* 5, ra38 (2012).
30. Wu Q, Wang Y, Wang J, Hedgeman EO, Browning JL, Fu YX. The requirement of membrane lymphotoxin for the presence of dendritic cells in lymphoid tissues. *J Exp Med* 190, 629-638 (1999).
31. Loria AS, Brands MW, Pollock DM, Pollock JS. Early life stress sensitizes the renal and systemic sympathetic system in rats. *Am J Physiol Renal Physiol* 305, F390-395 (2013).
32. Brenhouse HC, Danese A, Grassi-Oliveira R. Neuroimmune Impacts of Early-Life Stress on Development and Psychopathology. *Curr Top Behav Neurosci*, (2018).
33. Wohleb ES, et al. beta-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. *J Neurosci* 31, 6277-6288 (2011).
34. Silverman MN, Pearce BD, Biron CA, Miller AH. Immune modulation of the hypothalamic-pituitary-adrenal (HPA) axis during viral infection. *Viral Immunol* 18, 41-78 (2005).

35. Bellinger DL, Lorton D. Sympathetic Nerve Hyperactivity in the Spleen: Causal for Nonpathogenic-Driven Chronic Immune-Mediated Inflammatory Diseases (IMIDs)? *Int J Mol Sci* 19, (2018).
36. Tholanikunnel BG, Granneman JG, Malbon CC. The M(r) 35,000 beta-adrenergic receptor mRNA-binding protein binds transcripts of G-protein-linked receptors which undergo agonist-induced destabilization. *J Biol Chem* 270, 12787-12793 (1995).
37. Bouvier M, et al. Two distinct pathways for cAMP-mediated down-regulation of the beta 2-adrenergic receptor. Phosphorylation of the receptor and regulation of its mRNA level. *J Biol Chem* 264, 16786-16792 (1989).
38. Sekio M, Seki K. Lipopolysaccharide-induced depressive-like behavior is associated with alpha(1)-adrenoceptor dependent downregulation of the membrane GluR1 subunit in the mouse medial prefrontal cortex and ventral tegmental area. *Int J Neuropsychopharmacol* 18, (2014).
39. McCormick PN, Fletcher PJ, Wilson VS, Remington GJ. The adrenergic alpha2 antagonist atipamezole alters the behavioural effects of pramipexole and increases pramipexole concentration in blood plasma. *Life Sci* 151, 300-304 (2016).
40. Oberbeck R, van Griensven M, Nickel E, Tschernig T, Wittwer T, Pape HC. Influence of beta-adrenoceptor antagonists on hemorrhage-induced cellular immune suppression. *Shock* 18, 331-335 (2002).
41. Yang W, Shibamoto T, Kuda Y, Zhang T, Tanida M, Kurata Y. beta(2)-Adrenoceptor Blockade Deteriorates Systemic Anaphylaxis by Enhancing Hyperpermeability in Anesthetized Mice. *Allergy Asthma Immunol Res* 10, 52-61 (2018).

42. Coutellier L, Ardestani PM, Shamloo M. beta1-adrenergic receptor activation enhances memory in Alzheimer's disease model. *Ann Clin Transl Neurol* 1, 348-360 (2014).
43. Limmroth V, Putzki N, Kachuck NJ. The interferon beta therapies for treatment of relapsing-remitting multiple sclerosis: are they equally efficacious? A comparative review of open-label studies evaluating the efficacy, safety, or dosing of different interferon beta formulations alone or in combination. *Ther Adv Neurol Disord* 4, 281-296 (2011).
44. Grossman I, et al. Pharmacogenetics of glatiramer acetate therapy for multiple sclerosis reveals drug-response markers. *Pharmacogenet Genomics* 17, 657-666 (2007).
45. Villar LM, et al. Immunological markers of optimal response to natalizumab in multiple sclerosis. *Arch Neurol* 69, 191-197 (2012).
46. Murgatroyd C, Spengler D. Epigenetic programming of the HPA axis: early life decides. *Stress* 14, 581-589 (2011).
47. Heim C, et al. Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. *JAMA* 284, 592-597 (2000).
48. O'Mahony SM, et al. Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biol Psychiatry* 65, 263-267 (2009).
49. Fagundes CP, Glaser R, Kiecolt-Glaser JK. Stressful early life experiences and immune dysregulation across the lifespan. *Brain Behav Immun* 27, 8-12 (2013).

50. Hyland NP, O'Mahony SM, O'Malley D, O'Mahony CM, Dinan TG, Cryan JF. Early-life stress selectively affects gastrointestinal but not behavioral responses in a genetic model of brain-gut axis dysfunction. *Neurogastroenterol Motil* 27, 105-113 (2015).
51. Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci* 10, 434-445 (2009).
52. Warren S, Greenhill S, Warren KG. Emotional stress and the development of multiple sclerosis: case-control evidence of a relationship. *J Chronic Dis* 35, 821-831 (1982).
53. Vetulani J. Early maternal separation: a rodent model of depression and a prevailing human condition. *Pharmacol Rep* 65, 1451-1461 (2013).
54. Meaney MJ, et al. The effects of neonatal handling on the development of the adrenocortical response to stress: implications for neuropathology and cognitive deficits in later life. *Psychoneuroendocrinology* 16, 85-103 (1991).
55. Girardi CE, Zanta NC, Suchecki D. Neonatal stress-induced affective changes in adolescent Wistar rats: early signs of schizophrenia-like behavior. *Front Behav Neurosci* 8, 319 (2014).
56. Hsu FC, Zhang GJ, Raol YS, Valentino RJ, Coulter DA, Brooks-Kayal AR. Repeated neonatal handling with maternal separation permanently alters hippocampal GABAA receptors and behavioral stress responses. *Proc Natl Acad Sci U S A* 100, 12213-12218 (2003).
57. Burenkova OV, Aleksandrova EA, Zarayskaya IY. Effects of early-life stress and HDAC inhibition on maternal behavior in mice. *Behav Neurosci* 133, 39-49 (2019).

58. Wilber AA, Lin GL, Wellman CL. Neonatal corticosterone administration impairs adult eyeblink conditioning and decreases glucocorticoid receptor expression in the cerebellar interpositus nucleus. *Neuroscience* 177, 56-65 (2011).
59. Codarri L, et al. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* 12, 560-567 (2011).
60. Sonderegger I, Iezzi G, Maier R, Schmitz N, Kurrer M, Kopf M. GM-CSF mediates autoimmunity by enhancing IL-6-dependent Th17 cell development and survival. *J Exp Med* 205, 2281-2294 (2008).
61. Wang C, Wen T, Routy JP, Bernard NF, Sekaly RP, Watts TH. 4-1BBL induces TNF receptor-associated factor 1-dependent Bim modulation in human T cells and is a critical component in the costimulation-dependent rescue of functionally impaired HIV-specific CD8 T cells. *J Immunol* 179, 8252-8263 (2007).
62. Lanier LL, et al. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. *J Immunol* 154, 97-105 (1995).
63. Agasing AM, Gawde S, Kumar G, Turner E, Axtell RC. B cell function impacts the efficacy of IFN-beta therapy in EAE. *J Neuroimmunol* 338, 577106 (2020).
64. Axtell RC, et al. T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis. *Nat Med* 16, 406-412 (2010).
65. Boro M, Balaji KN. CXCL1 and CXCL2 Regulate NLRP3 Inflammasome Activation via G-Protein-Coupled Receptor CXCR2. *J Immunol* 199, 1660-1671 (2017).

66. Jurgens T, et al. Reconstruction of single cortical projection neurons reveals primary spine loss in multiple sclerosis. *Brain* 139, 39-46 (2016).
67. Khaw YM, Cunningham C, Tierney A, Sivaguru M, Inoue M. Neutrophil-selective deletion of *Cxcr2* protects against CNS neurodegeneration in a mouse model of multiple sclerosis. *J Neuroinflammation* 17, 49 (2020).
68. Bellier JP, Kimura H. Acetylcholine synthesis by choline acetyltransferase of a peripheral type as demonstrated in adult rat dorsal root ganglion. *J Neurochem* 101, 1607-1618 (2007).
69. Murphy AC, Lalor SJ, Lynch MA, Mills KH. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. *Brain Behav Immun* 24, 641-651 (2010).
70. Zhang B, Yang N, Mo ZM, Lin SP, Zhang F. IL-17A Enhances Microglial Response to OGD by Regulating p53 and PI3K/Akt Pathways with Involvement of ROS/HMGB1. *Front Mol Neurosci* 10, 271 (2017).
71. Browne TC, McQuillan K, McManus RM, O'Reilly JA, Mills KH, Lynch MA. IFN- γ Production by amyloid beta-specific Th1 cells promotes microglial activation and increases plaque burden in a mouse model of Alzheimer's disease. *J Immunol* 190, 2241-2251 (2013).
72. Irwin M. Stress-induced immune suppression. Role of the autonomic nervous system. *Ann N Y Acad Sci* 697, 203-218 (1993).
73. Zoukos Y, Kidd D, Woodroffe MN, Kendall BE, Thompson AJ, Cuzner ML. Increased expression of high affinity IL-2 receptors and beta-adrenoceptors on peripheral blood

- mononuclear cells is associated with clinical and MRI activity in multiple sclerosis. *Brain* 117 (Pt 2), 307-315 (1994).
74. Mackenzie FJ, Leonard JP, Cuzner ML. Changes in lymphocyte beta-adrenergic receptor density and noradrenaline content of the spleen are early indicators of immune reactivity in acute experimental allergic encephalomyelitis in the Lewis rat. *J Neuroimmunol* 23, 93-100 (1989).
 75. Araujo LP, et al. The Sympathetic Nervous System Mitigates CNS Autoimmunity via beta2-Adrenergic Receptor Signaling in Immune Cells. *Cell Rep* 28, 3120-3130 e3125 (2019).
 76. Estrada LD, Agac D, Farrar JD. Sympathetic neural signaling via the beta2-adrenergic receptor suppresses T-cell receptor-mediated human and mouse CD8(+) T-cell effector function. *Eur J Immunol* 46, 1948-1958 (2016).
 77. Loza MJ, Foster S, Peters SP, Penn RB. Beta-agonists modulate T-cell functions via direct actions on type 1 and type 2 cells. *Blood* 107, 2052-2060 (2006).
 78. Qian L, et al. beta2-adrenergic receptor activation prevents rodent dopaminergic neurotoxicity by inhibiting microglia via a novel signaling pathway. *J Immunol* 186, 4443-4454 (2011).
 79. Tanaka KF, Kashima H, Suzuki H, Ono K, Sawada M. Existence of functional beta1- and beta2-adrenergic receptors on microglia. *J Neurosci Res* 70, 232-237 (2002).
 80. Yamasaki R, et al. Differential roles of microglia and monocytes in the inflamed central nervous system. *J Exp Med* 211, 1533-1549 (2014).
 81. Huang YM, et al. Altered phenotype and function of blood dendritic cells in multiple sclerosis are modulated by IFN-beta and IL-10. *Clin Exp Immunol* 124, 306-314 (2001).

82. Tabansky I, et al. Targeting DEC-205(-)DCIR2(+) dendritic cells promotes immunological tolerance in proteolipid protein-induced experimental autoimmune encephalomyelitis. *Mol Med* 24, 17 (2018).
83. Mani BK, Osborne-Lawrence S, Vijayaraghavan P, Hepler C, Zigman JM. beta1-Adrenergic receptor deficiency in ghrelin-expressing cells causes hypoglycemia in susceptible individuals. *J Clin Invest* 126, 3467-3478 (2016).
84. Davis BM, Salinas-Navarro M, Cordeiro MF, Moons L, De Groef L. Characterizing microglia activation: a spatial statistics approach to maximize information extraction. *Sci Rep* 7, 1576 (2017).

CHAPTER 4: LYMPH NODE-DERIVED ESTROGEN MODULATES DISEASE

PHENOTYPE VIA ESTROGEN RECEPTOR ALPHA³

4.1 INTRODUCTION

In the last three decades, the perception of multiple sclerosis (MS) has changed from an idiopathic disease with symptomatic treatments to a complex disorder with profound heterogeneity. Individuals with MS present distinct clinical characteristics, disease trajectory, immune cell profiles, and responses to drug treatment^{1,2}. Because MS results in permanent damage of the central nervous system (CNS), accurate diagnosis and treatment based on a comprehensive understanding of MS pathogenesis and heterogeneity will prevent time wasted on ineffective prescription and improve treatment outcome. MS can be replicated in animal models, such as experimental autoimmune encephalomyelitis (EAE), that exhibits a wide array of heterogeneity in response to environmental and biological factors^{3,4}, providing an avenue to better understand this complex disease. Estrogens are biological factors that can influence MS clinical trajectory. People with MS suffer significantly lower relapse rates during pregnancy when estrogen levels rise. Interestingly, relapse rate is lowest during the third trimester when circulating estrogen is the most abundant⁵. The inverse relationship between endogenous estrogen level and MS development held true in post-partum women who report low endogenous estrogen levels and greater than baseline susceptibility to relapse episodes⁶.

Immune cells are thought to be exposed to endogenous estrogens in circulation^{7,8}. While gonads are the main source of circulating endogenous estrogen, our recent work demonstrated that lymph nodes (LNs) are sites of de novo estrogen synthesis in mice⁹ and pigs¹⁰. LNs are

³ This chapter contains unpublished data and is submitted to journal(s) for peer review as of November 2021.

critical regions where immune cells exchange information and program cellular functions. In MS and EAE, events that occur in the LN, such as antigen-presentation and T cell activation, are thought to be key in disease pathophysiology. Most immune cells involved in MS and EAE development, such as T cells and dendritic cells, express estrogen receptors^{11,12}. Importantly, local estrogen levels in LNs are 3-5 times greater than that of circulating blood⁹. Thus, estrogen regulation of immune responses may be much more extensive than previously imagined.

The present study sought to understand MS heterogeneity by investigating the influence of endogenous estrogen signaling on EAE phenotypic alterations in the context of estrogen receptor α (ER α) signaling. Our disease phenotype-focused investigation involves monitoring [1] symptoms of paresis, [2] sensitivity to therapeutic drug treatment¹³, [3] CNS pathology, and [4] immune cell signatures¹⁴. Our findings identify estrogen synthesized in LNs, but not gonads, as a key factor in driving heterogeneity in EAE phenotypes.

4.2 METHODS

Animals

Six-to-eight-week old healthy WT male and female mice of the C57BL/6 background were purchased from Jackson Laboratories and maintained under pathogen-free conditions in closed breeding colonies. Rodent diet and water were provided ad libitum. ER α ^{-/-} mice, ER α -floxed mice, and Cyp19^{-/-} mice were previously reported given to us by Dr. Chemyong Ko⁹. CD11c-ER α ^{-/-} (cER α ^{-/-}) mice, which are DC-specific ER α ^{-/-} mice, were generated by crossing ER α -floxed mice with Itgax-Cre mice (CD11c-Cre, Jackson Laboratory #7567). Lck-ER α ^{-/-} mice, which are T cell-specific ER α ^{-/-} mice, were generated by crossing ER α -floxed mice with Lck-Cre mice (Jackson Laboratory #3802). In the absence of cre recombinase activity, mice homozygous

for ER α -flox were indistinguishable from wild-type littermates. Cyp19-RFP mice were generated by crossing Cyp19-cre mice with RFP(Tomato)-floxed mice. Tcra mice were purchased from Jackson Laboratories (#002116). All the mice were kept group-housed (3-5 mice per cage) in a pathogen-free facility with 12h each of a light-dark cycle in Veterinary Medicine Basic Sciences Building at University of Illinois at Urbana-Champaign. This study was approved by the University of Illinois at Institutional Animal Care and Use Committee (Protocol number 19171).

Reagents

Recombinant IFN β used in EAE treatment was human recombinant interferon-1b (Betaseron, Bayer). Lymphotoxin β receptor (mLT β R)-Fc fusion protein was purchased from BPS Bioscience. MOG35-55 peptide was synthesized by United Peptides. LPS was obtained from InvivoGen. Heat-killed Mycobacteria Tuberculosis (Mtb) was purchased from BD Difco. Enzyme-linked immunosorbent assay (ELISA) kit for the detection of estradiol was purchased from DRG International Inc. Antibodies for flow cytometry were purchased from Biolegend or eBioscience.

Induction of active EAE

In six- to eight-week-old mice, EAE was induced by subcutaneous (s.c) injection of myelin oligodendrocyte glycoprotein (MOG)35-55 peptide (100 μ g/mouse) emulsified in complete Freund's adjuvant (CFA) including heat-killed Mycobacteria Tuberculosis (BD Difco, #DF3114-33-8, 200 μ g/mouse) on day 0, along with i.p. injection of pertussis toxin (List Biological 181, 200 ng/mouse) on days 0 and 2. EAE score is defined as follows: 0.5: partial tail limpness, 1:

tail limpness, 1.5: reversible impaired righting reflex, 2: impaired righting reflex, 2.5: one hind limb paralysis, 3: both hind limb paralysis, 3.5: both hind and one forelimb paralysis, 4: hind and fore limb paralysis, 5: death.

Pharmaceutical treatment of mice

Mice were i.p. injected with IFN β (47 μ g/kg) every other day from day 0 to day 10. LT β R-Fc (7.5 μ g/kg) was i.p. injected every other day from day 0.

Golgi-cox staining and analyses

At chronic- and late-phase disease (~33 and 60 dpi), animals were perfused with 4% paraformaldehyde (PFA) with no post-fixing then processed according to FD Rapid Golgi staining kit protocol (FD Neurotechnologies, #PK401A) which involves 17 days of incubation in metallic solution in the dark at RT. Spinal cords were submerged in 30% sucrose solution for 12-24 hours, frozen in Tissue-tek OCT compound (Sakura Finetek), and stored in -80°C until sectioning. Spinal cords were transversely cut into 50 μ m sections using a cryostat (Reichert Jung Cryocut 1800 Cryostat) and mounted onto poly-L-lysine-coated glass slides. At least eight sections were developed for Golgi-Cox neuronal staining performed according to the manufacturer's protocol. After drying, slides were covered with resinous promount and 0.17 μ m coverslips. Golgi-Cox area stained analysis: Bright field images were taken using DM2000 (Leica). To obtain percentage area stained, 10x brightfield micrographs of selected region (ventral horns) were analyzed using ImageJ. Spine density analysis: To obtain spine density data, z-stacks of ventral horn region of Golgi-Cox-stained lumbar spinal cord sections were collected in a random fashion using a Nikon A1 confocal scanning microscope under the CRSR

modality with minimized pinhole at 0.3 AU. A 405-nm continuous wave laser was used, and the reflectance mirror (BS 20/80) was applied. Images were acquired using Plan-Apochromat 100x/1.49 NA Oil objective. Pixel dimensions were: x: 0.0628 μm ; y: 0.0628 μm ; z: 0.075 μm . Using IMARIS, Gaussian filter and background subtraction were applied to z-stacks of cropped individual dendrites prior to tracing filaments. To generate the 3D reconstructions for spine analysis, dendritic regions that was 40-60 μm in length and void of dendritic branch points and crossing neurites were selected. To validate the automated spine detection, spine density was calculated within the same dendritic regions using manual analyses. Twenty dendrites in the lumbar spinal cord ventral horn regions in each animal were randomly selected for dendritic spine analysis. Sholl analysis: Microscopic images for analysis were captured using a 40 $\times$ objective lens (Leica DM2000). Twenty individual neurons in the lumbar spinal cord ventral horn regions in each animal were selected for Sholl analysis. The software ImageJ was used for quantification. Neuronal soma was selected manually using the 'polygon selection' tool to remove background signals and signals derived from cells other than the selected neuronal soma. To initiate Sholl analysis plugin, image was converted into 8-bit and a standard threshold was applied. Distance between rings were set at 10 micron with the end radius being at 200 micron.

Luxol fast blue staining and analysis

Transverse sections (30 μm) of frozen spinal cord from mice were mounted onto poly-L-lysine coated slides and left to dry overnight. Sections were hydrated with 95% ethyl alcohol then placed in 1:1 ethanol/chloroform for 5 minutes. Sections were rehydrated with 95% ethyl alcohol then incubated in 0.1% Luxol fast blue solution for 4 hours. 95% ethanol was used to wash away excess dye solution, followed by rinsing step with distilled water. 0.05% Li_2CO_3

solution was applied to sections for 30 seconds, followed by a wash step with 70% ethanol until a sharp contrast between gray and white matter becomes apparent. The sections were immersed in 90%, 95%, and 100% ethanol gradient dehydration washes, followed by the application of a coverslip. For image analysis, grey scale images were opened in ImageJ and an automatic threshold was applied. Region of interest was manually drawn to restrict analysis to the area of white matter regions. Quantitative analyses measured the area of Luxol fast blue staining and normalized this area to the total analysis area. Percentage demyelination was calculated by dividing percentage area stained within ROI over total ROI area.

Flow cytometry

LNs (axillary, bronchial, and inguinal) and spleen were removed, and then mashed with slide glass. Minced spleen and lymph nodes were passed through a mesh filter. After washing, cells were suspended in ice-cold staining buffer (sterile PBS containing 2% FBS). Cells were blocked with anti-CD16/32 on ice for 10 min. After blocking, 1×10^6 cells were suspended in 0.1 ml of ice-cold staining antibody buffer and stained on ice in the dark for 20 min. After washing twice in washing buffer, samples were acquired on a Cytex Aurora flow cytometer. Gates were determined using unstained and single-stained samples obtained from the same tissue of origin. Results were analyzed using FCS Express 6 flow cytometry software (De Novo Software).

Generation of bone marrow-derived DC

Bone marrow was isolated from naïve C57BL/6 mice. Isolated cells were treated with recombinant GM-CSF (200 ng/ml) in complete RPMI for 7 days to generate bone marrow-derived DCs (BMDCs). Half of the solution was reconstituted at day 4. For qPCR experiments,

BMDC (10⁶ cells) were treated with 4,4'-[4-Propyl-[1H]-pyrazole-1,3,5-triyl] (PPT) (TOCRIS Bioscience 1426) for 18 h at 37°C and then treated with LPS (B4 Sigma, #L4391) for 3 h at 37°C. As control, BMDCs were treated with vehicle (DMSO 5%) instead of PPT and vehicle (PBS) instead of LPS. For IHC experiments, BMDC (2.5x10⁵ cells) were pretreated with vehicle or PPT for 18 h and then treated with vehicle or LPS for 2 h. For immunoprecipitation, BMDC (10⁷ cells) were treated with vehicle or PPT for 3, 6, or 9 h. For western blotting analysis, BMDC (5x10⁶ cells) were treated with vehicle or PPT for 18 h.

Immunoprecipitation and western blot analysis

Immunoprecipitation studies of BMDCs (10⁷ cells) were performed using Dynabeads Protein G Immunoprecipitation Kit (Invitrogen, 10007D) according to manufacture protocol with an antibody against TRAF3 (Proteintech, #18099-1-AP, 2 μ g). ER α antibody was then used against immunoprecipitated samples (MilliporeSigma, #06935, 1:2000) to detect ER α protein by Western blotting. Pre-immunoprecipitation samples were used for measuring housekeeping gene, GAPDH (Santa Cruz Biotechnology, #sc-32233, 1:2000). Western blots of TRAF3 and actin were performed using specific antibodies (Abnova Corporations, #89-114-098, 1:1000 for TRAF3 and MyBiosource, #MBS8507421, 1:500 for actin). Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (MyBioSource, #MBS440123, 1:20,000) and HRP-conjugated anti-mouse secondary antibody (MyBioSource, #MBS440122, 1:10,000) were used to detect signals, which were visualized by enhanced chemiluminescence (Peace). Chemiluminescence signal was captured by FluorChem R Imager (ProteinSimple). ER α and Ub expression was normalized by GAPDH expression, because TRAF3 expression alters after PPT treatment.

qPCR analysis

Dendritic cells were isolated by positive selection using selection beads (STEM CELL, EasySep™ Mouse CD11c Positive Selection Kit II). Total RNA was extracted from cells with TRIsure (Bioline, #BIO-38033). cDNA synthesis was performed with qScript cDNA SuperMix (Quanta). qPCR analysis was performed with KAPA (KAPA Bio Systems) with an initial denaturing step at 95°C for three minutes, followed by 35 cycles of denaturation phase at 94°C for 3 seconds and annealing and extension phase at 60°C for 30 seconds. The relative amounts of qPCR products were determined with the $\Delta\Delta C_t$ method to compare relative expression of target genes and housekeeping genes. Expression of β -actin was used as internal control.

Lentivirus transfection

BMDC were lentivirally transfected with Traf3 shRNA (Santacruz, #sc-36712-V) or control shRNA (Santacruz, #sc-108080) expression vector, according to the manufacture's protocol. Three days after the transfection, cells were treated with LPS (100 pg/ml) for 3 h.

IRF3 nucleus translocation

BMDC (5x10⁵ cells) were harvested in 24-well plates on top of RPMI coated glass cover slides. Cells were either pretreated with PPT (10 μ M) or vehicle for 18 h at 37°C and 5% CO₂. Cells were then treated with LPS (100 ng/mL) or vehicle for 2 h. BMDCs were fixed with 4% PFA for 15 minutes at 37°C and permeabilized in 0.25% TritonX in 0.05% Tween-20 in 1xTBS (TBST) for 15 minutes at room temperature. Cells were blocked for 1 h in 2% Bovine Serum Albumin (BSA) in TBST. Cells were then stained with primary antibody IRF3 (Thermo Fisher Scientific, #PA5-87506, 1:200) in 2% BSA in TBST overnight at 4°C. The following day, cells

were stained with secondary antibody Donkey Anti Rabbit Alexa488 (Invitrogen, #R37118, 1:500) in 2% BSA in TBST for 1 h at room temperature. Finally, cells were stained with DAPI (1:10,000) for 1 minute at room temperature. The glass cover slips containing the cells were mounted on glass slides using Prolong Gold (Thermo Fisher Scientific, #P36930) and stored long term at -20°C. Slides were visualized using a Nikon A1 confocal microscope at 60x. Slides were visualized using a Nikon A1 confocal microscope at 60x. To quantify IRF3 nuclear subcellular distribution, DAPI-stained nuclear regions of individual cells were each selected using a polygon drawing tool in ImageJ. Background FITC signal was calculated from cell-free regions and subtracted from calculations. Number of IRF3+ nucleus was determined by manual counting by a single blinded experimenter.

Passive EAE induction by intrathecal transfer of CD4⁺T cells

Six-to eight-week-old WT or ER α ^{-/-} mice were subjected to EAE simultaneously. At 9 dpi, WT or ER α ^{-/-} EAE mice were sacrificed to allow harvesting of splenic CD4⁺T cells. LNs and spleens were dissected and passed through an 80- μ m filter (Falcon) to obtain a single-cell suspension in PBS supplemented with 2% FBS. Cells were centrifuged at 277 g for 5 min at 4°C, and supernatant was discarded. Cells were resuspended in red blood cell lysis buffer [155 mM ammonium chloride (NH₄Cl), 10 mM potassium bicarbonate (KHCO₃), 0.01 mM EDTA pH 8.0] for 3 min at room temperature. CD4⁺T cells were isolated by first removing CD8⁺T cells, B cells, neutrophils, and dendritic cells using biotin-labeled anti-CD8 (#100704, Biolegend), anti-CD19 (#115504, Biolegend), anti-Ly6G (#127604, Biolegend), and anti-CD11c antibodies (#117304, Biolegend) with streptavidin beads (#19860, Stemcell Technologies). Then, CD4⁺T cells were selected using a biotin-labeled anti-CD4 antibody (#127604, Biolegend)

and dextran-coated magnetic particles (#18556, Stemcell Technologies). Isolated CD4⁺T cells (10⁶ cells in 10 μ l per mouse) were adoptively transferred by intrathecal injection to Tcr α ^{-/-} recipient mice that subject EAE induction for 14 days. Mice were also intraperitoneally injected with pertussis toxin on days 0 and 2. On day 14 after CD4⁺T cell transfer, mice were anesthetized and perfused with 4% PFA. Spinal cords were processed for Golgi-Cox staining and section analysis.

RNA-seq analysis

Total RNA was extracted from CD4⁺ T cells isolated from LNs and spleen of WT and ER α ^{-/-} EAE mice at 9 dpi by FACS (CD4⁺CD3⁺DAPI⁻) for RNAseq analysis. RNA from the samples was extracted with QIAGEN RNeasy kit. Library construction and sequencing was done at the Roy J. Carver Biotechnology Center at the University of Illinois Urbana-Champaign using the Ovation RNASeq kit from Tecan and sequencing 100 bp single end reads on one SP lane in a NovaSeq 6000. Samples were demultiplexed and adapter sequences were removed along with any reads shorter than 35 nt. Sequencing quality was assessed by FastQC (version 0.11.8) and average per-base read quality scores were over 30 in all samples so no further trimming/filtering was performed. Salmon version 1.2.0 was used to quasi-map reads to the GRCm39 Annotation 109 transcriptome and quantify the counts for each transcript, then summed to gene level after adjusting for transcript lengths using the tximport package (v1.18.0) in R (v4.0.3). Gene detection threshold was set at 2 cpm (counts per million) in at least two samples. After filtering genes, TMM (trimmed mean of M values) normalization was performed and normalized log2-based count per million values (logCPM) were calculated using edgeR (v3.32.0). Batch effect

was added to the statistical model and differential gene expression was tested using the limma-trend method (limma package v3.46.0).

N2a neuronal cell co-culture with T lymphocyte

N2a cells were a gift from Dr. Keith Kelly (UIUC). N2a cells (1×10^3 cells/well) were cultured on cover slips in 24-well plates in 1% FBS/DMEM before initiating co-culture to initiate neuron differentiation. After 3 days, CD4⁺T cells isolated from spleen and lymph nodes of WT and ER $\alpha^{-/-}$ mice at EAE 9 dpi were added to N2a neuron culture triplicates at a 3:1 cell ratio and incubated for 18 h at 37 °C with 5% carbon dioxide circulation in a sterile incubator. After 24 h, cells were stained with tubulin-III (Tuj1) (Novus Biologicals, #NBP130048, 1:200) cytoskeletal staining with fluorescent secondary antibody. Cells were post-fixed in 4% paraformaldehyde. Coverslips were mounted on glass slides with Prolong Gold and stored at 4 °C until image acquisition. A total of 1412 N2a cells were included in the analyses by using ImageJ by a blinded experimenter.

Surgery

Ovariectomy and ovary transplantation were performed as has been previously reported^{15,16}. At two weeks after surgery, EAE was induced as described above.

Ex vivo imaging of endogenous fluorescence

Cyp19-RFP mice were sacrificed using CO₂ overdose. Mice were dissected to reveal internal organs and visualized within 20 minutes since death. Distribution of RFP fluorescence was

acquired using In-Vivo Xtreme II (Bruker). Data was processed to apply spectral corrections to fluorescence intensity.

Aromatase expression by immunohistochemistry

Transverse sections (30 μm) of LNs were mounted onto poly-L-lysine coated slides. After drying overnight, samples were permeabilized with 0.5% Triton-X for 15 minutes, washed with 0.05% Tween-20 solution, then blocked with 2% FBS solution. Samples were then incubated in 2% BSA solution with biotin MECA-79 at (1:200) (Biolegend, #120804) and/or polyclonal rabbit aromatase (1:50) (Cell Signaling, #14528S) primary antibodies overnight at 4°C. Following that, samples were washed and incubated with 2% BSA that contained a species-specific secondary antibody: streptavidin alexa-488 (Biolegend, #405235), donkey anti-rabbit A568 (Fisher, #A10042). Samples were washed again before they are left to dry. After drying, a cover slip was applied after prolong gold antifade reagent application (Biolegend, #P36930). Images were taken using the Nikon A1 confocal systems using 10x and 20x objectives.

Albumin-coated nanoparticle preparation and treatment

NP were prepared using nanoprecipitation method. PLA solution (50 mg/mL in DMF, 1 mL), exemestane solution (10 mg/mL in DMF, 0.225 mL), and DMF (1 mL) were mixed and added dropwise into a glass vial prefilled with BSA aqueous solution (10 mL, 2.5 mg/mL) under vigorous stirring. For control NP, no exemestane solution was added. The resulting emulsion was dialyzed against DI water for 48 h with water changed every 8 h (MWCO = 3.5 kDa) and then lyophilized. Exemestane-loaded A-NPs were dissolved in PBS (7.5 $\mu\text{g/mL}$) prior to injection and 200 μL of the solution were injected to the mice via s.c. every other day 4 days

before to 8 days after EAE induction. Estrogen-loaded A-NPs were dissolved in PBS (0.1 $\mu\text{g/mL}$) prior to injection and 200 μL of the solution were injected to the mice via s.c. every other day from day 0 to day 10.

Patient specimen

Experimental protocol was approved by the institutional review board at Akita University Hospital (Permit number: 2422). Normal (or Non-malignant) cervical LN samples were isolated from patients with a benign thyroid disease such as Graves' disease and follicular adenoma. Written informed consent for use of samples was obtained from all patients at each hospital. The methods in this study were carried out in accordance with the approved guidelines. Resected human LNs were determined to be normal by pathological diagnosis when underwent the neck surgery. For aromatase staining, sections were washed with PBS after deparaffinization and treated with 0.3% hydrogen peroxidase in methanol for 20 min. Bovine serum albumin (1%) was applied to the sections for 20 min and primary antibody (Aromatase (D5Q2Y) Rabbit mAb, #14528, Cell Signaling) was applied to the tissue sections for 18 h at 4C. Reacted sections were subsequently incubated with anti-mouse immunoglobulin for 20 min, followed by exposure to peroxidase-conjugated streptavidin for 20 min at room temperature. Immunoreactivity was detected by immersing the tissue sections in 3,30-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM tris-HCl buffer (pH 7.6), 10 mM sodium azide, 0.006% hydrogen peroxidase). Sections were counterstained with hematoxylin. Samples were fixed with formalin, embedded in paraffin, and sectioned into 4 μm slices. After deparaffinization, sections were stained for aromatase and counterstained with hematoxylin.

Statistical analysis

Statistical analysis in all results was evaluated with two-tailed unpaired Student's t tests and t-values. The criterion of significance was set as $P < 0.05$. Animals were randomly used for experiments under the criteria aforementioned in the section of "Animals." All behavior experiments were performed in a blinded and randomized fashion. No animals or data points were excluded. Quantifications were performed from at least two experimental groups in a blinded fashion. Measurements were taken from distinct samples for each experiment. For each test, the experimental unit was an individual animal. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. Data distribution was assumed to be normal, but this was not formally tested. Statistical analyses and graphical presentations were computed with GraphPad Prism software (GraphPad, La Jolla, United States). According to previous report, data significance in Sholl analysis was considered only if more than two adjacent points showed P values less than 0.05. All bar graphs are presented as the mean $\pm$ SEM.

4.3 RESULTS

IFN β is ineffective against EAE in mice that lack extragonadal estrogens

Like in MS, EAE develops with heterogeneous symptomology and disease mechanisms that are dependent on biological and environmental factors. Here, we sought to identify if endogenous estrogen is a disease-phenotype determining factor using the EAE model. Interferon- β (IFN β) is a first line therapy for individuals with relapse-remitting form of MS¹⁴. However, treatment is ineffective in a subset of MS patients and in several EAE models that show severe CNS neuronal damage, likely as a result heterogeneity in disease mechanisms³. As such, we used

responsiveness to IFN β in EAE mice as a proxy for EAE phenotype. Because aromatase is an essential enzyme for the synthesis of endogenous estrogens¹⁷, we determined whether endogenous estrogen contributes to heterogeneity in IFN β sensitivity using aromatase (CYP19)-deficient (Cyp19^{-/-}) mice. While IFN β suppressed EAE development in wild-type (WT) mice, it did not suppress EAE development in Cyp19^{-/-} mice (Fig. 4.1 A), suggesting that endogenous estrogen synthesis is necessary for the development of EAE phenotype that is sensitive to IFN β treatment.

To identify the source of endogenous estrogens that determine IFN β sensitive EAE phenotype, ovary-derived estrogen was explored because ovary is primary source in estrogen synthesis. EAE was induced in ovariectomized (OVX) mice that were then treated with IFN β . Interestingly, IFN β treatment suppressed EAE disease in OVX mice similar to sham-operated control mice (Fig. 4.1 B), suggesting that ovaries are not the source of estrogens that determine IFN β sensitivity during EAE. Estrogen is also produced in extra-gonadal organs⁹. To evaluate involvement of extra-gonadal estrogens, we conducted an ovary transplantation study (Fig. 4.1 C). WT ovaries were transplanted to OVX Cyp19^{-/-} mice, thereby limiting estrogen production in gonadal sites only. Ovaries of Cyp19^{-/-} mice were transplanted to OVX WT mice, thereby limiting estrogen production in extragonadal organs only. Similar to OVX WT mice, WT mice with Cyp19^{-/-} ovaries were sensitive to IFN β treatment (Fig. 4.1.D). In contrast, Cyp19^{-/-} mice with WT ovaries were resistant to IFN β treatment (Fig. 4.1 D). These results suggest that estrogens of extra-gonadal origin are determinants of disease phenotype, such as IFN β sensitivity, in EAE pathogenesis.

Lymph nodes are de novo sites of estrogen synthesis via local expression of aromatase

To identify non-gonadal sites of de novo estrogen synthesis, we used a transgenic mouse line in which a reporter gene (red fluorescent protein, RFP) was expressed in the Cyp19-lineage cells⁹. Ex vivo imaging of dissected Cyp19-RFP female and male mice demonstrated positive RFP signals in ovary and testes, respectively (Fig. 4.2 A). Remarkably, high intensity RFP signals were detected in all LNs including axillary, brachial, cervical, and inguinal lymph nodes, which are important sites in MS^{18,19} and EAE^{20,21} pathogenesis. Aromatase expression in LNs of adult mice was further confirmed by immunohistochemistry (Fig. 4.2 B). Specifically, we show that aromatase expression was localized to high endothelial venules (HEV) stained by monoclonal antibody against MECA-79 that recognizes sulfated L-selectin ligands of HEVs²⁵ (Fig. 4.2 B). Importantly, like in adult mice, aromatase expression was also detected in human cervical LNs (Fig. 4.2 C). To investigate if LN aromatase is involved in de novo synthesis and tissue-specific retention of estrogens, 17 β -estradiol amounts in LNs were compared between WT mice with sham- or OVX-operation. Levels of 17 β -estradiol in axillary, branchial, cervical, and inguinal LNs were not different between these mice (Fig. 4.2 D), suggesting de novo synthesis and tissue-specific retention of 17 β -estradiol in LNs. These results suggest that LNs produce and retain estrogen independent of circulating gonad-derived estrogen.

Inhibition of aromatase in the lymph nodes causes prolonged IFN β -resistant EAE

LNs are key locations where T cells become autoreactive upon contact with dendritic cells in MS and its animal model of EAE²⁶. The potential significance of estrogen synthesized in LNs during EAE pathogenesis was assessed. To do so, we developed a LN-targeted nanoparticle drug-delivery vehicle to inhibit aromatase function in LNs of EAE mice. As a natural

biomacromolecule, albumin possesses numerous positive characteristics of an ideal carrier for drug delivery such as good water-solubility, biodegradability, lack of toxicity, and minimal immunogenicity²⁷. Compounds that bind to albumin can be effectively transported to draining LNs, making albumin an ideal moiety for targeting lymph nodes^{28,29}. Leveraging the nature of albumin, albumin-coated nanoparticles (A-NPs; Fig. 4.3. A) were synthesized as a LN-targeted drug-delivery vehicle. Upon subcutaneous injection of rhodamine-loaded A-NPs, fluorescence was observed in ex vivo LNs including the axillary and inguinal LNs 6 hours after injection (Fig. 4.3.B). No off-targeting signals were observed in the intact brain, spleen, or ovary, which are other tissues that express aromatase (data not shown). These results confirm that A-NP is specifically delivered to and retained in LNs with no significant off-targeting effect.

To determine if LN-synthesized estrogen plays a role in EAE pathogenesis, A-NPs were loaded with exemestane, an aromatase inhibitor³⁰, and used to selectively inhibit aromatase activity in the LNs. As proof of A-NP design efficacy, we show that estradiol content in the LNs, but not ovary, significantly decreased 4 days post two subcutaneous injections with exemestane-loaded A-NPs (Fig. 4.3 C).

To examine if inhibition of LN synthesis of estrogen is sufficient to induce phenotype alterations in EAE mice, WT mice were injected with exemestane-loaded A-NPs or non-loaded A-NPs (as vehicle control) before and after EAE induction. While IFN β suppressed disease development in the vehicle-A-NP-treated mice, mice treated with exemestane-A-NP did not respond to IFN β treatment (Fig. 4.3 D). Because prolonged motor paralysis in response to EAE induction is associated with CNS neuronal damage in spinal cord ventral horn regions where lower motor neurons reside^{3,33,34}, morphology of neuronal populations in lumbar spinal cord were examined by using Golgi-Cox neuron staining³⁵. Sholl analysis^{36,37} showed a significant decrease

in dendritic length and number of intersections from exemestane-loaded A-NP-treated mice when compared to control (Fig. 4.3 E). These results suggest that estrogen levels in LNs determines EAE outcome, the absence of which results in EAE with severe neurodegeneration and IFN β -resistance.

Aging elicits lymph node estradiol reduction and EAE phenotype alteration

Aging is a significant factor that influences MS development and pathogenicity³⁸. Estradiol contents of mice aged 7-8 months, which is equivalent to around 38-40 years in humans³⁹, were compared to those of younger mice (2 months). While estradiol contents in both testes and ovaries were similar in 2-month-old and 8-month-old mice, estradiol levels in inguinal and axillary LNs were significantly decreased in middle-aged (8-month) male mice but not female mice compared to those of young (2-months) mice, respectively (Fig. 4.4 A). Interestingly, middle-aged male EAE mice with significantly reduced LN production of estradiol were IFN β -resistant (Fig. 4.4 B). We sought to examine if restoration of estradiol in LNs is sufficient to overcome IFN β -resistance. To do so, we subjected middle-aged male EAE mice to treatments of estradiol-loaded ANP and IFN β . Treatment of estradiol-loaded ANP to middle-aged male EAE mice rendered these mice receptive to IFN β (Fig. 4.4 C). These results show for the first time that aging results in reduced LN synthesized estrogen and that aging-driven reduction in LN estradiol synthesis plays a key disease phenotype-altering role in EAE pathogenesis.

ER α ^{-/-} mice show altered EAE phenotypes

We next sought to identify if endogenous estrogen receptor alpha (ER α) signaling is mechanism by which LN-derived estrogens exert influence as a disease-phenotype determining factor. To

do so, we treated IFN β to male and female adult ER α -deficient (ER $\alpha^{-/-}$) C57BL/6J mice and wild-type (WT) mice with EAE and monitored daily disease scores. While IFN β successfully prevented EAE-induced motor paralysis in WT mice, it did not prevent EAE in ER $\alpha^{-/-}$ mice (Fig. 4.5 A). Further, at 33 dpi, Sholl analysis of neurons in the ventral horns of lumbar spinal cord indicates a significant decrease in dendritic length of neurons in ER $\alpha^{-/-}$ mice when compared to control (Fig. 4.5 B). Additionally, ~36% reduction in dendritic spines of neurons in the ventral horn region of the lumbar spinal cord were observed in ER $\alpha^{-/-}$ mice when compared to WT mice (Fig. 4.5 C). Interestingly, the degree of demyelination was similar between ER $\alpha^{-/-}$ and WT mice at 33 dpi (data not shown) as examined by Luxol fast blue staining, suggesting that neuronal damage occurred independent of demyelination in the white matter, as seen in some patients with progressive-type MS disease⁴⁰. These findings suggest that the disease phenotype-modifying role of LN-derived estrogen is mediated by ER α .

Dendritic cell activation of ER α regulates lymphotoxin activity, which is critical for altered EAE phenotypes

Human and mouse studies suggest that dendritic cell properties play a major role in disease phenotype in MS and EAE diseases^{41,42}. To evaluate involvement of dendritic cell function in regulating EAE pathogenesis by ER α signaling, we used dendritic cell-specific ER $\alpha^{-/-}$ mice (cER $\alpha^{-/-}$ mice). While IFN β suppressed EAE disease in control ER α floxed mice, cER $\alpha^{-/-}$ EAE mice were resistant to IFN β -treatment throughout testing period of 20 days (Fig. 4.6A). These results suggest that the impact of ER α signaling on EAE disease phenotype alternation can be primarily attributed to its activity in dendritic cells.

Our previous work demonstrated that upregulated membrane-bound lymphotoxin in dendritic cells is involved in EAE phenotypes of IFN β resistance and severe CNS neuronal damage^{3,33}. To examine if IFN β resistance in ER $\alpha^{-/-}$ and cER $\alpha^{-/-}$ mice is linked to lymphotoxin function³³, we quantitated membrane-bound lymphotoxin expression on dendritic cells using flow cytometry in EAE mice at 9 dpi. ER $\alpha^{-/-}$ and cER $\alpha^{-/-}$ EAE mice had ~15 times and ~8 times, respectively, significantly greater numbers of lymphotoxin-expressing dendritic cells in LNs when compared to WT control mice (Fig. 4.6B).

Next we sought to determine if the inhibition of lymphotoxin receptor signaling is sufficient to suppress prolonged disease observed in ER $\alpha^{-/-}$ and cER $\alpha^{-/-}$ mice. To do so, ER $\alpha^{-/-}$ and cER $\alpha^{-/-}$ EAE mice were treated with lymphotoxin β receptor (LT β R) Fc blocker. While LT β R Fc blocker treatment did not affect EAE disease in control WT mice (Fig. 4.6 C), as previously reported³³, it significantly suppressed the disease severity in cER $\alpha^{-/-}$ mice (Fig. 4.6 C). These results identified ER α signaling in dendritic cell as a determinant of EAE phenotype, the limitation of which results in IFN β -resistant EAE that is driven by the membrane-bound lymphotoxin-LT β R signaling pathway.

ER α signaling suppresses lymphotoxin expression in dendritic cells

To determine the mechanism by which the disruption of ER α signaling results in heightened expression of membrane-bound lymphotoxin in dendritic cells, bone marrow-derived dendritic cells (BMDCs) were studied in vitro. LT α (encoded by Lta) is crucial for the transport of LT β to the cell surface, which results in the expression of the cell surface LT α 1 β 2 complex that binds to the LT β R⁴⁶. Thus, we evaluated Lta expression level in BMDCs. Pretreatment of BMDCs with PPT (1,3,5-tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole), an ER α agonist, significantly

suppressed upregulation of *Lta* by treatment of heat-killed *Mycobacterium tuberculosis* (Mtb) or lipopolysaccharide (LPS) (Fig. 4.7 A). These results suggest that ER α signaling is suppressive of LT production downstream of TLR4 activation that plays an essential role in successful EAE induction⁴⁷.

To determine the pathway by which ER α activation leads to the suppression of *Lta* transcription, we assessed the impact of ER α selective agonist PPT on *Il1b* and *Ifnb* transcription which are distinctively regulated by MyD88-TRAF6 and TRIF-TRAF3 pathways, respectively, in response to TLR4 activation⁴⁸. Interestingly, pretreatment of BMDCs with PPT did not alter *Il1b* mRNA levels but significantly downregulated *Ifnb* mRNA amount (data not shown). This result suggests that ER α signaling in dendritic cells suppresses *Lta* transcription by inhibiting the TRIF-TRAF3 pathway but not the MyD88-TRAF6 pathway of TLR4 activation. To identify the mechanism by which the activation of ER α inhibits TRIF-TRAF3 pathway, we examined the presence of physical interactions between ER α and TRAF3 using a co-immunoprecipitation assay. ER α was found to directly bind to TRAF3 after PPT treatment (Fig. 4.7 B). Because ER α binding to TRAF3 may result in the downregulation of TRAF3 via protein ubiquitination as previously reported in macrophages⁴⁹, ubiquitination status of TRAF3 was determined under PPT treatment in BMDCs. In the PPT-pretreated BMDC, TRAF3 was markedly ubiquitinated compared with control group (Fig. 4.7 B). Notably, ubiquitination of TRAF3 was most apparent in BMDC that were pre-treated with PPT for 6 hours. Furthermore, TRAF3 expression was decreased in BMDC after 18-hour PPT treatment, compared with controls (Fig. 4.7 C). In order to establish if ER α -mediated downregulation of TRAF3 was able to block the translocation of activated IRF3 to the nucleus, IRF3 nuclear translocation was evaluated under the treatment of LPS with or without PPT pre-treatment. As previously reported with GFP-fusion IRF3⁵⁰, IRF3

puncta were detected in the nucleus when LPS was treated to vehicle-treated BMDC (Fig. 4.7 D). This IRF3 translocation to the nucleus was significantly reduced in PPT-pretreated BMDCs (Fig. 4.7 D). Taken together, these results suggest that ER α activation in dendritic cells non-genomically suppresses membrane-lymphotoxin expression and endogenous interferon response.

ER α signaling in dendritic cells regulates T cell neurotoxic profile

Finally, we asked if T cell neurotoxic effector function can result from deficient ER α signaling in dendritic cell. We first compared transcriptional profiles of splenic CD4⁺ T cells isolated from ER α ^{-/-} mice with EAE to those of WT mice with EAE at 9 dpi. Using high-throughput RNA-seq analysis, we found 100 significantly upregulated genes ($P < 0.05$) in CD4⁺ T cells from ER α ^{-/-} EAE mice compared to those from WT EAE mice as well as 105 downregulated genes ($P < 0.05$). Notably, highly expressed genes in CD4⁺ T cells from ER α ^{-/-} EAE included those that encode neuronal damage-related molecules (Fig. 4.8 A). To identify whether ER α in dendritic cell is necessary for changes in CD4⁺ T cell neurotoxic function, we conducted intrathecal passive transfer of CD4⁺ T cells that were derived from 10 dpi ER α ^{-/-} EAE, cER α ^{-/-} EAE, and control EAE animals, into Tcr α ^{-/-} mice, which intrinsically lack T cells, at 14 dpi, as recently reported (Model schema shown in Fig. 4.8 B)⁵¹. Sholl analysis of neurons that reside in the ventral horns of lumbar spinal cord showed a significant decrease in neuron dendritic length and number of intersections in Tcr α ^{-/-} mice with ER α ^{-/-} CD4⁺ T cell transfer when compared to Tcr α ^{-/-} mice with WT CD4⁺ T cell transfer (Fig. 4.8 C). Significant decrease in dendritic length was also observed in mice that received T cells from cER α ^{-/-} EAE donors (neurons that reside in the ventral horns of lumbar spinal cord). Together, these results suggest that ER α signaling in dendritic cells is a determining factor of EAE pathogenesis via CD4⁺ T cell neurotoxicity.

4.4 DISCUSSION

Estrogens are primarily produced in gonads and are historically thought to be long-range circulating endocrine signals that regulate the immune system. It is well established that estrogens are also synthesized in extra-gonadal sites such as liver and brain²²⁻²⁴. More recently, we reported de novo estrogen synthesis in the mesenteric LNs and Peyer's patches⁹. In this work, we show for the first time that human LNs, too, express aromatase. This highlights the impact of LN-derived estrogen on immune regulation. Ovariectomy in mice does not alter LN estrogen content¹⁰, demonstrating that LNs are indeed sites of de novo estrogen synthesis and that estrogens produced in LNs are retained in its tissue microenvironment. Notably, LNs retain significantly higher estradiol concentrations than peripheral blood⁹ at a concentration that is higher or equivalent to that of gonadal levels⁹. In this study, we identified a novel role of LN-derived estrogen in regulating immune cell response that underlie EAE phenotypic differences characterized by sensitivity to MS treatment and gray matter pathology.

Treatment of patients with estriol, an endogenous form of estrogen, alone^{52,53} or in combination with an immune-modulatory drug⁵⁴ produces favorable therapeutic outcomes such as reduced lesion load⁵³ and relapse rate⁵⁴. However, while estrogen treatment produced promising results, clinicians and endocrinologists have significant concerns regarding the negative impact of prolonged systemic estrogen treatment on cancer risk, fertility, and mood^{55,56}. Therefore, this study serves as an important first milestone for the development of novel precision therapeutics to treat MS and other autoimmune disorders that can be specifically managed with immunosuppressive estrogens in LNs. Here we show that the lack of endogenous production of estrogen in LNs specifically is sufficient to induce remarkable disease phenotype changes such as IFN β resistance, non-remitting disease, and CNS neuron damage in mice.

Additionally, we identified mechanisms by which deficient dendritic cell ER α signaling results in each altered disease phenotype.

We show that animals lacking LN estrogen signaling developed EAE with IFN β resistance via multiple potential pathways described below. First, the absence of ER α signaling in dendritic cells increases endogenous IFN β production, via the lack of TRAF3 suppression, which has been clinically associated with blunted IFN β response in MS patients. As such, MS patients with active virus infections, that triggers endogenous IFN β production, have less response to IFN β therapy⁴⁵. Genome-wide expression profiling in peripheral blood mononuclear cells of MS patients demonstrated that endogenous IFN β level is associated with poor response to IFN β therapy^{43,44}. A second possible mechanism for IFN β resistance in animals that lack LN-derived estrogen is that the absence of ER α signaling increases membrane-bound lymphotoxin expression via the lack of TRAF3 suppression, thus triggering the development of an altered EAE disease pathway that is primarily driven by the membrane-bound lymphotoxin-LT β R signaling pathway. We previously reported that treatment of recombinant membrane-bound lymphotoxin, which activates LT β R, to EAE mice is shown to be sufficient to induce resistance to IFN β ³³, suggesting IFN β does not prevent membrane-bound lymphotoxin-LT β R signaling pathway.

Next we sought to understand the mechanism by which LN-derived is a determining factor of EAE phenotype in the context of neurodegeneration. Using RNAseq analysis and a T cell passive transfer study, we demonstrated that T cells are programmed by dendritic cells to become primary drivers of neuron damage during EAE. As such, we showed that CD4⁺ T cells from ER $\alpha^{-/-}$ and cER $\alpha^{-/-}$ mice have upregulation in genes that encode neurotoxic molecules. In particular, Npcd, which is commonly expressed by neuron lineages, is likely to be relevant to

axon growth and/or guidance⁵⁷. Additionally, genes that encode proteins that activate glia via increasing the secretion of signaling molecules, namely glutamate (encoded by *Asns*) are upregulated in CD4⁺ T cells from ER α ^{-/-} mice⁵⁸. *Naip6* which encodes IL-1 β and IL-18⁵⁹ is also significantly upregulated in isolated CD4⁺ T cells from ER α ^{-/-} mice when compared to WT control. In the passive EAE study, T cells were transferred intrathecally, instead of the more common intravenous route, to bypass T cell migration from the periphery to the CNS and avoid the possibility of cell property changes while in the periphery⁶⁰. Passive intrathecal transfer of equal numbers of T cells derived from WT, ER α ^{-/-}, and cER α ^{-/-} mice to Tcr α ^{-/-} recipient mice demonstrated that T cells derived from ER α ^{-/-} and cER α ^{-/-} mice were sufficient to result in significant neuronal loss, suggesting that altered T cell effector properties, but not cellularity, in ER α ^{-/-} and cER α ^{-/-} mice are reasons for severe CNS neurodegenerative pathology. It is likely that the T cell effector function that mediates neuronal atrophy is dependent on the expression of multiple factors that dynamically regulate cellular property and behavior. The identification of the molecular mechanism of T cell-mediated neuron damage will be our next area of investigation.

Human and mouse studies suggest that dendritic cell properties play a major role in disease phenotype in MS and EAE diseases^{41,42}. In the present study, we identified the activation of dendritic cell ER α in the LN as a disease-modifying factor in EAE mice within the contexts of treatment sensitivity and neurodegeneration via ER α regulation of lymphotoxin transcription. Estrogenic activation of ER α regulates immune cell activity through non-genomic⁶¹ and genomic responses⁶². In dendritic cells, we tested the involvement of nongenomic pathways because no estrogen-responsive elements were found in the promoter region of the lymphotoxin gene using a weight matrix-based program (<http://gene-regulation.com/pub/programs.html>). As such, the

impact of ER α activation was studied in the context of the TRAF3-IRF3 pathway, which is essential for transcription of lymphotoxin and IFN β genes.

There are limitations to this study that should be addressed. First, the choice of CD11c-cre mice to target gene expression in dendritic cells is not fully dendritic cell-specific because monocytes also express CD11c⁶³. Using the recently generated dendritic cell-restricted cre mouse with a zinc finger transcription factor, which is not expressed by monocytes or other immune cell populations, is an option, but it is limited in conventional dendritic cells⁶⁴. As the large majority of CD11c⁺ cells are DCs, our results strongly suggest critical crosstalk between estrogen and dendritic cells in responding to EAE induction. Second, our study indicates the possible involvement of T cell ER α signal in the development of IFN β -resistant EAE disease during disease late phase. The identification of mechanism by which lacking ER α signal in T cells trigger IFN β resistance in late phase will be our next area of investigation.

The significance of estrogen synthesis in the LNs during aging is unexplored. Here we show that middle-aged male mice, but not female mice, have reduced estradiol levels in the LNs and show non-remitting and IFN β -resistant EAE pathogenesis. Supplementation to LNs specifically in middle-aged male mice was sufficient to convert the phenotype of IFN β -resistance to IFN β -sensitive, as seen in young WT mice, though the effect is partial. Interestingly, disease remittance did not occur in middle-aged mice despite LN estrogen supplementation. Due to the complex nature of the aging process, it is highly likely that the partial efficacy of LN-targeted estrogen delivery in reverting altered EAE phenotypes (IFN β -resistance and non-remitting disease) to normal EAE phenotypes can be attributed to another pathway involved in altered EAE phenotype development in middle-aged mice. Incidentally, middle-age male MS patients tend to develop a progressive form of MS, which is non-remitting,

IFN β -insensitive, and more burdened with CNS neuronal damage than relapse-remitting MS^{65,66}. Thus, it is tempting to speculate that these observations may be partially reflected by lower LN estradiol levels in middle-age men. Interestingly, reduction of estrogens in LNs is independent of levels of gonadal estrogens, suggesting its unique synthetic machinery. Synthetic machinery of LN estrogen and mechanism by which aging reduces LN estrogen are future topics of investigation.

In summary, our studies suggest that estrogen content or aromatase expression levels in human LNs may serve as a biomarker for the IFN β -resistant MS phenotype with severe CNS neurodegeneration. In addition, the findings indicate that a LN-specific treatment with estrogen, ER α agonists, or LT β R inhibitors may be considered a therapeutic approach for treating patients with progressive MS that has non-remitting, IFN β -resistant, or severe gray matter atrophy phenotypes. Such a targeted therapeutic approach may reduce or even eliminate the inevitable side effects resulting from systemic treatment regimens.

4.5 FIGURES

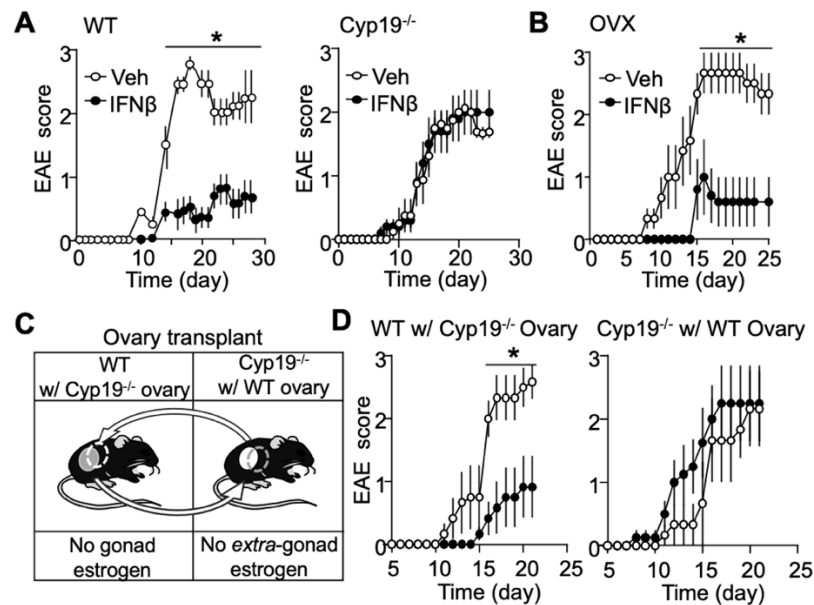


Fig. 4.1. IFN β is ineffective against EAE in mice that lack extragonadal estrogens. (A) Sensitivity of EAE to IFN β in 6-to-8-week-old wild-type (WT) and Cyp19^{-/-} mice. Vehicle (veh) or IFN β (47 μ g/kg) were administered intraperitoneally (i.p.) every other day from 0 to 10 dpi (n=5-7 mice per group). (B) Sensitivity of EAE to IFN β in 6-to-8-week-old ovariectomized (OVX) mice (n=5-6 mice per group). (C) Schematic for ovary transplantation experiment. (D) Sensitivity of EAE to IFN β in 6-to-8-week-old Cyp19^{-/-} ovary-transferred WT mice and WT ovary-transferred Cyp19^{-/-} mice. Vehicle or IFN β were administered i.p. every other day from 0 to 10 dpi (n=5-6 mice per group). Data are representative of two independent experiments. Data are presented as mean $\pm$ SEM. Statistical significance was assessed by an unpaired Student's t-test.

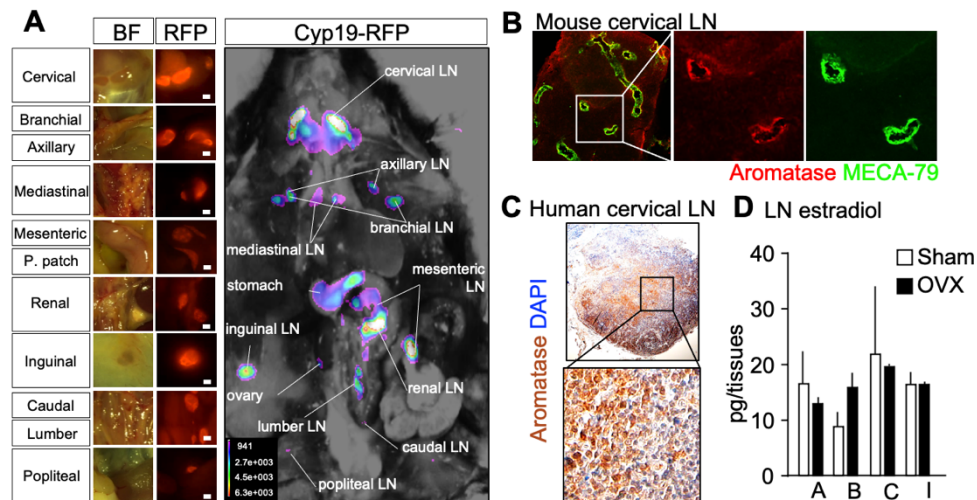


Fig. 4.2. Lymph nodes are de novo sites of estrogen synthesis via local expression of aromatase. (A) Representative image of Cyp19 promoter-derived RFP fluorescence in a female mouse (8 weeks old). (B) Representative images of immunohistochemistry staining for aromatase (red) and MECA-79 (green) in cervical LNs of a female mouse (8 weeks old). (C) Representative images of aromatase immunohistochemistry staining in cervical LNs of a human patient. (D) Estradiol levels in LNs (A: axillary, B: branchial, C: cervical, and I: inguinal) in sham-operated and OVX mice (n=8 mice per group). Data are presented as mean $\pm$ SEM. Data are representative of two independent experiments. Statistical significance was assessed by an unpaired Student's t-test.

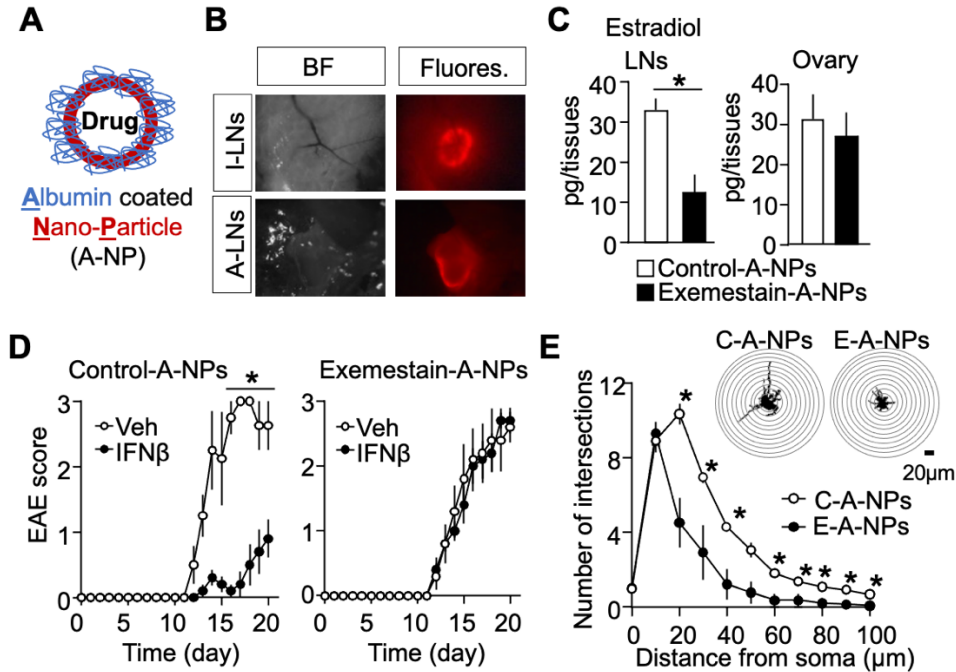


Fig. 4.3. Inhibition of aromatase in the lymph nodes causes IFN β -resistant and CNS neurotoxic EAE. (A) Graphical design of albumin-coated nanoparticle (A-NP). (B) Representative brightfield (BF) and fluorescence (Fluores.) images of intact inguinal (I-LNs) and axillary (A-LNs) LNs 6 h after subcutaneous (s.c.) treatment of rhodamine-loaded-A-NP. (C) Estradiol level in LNs (axillary and inguinal) and ovary after treatment with exemestane (1.5 $\mu\text{g}/\text{mouse}$)-A-NPs by s.c. injections given at day 0 and 2 followed by tissue harvesting at day 4 (n=4 mice per group). (D) Sensitivity of EAE to IFN β in 6-to-8-week-old control- and exemestane-A-NP-treated mice (n= 5 mice per group). (E) Neurite outgrowth of the neurons in Golgi-stained spinal sections of EAE mice treated with control- or exemestane-A-NP at 33 dpi quantified using Sholl analysis method. Sholl rings are separated by 10 μm (n=5 mice per group, 20 neurons analyzed per animal) with end radius of 100 μm . Data are representative of two to three independent experiments. Data are presented as mean $\pm$ SEM. Statistical significance was assessed by an unpaired Student's t-test.

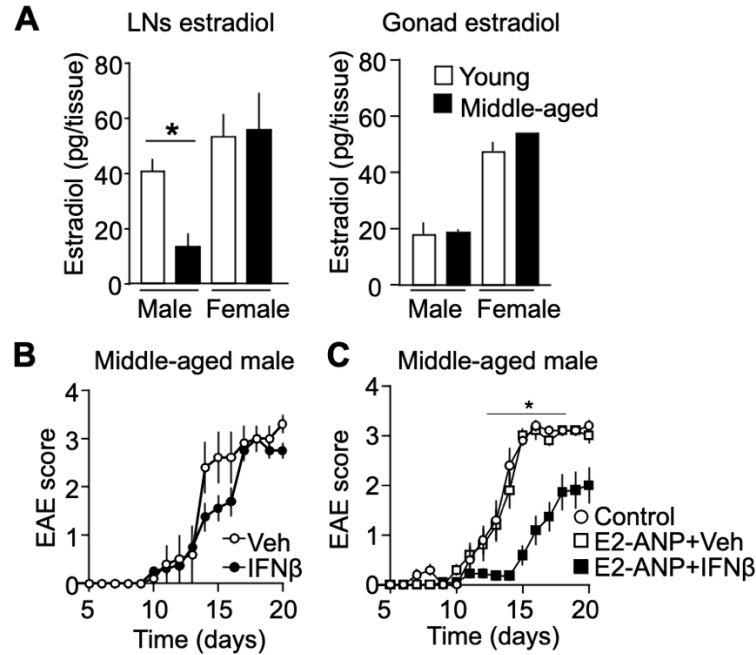


Fig. 4.4. Aging elicits lymph node estradiol reduction and EAE phenotype alteration. (A) Estradiol levels in LNs (axillary and inguinal) and gonads in male and female young (2-month-old) and middle-aged (8-month-old) mice ($n = 10-11$ mice per group). (B) EAE sensitivity to IFN β in middle-aged male. Vehicle (Veh) or IFN β (47 $\mu\text{g/kg}$) were administered i.p. every other day from 0 to 10 dpi ($n = 5-8$ per group). (C) EAE sensitivity to IFN β in middle-aged male mice treated with estradiol-loaded ANP (E2-ANP). Vehicle (Veh) or IFN β were administered i.p. every other day from 0 to 10 dpi ($n = 5-10$ per group). Vehicle- or estrogen-ANP (200 ng/mouse) were administered s.c. every other day from 0 to 10 dpi. Data are representative of two independent experiments. Data are presented as mean $\pm$ SEM. Statistical significance was assessed by an unpaired Student's t -test.

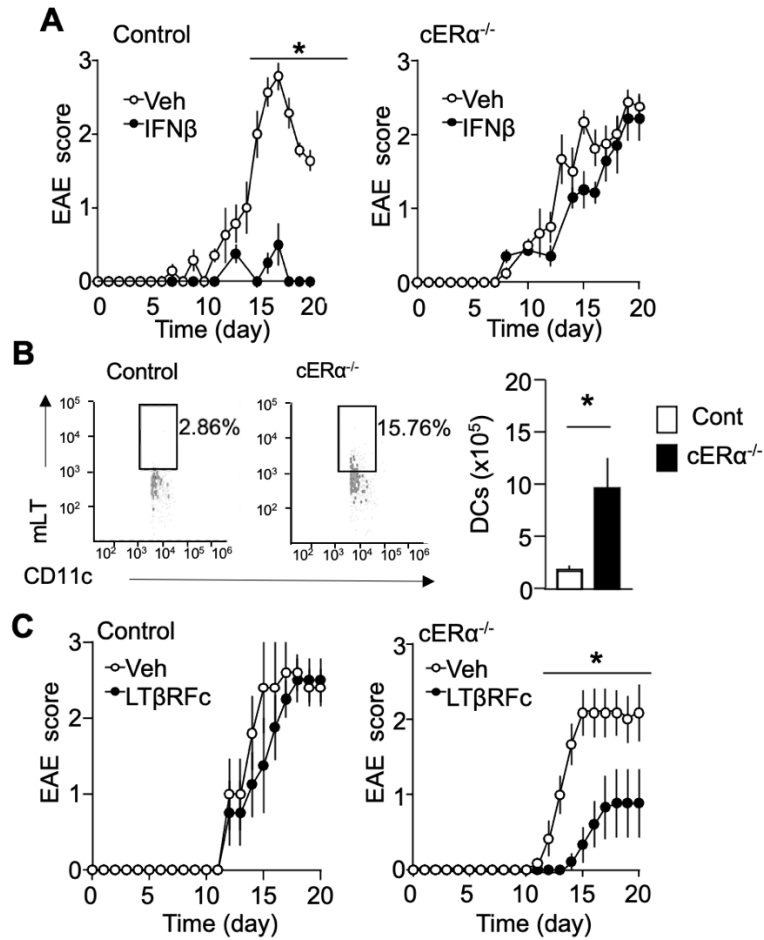


Fig. 4.6. Dendritic cell expression of $ER\alpha$ regulates $IFN\beta$ sensitivity likely via modulating lymphotoxin activity. (A) Sensitivity of EAE to $IFN\beta$ in 6-to-8-week-old male $ER\alpha^{fl/fl}$ (control) and $CD11c-ER\alpha^{-/-}$ ($cER\alpha^{-/-}$) mice. Vehicle or $IFN\beta$ (47 μ g/kg) were administered i.p. every other day from 0 to 10 dpi (n=8 mice per group). (B) Number of membrane-bound lymphotoxin (mLT) $^{+}$ DCs (derived from spleen and LNs) in $cER\alpha^{-/-}$ EAE mice at 9 dpi (n=5 mice per group). (C) Sensitivity of EAE to $LT\beta$ R-blocking Fc in 6-to-8-week-old WT or $cER\alpha^{-/-}$ mice. Vehicle or $LT\beta$ R-blocking Fc (7.5 μ g/kg) were administered i.p. every other day from 0 to 10 dpi (n=8 mice per group). Data are representative of two independent experiments. Data are presented as mean $\pm$ SEM. Statistical significance was assessed by an unpaired Student's t-test.

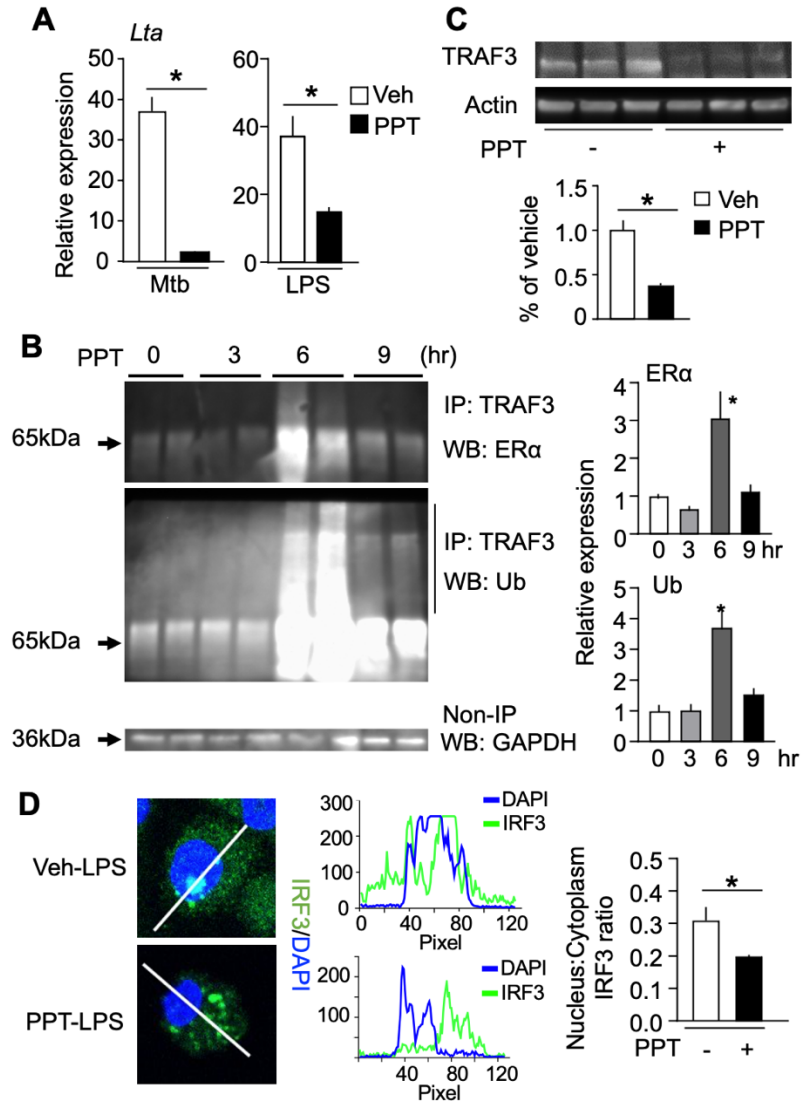


Fig. 4.7. ER α suppresses lymphotoxin expression in dendritic cells. (A) *Lta* mRNA expression in BMDCs 3 h after treatment with heat-killed Mtb (100 μ g/ml) or LPS (100 pg/ml) with vehicle (Veh) or PPT (10 μ M) pre-treatment for 18 h (n=5 mice per group). (B) Representative Western blot of co-immunoprecipitation of ER α and TRAF3 in BMDCs 0, 3, 6, or 9 h after PPT (10 μ M) treatment. Ubiquitination of TRAF3 in BMDCs following PPT (10 μ M) treatment for 0, 3, 6, or 9 h (n=5 per group). Western blot of GAPDH in pre-IP sample as control. ER α and Ub expression was normalized by GAPDH expression because of alteration of TRAF3 expression after PPT treatment. (C) TRAF3 expression in BMDCs following vehicle (Veh) or PPT (10 μ M) pre-treatment for 18 h (n=5 per group). (D) Nuclear localization of IRF3 in BMDCs treated with LPS for 2 h prior with vehicle (Veh) or PPT (10 μ M) pre-treatment for 18 h. The results presented here are representatives of at least three biological repeats. Data are presented as mean $\pm$ SEM. Data are representative of two independent experiments. Statistical significance was assessed by an unpaired Student's t-test.

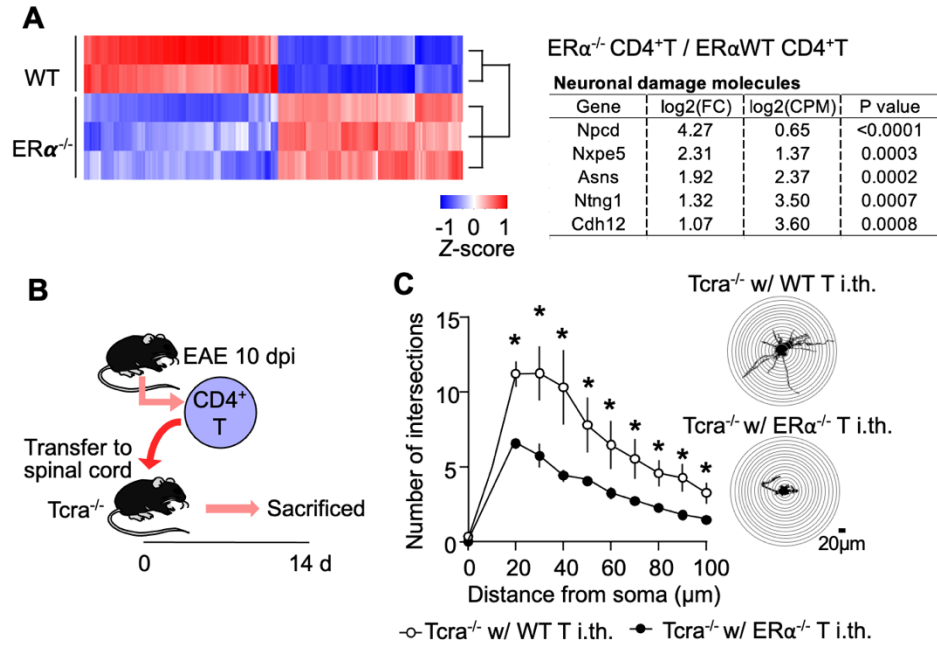


Fig. 4.8. T cell neurotoxic profile in ERα^{-/-} EAE mice. (A) Heatmap of RNA-Seq expression z-scores computed for all genes that are differentially expressed (FDR p-value < 0.1 from limma-trend model with batch effect) (left). Selected genes that encode neuronal damage molecules with p-value < 0.05 (right). Splenic CD4⁺ T cells from WT EAE and ERα^{-/-} EAE mice at 9 dpi were RNA sequenced. Data are presented as mean ± SEM. (B) Schematic for intrathecal passive EAE model. CD4⁺ T cells were isolated from spleen and LNs of WT or ERα^{-/-} EAE mice at 9 dpi. Isolated CD4⁺ T cells (10⁶ cells per mouse) were adoptively transferred by intrathecal injection to Tcrα^{-/-} recipient mice that were induced with EAE (14 dpi). Tcrα^{-/-} recipient mice were sacrificed 14 days after i.th. injection. (C) Neurite outgrowth of the neurons in Golgi-stained spinal sections of Tcrα^{-/-} recipient mice that received passive intrathecal injections of CD4⁺ splenic cells from WT EAE and ERα^{-/-} EAE mice at 14 dpi quantified using Sholl analysis method. Sholl rings are separated by 10 μm (n=4 mice per group, 20 neurons analyzed per animal) with end radius of 100 μm. Statistical significance was assessed by an unpaired Student's t-test.

4.6 REFERENCES

1. Lassmann, H., Bruck, W. & Lucchinetti, C. Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. *Trends Mol Med* 7, 115-121 (2001).
2. Petzold, A., et al. Treatment response in relation to inflammatory and axonal surrogate marker in multiple sclerosis. *Mult Scler* 10, 281-283 (2004).
3. Khaw, Y.M., Majid, D., Oh, S., Kang, E. & Inoue, M. Early-life-trauma triggers interferon-beta resistance and neurodegeneration in a multiple sclerosis model via downregulated beta1-adrenergic signaling. *Nat Commun* 12, 105 (2021).
4. Simmons, S.B., Pierson, E.R., Lee, S.Y. & Goverman, J.M. Modeling the heterogeneity of multiple sclerosis in animals. *Trends Immunol* 34, 410-422 (2013).
5. Vukusic, S., et al. Pregnancy and multiple sclerosis (the PRIMS study): clinical predictors of post-partum relapse. *Brain* 127, 1353-1360 (2004).
6. Birk, K., et al. The clinical course of multiple sclerosis during pregnancy and the puerperium. *Arch Neurol* 47, 738-742 (1990).
7. Giglio, T., et al. Immune cell circulating subsets are affected by gonadal function. *Life Sci* 54, 1305-1312 (1994).
8. Vermillion, M.S., Ursin, R.L., Attreed, S.E. & Klein, S.L. Estriol Reduces Pulmonary Immune Cell Recruitment and Inflammation to Protect Female Mice From Severe Influenza. *Endocrinology* 159, 3306-3320 (2018).
9. Oakley, O.R., et al. Estradiol Synthesis in Gut-Associated Lymphoid Tissue: Leukocyte Regulation by a Sexually Monomorphic System. *Endocrinology* 157, 4579-4587 (2016).
10. Park, C.J., et al. Porcine intestinal lymphoid tissues synthesize estradiol. *J Vet Sci* 19, 477-482 (2018).

11. Cunningham, M. & Gilkeson, G. Estrogen receptors in immunity and autoimmunity. *Clin Rev Allergy Immunol* 40, 66-73 (2011).
12. Khan, D. & Ansar Ahmed, S. The Immune System Is a Natural Target for Estrogen Action: Opposing Effects of Estrogen in Two Prototypical Autoimmune Diseases. *Front Immunol* 6, 635 (2015).
13. Hegen, H., et al. Cytokine profiles show heterogeneity of interferon-beta response in multiple sclerosis patients. *Neurol Neuroimmunol Neuroinflamm* 3, e202 (2016).
14. Palace, J., et al. Assessing the long-term effectiveness of interferon-beta and glatiramer acetate in multiple sclerosis: final 10-year results from the UK multiple sclerosis risk-sharing scheme. *J Neurol Neurosurg Psychiatry* 90, 251-260 (2019).
15. Souza VR, Mendes E, Casaro M, Antiorio AT, Oliveira FA, Ferreira CM. Description of ovariectomy protocol in mice. *InPre-Clinical Models* (2019) Humana Press, New York, NY.
16. Torrents E, Boiso I, Barri PN, Veiga A. Applications of ovarian tissue transplantation in experimental biology and medicine. *Human reproduction update* (2003) Sep 1;9(5):471-81.
17. Stocco, C. Tissue physiology and pathology of aromatase. *Steroids* 77, 27-35 (2012).
18. Pelletier, D. & Hafler, D.A. Fingolimod for multiple sclerosis. *N Engl J Med* 366, 339-347 (2012).
19. Di Giuliano, F., et al. Abnormal cervical lymph nodes in multiple sclerosis: a preliminary ultrasound study. *Radiol Med* 123, 202-208 (2018).
20. Tostanoski, L.H., et al. Reprogramming the Local Lymph Node Microenvironment Promotes Tolerance that Is Systemic and Antigen Specific. *Cell Rep* 16, 2940-2952 (2016).

21. van Zwam, M., et al. Surgical excision of CNS-draining lymph nodes reduces relapse severity in chronic-relapsing experimental autoimmune encephalomyelitis. *J Pathol* 217, 543-551 (2009).
22. Saldanha, C.J., Duncan, K.A. & Walters, B.J. Neuroprotective actions of brain aromatase. *Front Neuroendocrinol* 30, 106-118 (2009).
23. Oz, O.K., et al. Aromatase deficiency causes altered expression of molecules critical for calcium reabsorption in the kidneys of female mice *. *J Bone Miner Res* 22, 1893-1902 (2007).
24. Jones, M.E., Boon, W.C., Proietto, J. & Simpson, E.R. Of mice and men: the evolving phenotype of aromatase deficiency. *Trends Endocrinol Metab* 17, 55-64 (2006).
25. Hayasaka, H., Taniguchi, K., Fukai, S. & Miyasaka, M. Neogenesis and development of the high endothelial venules that mediate lymphocyte trafficking. *Cancer Sci* 101, 2302-2308 (2010).
26. Faries, M.B., et al. Active macromolecule uptake by lymph node antigen-presenting cells: a novel mechanism in determining sentinel lymph node status. *Ann Surg Oncol* 7, 98-105 (2000).
27. Sleep, D. Albumin and its application in drug delivery. *Expert Opin Drug Deliv* 12, 793-812 (2015).
28. Zhu, G., et al. Albumin/vaccine nanocomplexes that assemble in vivo for combination cancer immunotherapy. *Nat Commun* 8, 1954 (2017).
29. Kratz, F. Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. *J Control Release* 132, 171-183 (2008).
30. di Salle, E., et al. Exemestane (FCE 24304), a new steroidal aromatase inhibitor. *J Steroid Biochem Mol Biol* 43, 137-143 (1992).

31. Swartz, M.A. The physiology of the lymphatic system. *Adv Drug Deliv Rev* 50, 3-20 (2001).
32. Xie, Y., Bagby, T.R., Cohen, M.S. & Forrest, M.L. Drug delivery to the lymphatic system: importance in future cancer diagnosis and therapies. *Expert Opin Drug Deliv* 6, 785-792 (2009).
33. Inoue, M., et al. An interferon-beta-resistant and NLRP3 inflammasome-independent subtype of EAE with neuronal damage. *Nat Neurosci* 19, 1599-1609 (2016).
34. Bannerman, P.G., et al. Motor neuron pathology in experimental autoimmune encephalomyelitis: studies in THY1-YFP transgenic mice. *Brain* 128, 1877-1886 (2005).
35. Sivaguru, M., Khaw, Y.M. & Inoue, M. A Confocal Reflection Super-Resolution Technique to Image Golgi-Cox Stained Neurons. *J Microsc* 275, 115-130 (2019).
36. Koch, J.C., et al. Alpha-Synuclein affects neurite morphology, autophagy, vesicle transport and axonal degeneration in CNS neurons. *Cell Death Dis* 6, e1811 (2015).
37. Michaelsen, K., et al. Fine-tuning of neuronal architecture requires two profilin isoforms. *Proc Natl Acad Sci U S A* 107, 15780-15785 (2010).
38. Minden, S.L., Frankel, D., Hadden, L.S., Srinath, K.P. & Perloff, J.N. Disability in elderly people with multiple sclerosis: An analysis of baseline data from the Sonya Slifka Longitudinal Multiple Sclerosis Study. *NeuroRehabilitation* 19, 55-67 (2004).
39. Dutta, S. & Sengupta, P. Men and mice: Relating their ages. *Life Sci* 152, 244-248 (2016).
40. Haines, J.D., Inglese, M. & Casaccia, P. Axonal damage in multiple sclerosis. *Mt Sinai J Med* 78, 231-243 (2011).

41. Quintana, F.J., Yeste, A. & Mascanfroni, I.D. Role and therapeutic value of dendritic cells in central nervous system autoimmunity. *Cell Death Differ* 22, 215-224 (2015).
42. Nuyts, A.H., Lee, W.P., Bashir-Dar, R., Berneman, Z.N. & Cools, N. Dendritic cells in multiple sclerosis: key players in the immunopathogenesis, key players for new cellular immunotherapies? *Mult Scler* 19, 995-1002 (2013).
43. Comabella, M., et al. A type I interferon signature in monocytes is associated with poor response to interferon-beta in multiple sclerosis. *Brain* 132, 3353-3365 (2009).
44. Killestein, J. & Polman, C.H. Determinants of interferon beta efficacy in patients with multiple sclerosis. *Nat Rev Neurol* 7, 221-228 (2011).
45. Petersen, T., Moller-Larsen, A., Ellermann-Eriksen, S., Thiel, S. & Christensen, T. Effects of interferon-beta therapy on elements in the antiviral immune response towards the human herpesviruses EBV, HSV, and VZV, and to the human endogenous retroviruses HERV-H and HERV-W in multiple sclerosis. *J Neuroimmunol* 249, 105-108 (2012).
46. Androlewicz, M.J., Browning, J.L. & Ware, C.F. Lymphotoxin is expressed as a heteromeric complex with a distinct 33-kDa glycoprotein on the surface of an activated human T cell hybridoma. *J Biol Chem* 267, 2542-2547 (1992).
47. Lu, Y.C., Yeh, W.C. & Ohashi, P.S. LPS/TLR4 signal transduction pathway. *Cytokine* 42, 145-151 (2008).
48. Guven-Maiorov, E., et al. The Architecture of the TIR Domain Signalosome in the Toll-like Receptor-4 Signaling Pathway. *Sci Rep* 5, 13128 (2015).
49. Wang, C., Huang, Y., Sheng, J., Huang, H. & Zhou, J. Estrogen receptor alpha inhibits RLR-mediated immune response via ubiquitinating TRAF3. *Cell Signal* 27, 1977-1983 (2015).

50. Courreges, M.C., Kantake, N., Goetz, D.J., Schwartz, F.L. & McCall, K.D. Phenylmethimazole blocks dsRNA-induced IRF3 nuclear translocation and homodimerization. *Molecules* 17, 12365-12377 (2012).
51. Khaw, Y.M., et al. Astrocytes lure CXCR2-expressing CD4(+) T cells to gray matter via TAK1-mediated chemokine production in a mouse model of multiple sclerosis. *Proc Natl Acad Sci U S A* 118(2021).
52. MacKenzie-Graham, A., et al. Estriol-mediated neuroprotection in multiple sclerosis localized by voxel-based morphometry. *Brain Behav* 8, e01086 (2018).
53. Sicotte, N.L., et al. Treatment of multiple sclerosis with the pregnancy hormone estriol. *Ann Neurol* 52, 421-428 (2002).
54. Voskuhl, R.R., et al. Estriol combined with glatiramer acetate for women with relapsing-remitting multiple sclerosis: a randomised, placebo-controlled, phase 2 trial. *Lancet Neurol* 15, 35-46 (2016).
55. Fillmore, C.M., et al. Estrogen expands breast cancer stem-like cells through paracrine FGF/Tbx3 signaling. *Proc Natl Acad Sci U S A* 107, 21737-21742 (2010).
56. Scarabin, P.Y., et al. Effects of oral and transdermal estrogen/progesterone regimens on blood coagulation and fibrinolysis in postmenopausal women. A randomized controlled trial. *Arterioscler Thromb Vasc Biol* 17, 3071-3078 (1997).
57. Figueiro-Silva, J., et al. Neuronal pentraxin 1 negatively regulates excitatory synapse density and synaptic plasticity. *J Neurosci* 35, 5504-5521 (2015).
58. Ruzzo, E.K., et al. Deficiency of asparagine synthetase causes congenital microcephaly and a progressive form of encephalopathy. *Neuron* 80, 429-441 (2013).

59. Tentorey, J.L., Kofoed, E.M., Daugherty, M.D., Malik, H.S. & Vance, R.E. Molecular basis for specific recognition of bacterial ligands by NAIP/NLRC4 inflammasomes. *Mol Cell* 54, 17-29 (2014).
60. Khaw YM, T.A., Cunningham C, Soto-Díaz K, Kang E, Steelman AJ, Inoue M. Astrocytes lure CXCR2-expressing CD4+T cells to gray matter via TAK1-mediated chemokine production in mouse model of multiple sclerosis. *Proc Natl Acad Sci U S A* in press(2021).
61. Kelly, M.J. & Levin, E.R. Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12, 152-156 (2001).
62. Nilsson, S., et al. Mechanisms of estrogen action. *Physiol Rev* 81, 1535-1565 (2001).
63. Wouters, K., et al. Circulating classical monocytes are associated with CD11c(+) macrophages in human visceral adipose tissue. *Sci Rep* 7, 42665 (2017).
64. Meredith, M.M., et al. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *J Exp Med* 209, 1153-1165 (2012).
65. Confavreux, C., Vukusic, S., Moreau, T. & Adeleine, P. Relapses and progression of disability in multiple sclerosis. *N Engl J Med* 343, 1430-1438 (2000).
66. Confavreux, C., Vukusic, S. & Adeleine, P. Early clinical predictors and progression of irreversible disability in multiple sclerosis: an amnesic process. *Brain* 126, 770-782 (2003).

CHAPTER 5: ASTROCYTES LURE CXCR2-EXPRESSING CD4⁺T CELLS TO GRAY MATTER VIA TAK1-MEDIATED CHEMOKINE PRODUCTION⁴

5.1 INTRODUCTION

Multiple sclerosis (MS) is a prevalent, chronic neurologic autoimmune disease that results in accumulating disability. Disease onset usually occurs at 20–50 years of age and is characterized by symptoms of numbness, pain, fatigue, and/or visual impairment ¹⁻³. Within 15-25 years of onset, 50% of MS patients require assistance with walking ^{4,5} and 50% of MS patients report neurocognitive impairment ⁶. Accumulation of debilitating symptoms is attributed to an episodically inflamed central nervous system (CNS) as a result from recurrent attacks by immune cells ⁷.

Demyelinated lesions are the classical hallmark of MS ^{8,9}, thus the disease is long considered a disease that primarily affects white matter of the CNS. In the past 20 years, a growing body of evidence suggests that inflammatory lesions in CNS are not restricted to white matter but are also observed in CNS gray matter ⁹⁻¹¹. In addition to the observation of myelin loss, gray matter lesions were shown to present with neuron damage characterized by axonal transection, synaptic loss, and even neuronal loss ¹²⁻¹⁶. Due to the irreversibility of neurodegeneration, neuron damage is proposed to underlie permanent neurological dysfunctions in persons with MS ^{17, 18}.

⁴ This chapter is published in its entirety in PNAS. This article is available from <https://www.pnas.org/content/118/8/e2017213118.short> and using DOI: <https://doi.org/10.1073/pnas.2017213118>

Antigen-specific lymphocytes such as T cells are implicated in gray matter damage observed in MS¹⁹ and an established mouse model, experimental autoimmune encephalomyelitis (EAE) (7-9). In the classical EAE model, T cells are mainly restricted to white matter of the spinal cord^{20,21} and are rarely found in spinal cord gray matter of EAE mice, with few exceptions²². Thus, genesis of T cells in the gray matter during MS pathogenesis is poorly understood. T cell-mediated neuronal damage would require trafficking from lymphoid organs of peripheral tissues, such as lymph nodes and spleen, and subsequent transmigration into the CNS. T cell migration can occur via a vascular route through the blood brain barrier (BBB), blood-cerebrospinal fluid, or meningeal lymphatic system^{23,24}.

Lymphocyte infiltration into the CNS is a tightly regulated process that is controlled by multiple factors that are cell intrinsic or extrinsic, including BBB status, adhesion molecule expression, and presence of migratory cues^{24,25}. During neuroinflammation, invading immune cells and local reactive glial cells create signaling gradients by secreting chemoattracting small peptide mediators to attract pathogenic cells to sites of inflammation. Of these cells, CNS-resident astrocytes have been identified to be a key cellular source of important chemokines to induce the migration of T cells^{26,27}.

Here, while exploring the drivers of severe neuron damage in the spinal cord gray matter of mice induced to have a neurodegenerative form of EAE (termed Type-B EAE), we made the serendipitous observation that Type-B EAE is characterized by massive infiltration of CD4⁺ T cells into gray matter of the spinal cord. Accumulation of CD4⁺ T cells in spinal cord gray matter was prevented by genetic ablation of T cell CXCR2 expression. Additionally, genetic ablation of astrocyte TAK1, an upstream molecule of CXCR2 ligand CXCL1, successfully prevented T cell migration to spinal cord ventral horns, neuronal damage, and motor dysfunction.

5.2 METHODS

Animals

Healthy male and female C57BL/6J (Jackson Laboratories, # 000664) mice were used for experiments. Tcra mice (# 002116), Cxcr2^{fl} mice (# 024638), tdTomato^{fl} mice (# 007908), Lckicre mice (# 012837), Gfapcre mice (# 024098), and Tak1^{fl} mice (# 011039) of C57BL/6J background were purchased from The Jackson Laboratories. The studies described here were approved by University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (protocol no. 19171).

Active EAE induction

Six- to eight-week-old mice were randomly subjected to either Type-A EAE or Type-B EAE. Type-A EAE induction consists of one subcutaneous (s.c) injection of 100 µg myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ peptide (MOG₃₅₋₅₅, United Peptides) emulsified in complete Freund's adjuvant (#F5881, Sigma) including 200 µg/mouse heat-killed Mycobacteria (Mtb, #DF3114-33-8, Fisher). Type-B EAE consists of two s.c. injection of 100 µg MOG₃₅₋₅₅ emulsified in complete Freund's adjuvant including 400 µg/mouse Mtb at 0 and 7 dpi. Pertussis toxin (#181, List Biological Laboratories, Inc, 200 ng/mouse) was i.p. injected on 0 and 2 dpi for Type-A EAE and 0, 2, and 7 dpi for Type-B EAE.

Passive EAE induction by intrathecal transfer of CD4⁺T cells

Six- to eight-week-old Tcra^{-/-} mice were randomly subjected to Type-A or Type-B EAE induction. Six- to eight-week-old B6 mice were subjected to Type-B EAE simultaneously. At 14 dpi, B6 mice with Type-B EAE are sacrificed to allow harvesting of splenic CD4⁺T cells.

Detailed method on splenic immune cell isolation is provided in SI Appendix. Isolated CD4⁺T cells (10⁵ cells per mouse) were adoptively transferred by intrathecal injection to Tcra^{-/-} recipient mice with Type-A or Type-B EAE induction at 14 dpi. Animals were monitored daily until 14 days-post passive transfer.

Golgi-Cox confocal microscopy and analysis

Animals were perfused with 4% PFA with no post-fixing then processed according to FD Rapid Golgi staining kit protocol. To obtain spine density data, 50 μ m Golgi-Cox-stained spinal cord sections were imaged using a Nikon A1 confocal scanning microscope under Confocal Reflection Super-Resolution (CRSR) modality and dendritic spines were analyzed using filament tracer autopath function (IMARIS). To obtain percentage area stained and size of Golgi-Cox⁺ cell values, 10x brightfield micrographs of selected region (ventral region or dorsal region) were analyzed using ImageJ (at least 4 images per region per animal). To co-stain Golgi-Cox and CD4 immunohistochemistry, 50 μ m Golgi-Cox-stained spinal cord sections were subjected to immunohistochemistry with extended incubation times.

CD4⁺ T cell count z-stack acquisition and analysis

15 μ m-thick z-stacks (distance between each of 17 images in a stack was 0.975 μ m) of halved spinal cord lumbar L4-L6 coronal sections were taken on a Nikon confocal A1 fluorescent microscope using a 20 $\times$ objective. In all analyses, six halved spinal cord lumbar coronal sections were analyzed based on selected areas per tissue section. Region of interests were manually traced using NIS-Elements software (Nikon) by a single-blinded experimenter. CD4⁺ T cells per selected region were manually counted by the same blinded experimenter in serial view.

Immunohistochemistry and antibodies

Animals were anesthetized at 28 dpi (unless otherwise mentioned) by isoflurane and intracardially perfused with PBS followed by 4% of paraformaldehyde (PFA). Lumbar spinal cord was carefully removed and post-fixed in 4% PFA for 24 h at 4°C. Tissue was then submerged in 30% sucrose solution for 12-24 hours before being embedded and snap-frozen in optimum cutting temperature gel on pulverized dry ice. Sample was stored at -80°C until it was cut into 30 μ m coronal sections (Cryostar NX50).

Fluorescence imaging and analyses

Images of half spinal cord lumbar coronal sections at L4-6 were taken on a Nikon confocal A1 fluorescent microscope using 20 $\times$ objective with numerical aperture of 0.75. All pictures in each set of micrographs were acquired using the same laser intensity and gain values. In all analyses, five halved spinal cord lumbar coronal sections were analyzed based on selected areas per tissue section (2 per section, 3 sections per mouse). Measurement of integrated density was obtained from selecting Region of Interest (ROI) on raw images followed by the measurement of integrated density/region of ROI using National Institutes of Health ImageJ software.

Ex vivo time-lapse imaging of t cells in the lumbar spinal cord

Mice were anaesthetized using isoflurane, and lumbar spinal cords were excised distally. Excised spinal cord tissue from LckCre-Tomato^{fl} mice was immediately placed in 4°C DMEM with 10% FBS to prevent hypoxia. Spinal cord was cut (2 mm-thick) coronally at L4 and L6 using a box cutter, encased 1% low-melting-point agarose solution in phosphate buffer saline (PBS), and mounted on the glass bottom of a 25-mm glass button dish. 37°C pre-warmed

DMEM media with 1% FBS were added to the dish. T cells were visualized with a confocal system (Nikon A1) using TRITC excitation. XYZ-stacks were typically collected using a 10x objective within a scan field of 512×512 -pixel resolution and a z-plane distance of 2 μm for 40 min. Nikon NIS Elements data files were processed in ImageJ. A maximum-intensity projection of each z-stack was generated. TrackMate plugin³⁰ for ImageJ was used to perform single-particle tracking of T cells over a 40–45 min interval. Detailed method is provided in SI Appendix. To confirm T cell liability in spinal cord tissue in ex vivo study, Live/Dead fixable blue dead cell stain kit (Thermofisher) was used to stain dead cells. As positive control of dead cell staining, we cultured spinal cord tissues in PBS for 3 days, and stimulate T cells with high doses of αCD3 (10 $\mu\text{g/ml}$) and αCD28 (30 $\mu\text{g/ml}$) for 3h.

Statistics

Sample sizes, numbers of animals, and factors used for statistical evaluations are indicated in figure legends and this Methods section. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field^{31–33}.

Datasets comprising two groups for comparison were tested for normal distribution (Shapiro-Wilk test, Kolmogorov-Smirnov test) and statistical significance using unpaired two-tailed Student's t tests for parametric data, or Mann–Whitney U tests for nonparametric data, as detailed in figure legends. Statistical analyses and graphical presentations were computed with GraphPad Prism software (GraphPad, La Jolla, United States). All bar graphs are presented as the mean $\pm$ SEM.

5.3. RESULTS

Severe neuronal damage in gray matter of Type-B EAE mice

C57BL/6J mice received two types of MOG peptide-specific EAE induction: “Type-A” EAE that consisted of the widely used one-time EAE immunization³⁴ with low dose heat-killed *Mycobacteria tuberculosis* (Mtb, 200 µg/mouse), or “Type-B” EAE that consisted of two immunizations with high dose Mtb (400 µg/mouse) (Fig. 5.1 A). To evaluate motor paralysis which is correlated with spinal cord inflammation in EAE^{35,36}, clinical signs were scored daily as follows: 0.5, partial tail limpness; 1, tail limpness; 1.5, reversible impaired righting reflex; 2, impaired righting reflex; 2.5, paralysis of one hindlimb; 3, paralysis of both hindlimbs; 3.5, paralysis of both hindlimbs and one forelimb; 4, hindlimb and forelimb paralysis; and 5, death. As previously observed³², clinical severity defined by motor paralysis started around 10 days post-immunization (dpi), peaked at 21 dpi, and tapered off into disease remission in Type-A EAE mice (Fig. 5.1 B). However, clinical severity of Type-B EAE mice did not taper off and instead chronically increased up to 60 dpi (Fig. 5.1 B). Notably, peak clinical severity of Type-B EAE mice was similar to that of Type-A EAE mice.

To determine whether there is a correlation between duration of motor dysfunction and degree of neuron damage in the CNS, we examined neuronal morphology in gray matter of the lumbar spinal cord of Type-A and Type-B EAE mice at 28 dpi using Golgi-Cox staining³⁷. We chose to evaluate histopathological manifestations at 28 dpi because it is a time in disease chronic phase when neurodegeneration or axonal loss is expected to have occurred. Secondly, EAE scores between Type-A and Type-B EAE animals are significantly different at 28 dpi which permits evaluation of cellular mechanism of diseases severity. A ~34% significant reduction in Golgi-Cox staining was observed in the gray matter of lumbar spinal cord (L4-L6)

of Type-B EAE mice when compared to Type-A EAE mice (Fig. 5.1 C). Interestingly, a ~25% significant reduction in Golgi-Cox staining was observed in the ventral gray matter (vGM) of Type-B compared to Type-A EAE mice (Fig. 5.1 C). In contrast, an insignificant reduction (~9%) in Golgi-Cox staining was observed in dorsal gray matter (dGM) of Type-B compared to Type-A EAE mice (Fig. 5.1 C). Further analysis of cell sizes of Golgi-impregnated cells in the vGM revealed enlargement of cell size, which reflects cell swelling in Type-B EAE mice when compared to Type-A EAE mice (Fig. 5.1 D). Because dendritic spine loss and abnormalities is reported to manifest in EAE ^{38,39}, we next quantitated spine density on dendrites in the vGM using Golgi-Cox-stained samples reconstructed in 3D via confocal reflection microscopy ³⁷. Number of vGM dendritic spines were significantly reduced by 33% in Type-B EAE mice compared to Type-A EAE mice (Fig. 5.1 E). Together, these results suggest that Type-B EAE triggers potential neuron loss and neuron morphological abnormalities in vGM of lumbar spinal cord, which provides a likely explanation for our observation of persistent motor dysfunction in these mice (Fig. 5.1 A). Further, these results suggest that Type-B EAE is a useful model to identify the mechanism of neuronal damage in spinal cord gray matter compared to the conventional Type-A EAE model.

CD4⁺ T cell migration to the gray matter in Type-B EAE is dependent on CXCR2 expression

CD4⁺ T cells are key immune cells for the induction of motor dysfunction and CNS neuronal damage during EAE ^{32,40,41}. We evaluated CD4⁺ T cell infiltration as visualized via anti-CD4 immunofluorescence in the spinal cord of Type-A and Type-B EAE mice at 28 dpi. CD4⁺ T cells were two-fold more abundant in the GM and white matter (WM) of Type-B EAE mice compared to Type-A EAE mice (Fig. 5. 2A). Strikingly, while CD4⁺ T cell accumulation in GM

was sparse and evenly distributed between dGM and vGM in Type-A EAE mice, CD4⁺ T cells were selectively and significantly enhanced in vGM of Type-B EAE mice (Fig. 5.2 A).

To study T cell dynamics in the spinal cord during EAE, we evaluated T cell movement in explanted lumbar spinal cord using EAE-induced lymphocyte protein tyrosine kinase (Lck)iCre-Tomato-floxed mice in which T cells endogenously express red fluorescence protein (RFP). RFP expression in grey and white matters was measured in ex vivo spinal cord tissue isolated at 28 dpi using time-lapse z-stack confocal imaging. Mean velocity of T cells in vGM was significantly slower than in vWM in both Type-A and Type-B EAE mice (Fig. 5.2 B). This suggests that T cells may have more lasting interactions with other cells, such as motor neurons, in the vGM compared to vWM.

To determine if T cells in the gray matter interact with CNS neuronal populations, we first conducted CD4 immunohistochemistry in Golgi-Cox samples. Using confocal reflection microscopy³⁷, we show that CD4⁺T cells adhered to Golgi-stained neuron in vGM of Type-B EAE mice at 28 dpi (Fig. 5.2 C). Adherence of CD4⁺T cell to neuron was also observed in immunohistochemistry with CD4 and Tuj1, a neuron marker (Fig. 5.2 D). These findings demonstrate that T cells may be conducting long term cell-to-cell interactions with neuronal bodies.

We next sought to identify necessary chemotactic signals needed to facilitate CD4⁺ T cell migration into vGM of the spinal cord in Type-B EAE mice. Because CXCR2 is a key molecule for motor dysfunction in Type-B but not Type-A EAE mice^{32, 43}, we evaluated the role of CXCR2 signaling in CD4⁺ T cell migration to vGM using T cell-specific conditional knockout of CXCR2 (cCxcr2^{-/-} mice) by breeding LckiCre mice with Cxcr2-floxed mice. The number of CD4⁺ T cells in vGM was reduced by 50% in Type B EAE-induced cCxcr2^{-/-} mice compared

with Type-B EAE-induced control (Cxcr2-floxed) mice (Fig. 5.2 E). Furthermore, Type B EAE-induced cCxcr2^{-/-} mice displayed less severe motor paralysis symptoms when compared to Type-B EAE-induced control mice (Fig. 5.2 F). This result suggests that CD4⁺ T cell expression of CXCR2 is essential for CD4⁺ T cell migration to vGM of the spinal cord and symptom development in Type-B EAE mice.

CXCR2 ligands CXCL1 and CXCL5 are upregulated in gray matter astrocytes within spinal cord ventral horn in Type-B EAE mice

We next investigated whether the migration of CXCR2-expressing CD4⁺ T cells in Type-B EAE-induced mice was dependent on signaling cues derived from spinal cord. For this purpose, CD4⁺ T cells were isolated from Type-B EAE mice at 14 dpi. We isolated T cells from Type-B EAE mice at 14 dpi because antigen-specific T cells are clonally proliferated and expressed CXCR2 at this time point ³², which allows abundant isolation of MOG antigen-specific and CXCR2-expressing CD4⁺ T cells. Isolated CD4⁺ T cells (10⁵ cells) were intrathecally transferred into the spinal cord of Type-B or Type-A EAE-induced Tcr α ^{-/-} mice, which intrinsically lack T cells, at 14 dpi (Fig. 5.3 A). Approximately two-fold of Type-B EAE-derived CD4⁺ T cells migrated to vGM of the spinal cord in Tcr α ^{-/-} mice with Type-B EAE mice when compared to Type-A EAE (Fig. 5.3 B). This suggests that Type-B EAE induced changes in the spinal cord, independent of T cells, which were essential for T cell migration to vGM.

To identify cellular players involved in attracting and maintaining T cell localization to the gray matter, we investigated glia activation status in the vGM of EAE-induced Tcr α ^{-/-} mice at 14 dpi without T cell transfer. By conducting immunohistochemistry experiments in lumbar spinal cord sections, we found that astrocytes are significantly more reactive in vGM of Type-B

EAE *Tcra*^{-/-} mice compared to Type-A EAE *Tcra*^{-/-} mice without CD4⁺T cell transfer, as indicated by heightened GFAP immunoreactivity ⁴⁴ (Fig. 5.3 C). On the other hand, there was no significant difference in microglia/macrophage density, as indicated by Iba1 immunoreactivity, in vGM between the two groups (Fig. 5.3 D). These findings identify astrocytes as being a potential key cellular player in the event of T cell localization in gray matter.

We next asked whether Type-B EAE differentially alters astrocyte component compared to Type-A EAE in B6 mice. Significantly greater integrated fluorescence density of reactive astrocytes was also observed in vGM of Type-B EAE compared to Type-A EAE mice (Fig. 5.3 E). Interestingly, in contrast to the absence of heightened microglia/monocyte reactivity in Type-B EAE *Tcra*^{-/-} mice without CD4⁺T cell transfer, significantly greater Iba1 integrated fluorescence density was observed in vGM of Type-B EAE compared to Type-A EAE mice (Fig. 5.3 F). This result suggests that CD4⁺T cells are required for microglia/macrophages activation in Type-B EAE mice. Together, all these results demonstrate that Type-B EAE model heightened astrocyte reactivity in the vGM independent of the presence of T cells which may be linked to local T cell invasion and aggregation.

Local activation of astrocytes in the vGM of Type-B EAE B6 and *Tcra*^{-/-} mice suggests that these astrocytes may be secreting chemotactic ligands to encourage T cell homing. To identify vGM-derived factors expressed in Type-B EAE that facilitate CD4⁺T cell localization to vGM, we evaluated CXCR2 ligand expression in the spinal cord of Type-B and Type-A EAE mice. CXCR2 ligands CXCL1 was highly upregulated in vGM of the spinal cord of Type-B compared to Type-A EAE mice (Fig. 5.3 G). CXCL1 was expressed by astrocytes, but not microglia nor neuron, as indicated by co-immunostaining of CXCL1 with GFAP and Iba1 or with Tuj1, a neuron-specific tubulin marker (Fig. 5.3 H). These results suggest that migration of

CXCR2-expressing CD4⁺ T cells to vGM may be mediated by increased site-specific CXCR2 ligands expressed by astrocytes.

Astrocyte-targeted TAK1 depletion prevents CD4⁺ T cell migration to gray matter, motor neuron damage, and motor dysfunction in Type-B EAE mice

Transforming growth factor β -activated kinase 1 (TAK1) is upstream of CXCL1 and CXCL5 chemokine production in astrocytes^{45,46}. To determine the role of astrocyte chemokine ligand production in CD4⁺ T cell trafficking to vGM of the spinal cord, we used a GFAP⁺ cell conditional knockout of TAK1 (cTak1^{-/-} mice) by breeding Glial fibrillary acidic protein (GFAP)-Cre mice with TAK1-floxed mice. GFAP-Cre mouse lines have been widely used to target astrocytes for genetic manipulation^{47,48}. cTak1^{-/-} mice showed ~40% reduction of CXCL1 expression in astrocyte in vGM of Type-B EAE mice (Fig. 5.4 A). cTak1^{-/-} mice showed significantly lower EAE symptoms upon Type-B EAE induction compared with control mice (Fig. 4B), suggesting less inflammation in the spinal cord. This suggests that astrocyte TAK1 signaling may be a key disease mechanism in Type-B but not Type-A EAE.

While high numbers of CD4⁺ T cells are observed in vGM of Tak1-floxed control mice with Type-B EAE, a significant 80% reduction in CD4⁺ T cell localization in vGM was observed in cTak1^{-/-} Type-B EAE mice (Fig. 5.4 C). Consistent with our observation that neuronal damage in the ventral horn was correlated with disease severity, area percentage of Golgi-Cox staining and number of dendritic spines in the vGM were ~100% greater in cTak1^{-/-} mice with Type-B EAE when compared to Tak1-floxed control mice with Type-B EAE (Fig. 5.4 D, E). These results suggest that TAK1-mediated chemokines such as CXCL1 expression in astrocytes

is necessary to facilitate CD4⁺T cell migration into vGM of the spinal cord of Type-B EAE mice and result in local neuronal damage.

5.4 DISCUSSION

Our studies highlight an important role for CD4⁺T cell chemokine receptor and astrocyte-derived chemokine ligands in determining spatial distribution of infiltrating peripheral CD4⁺T cells in gray matter of the CNS. Type-B EAE presents with prolonged motor dysfunction without remission and CD4⁺T cells were highly augmented in lumbar spinal cord gray matter, specifically the ventral horns, during disease chronic phase. The ventral horn is of interest because it is where lower motor neurons that innervate hindlimb muscles reside. Additionally, lesions are predominantly seen in the lumbar region of the spinal cord when using the classic EAE paradigm ^{49, 50}. However, based on recent observations by Cahalan group which revealed diffused T cell localization in whole spinal cord at disease chronic stage ⁵¹, we postulate that T cell localization in the gray matter may also be observed in spinal cord regions outside of lumbar regions. This hypothesis requires experimentation to be validated in the future. Here we show that the abundance of CD4⁺T cells in gray matter spatially correlates with pathologies of neuronal damage as defined here as reduction of neuronal bodies, swelling of neurons, and loss of dendritic spines. Increased neuronal cell size had been demonstrated by our group and others to potentially reflect cell swelling which is indicative of cell death processes such as necrosis ^{43, 52, 53}. While it is tempting to speculate that an increased cell size may be reconciled with the reduction in dendritic spine density, this is yet to be experimentally addressed.

Several studies demonstrated correlations between white matter lesions and gray matter atrophy, suggesting that degeneration of neurons can be a result of white matter demyelination

via retrograde degeneration ⁵⁴. Another theory suggests that white matter and gray matter damage are partially independent events. According to post-mortem studies, loss of neurons is observed within both gray matter demyelinated lesions and normal-appearing gray matter ^{55,56}, suggesting that neuronal loss might occur independent of demyelination. Several mechanisms have been proposed for how T cells damage neurons. These include neurotoxic cell contact-dependent interactions, such as IFN- γ -mediated induction of calcium-permeable complexes between IFN- γ receptor and glutamate receptor 1 expressed on neurons, induction of cellular apoptosis by Fas–FasL interactions, and initiation of neurite retraction by semaphorin glycoproteins ^{32,57}. Specifically, our previous report demonstrated CD4⁺T cells from Type-B EAE mice have significantly increased expression of semaphorin 6B, which induces neuronal damage in a co-culture system with primary neurons ³². Using mice that express RFP in Lck-expressing T cells, we found that T cells significantly accumulate in gray matter of Type-B EAE mice and that velocity of gray matter neurons is low. Notably, T cells analyzed for velocity in this experiment were not limited to CD4⁺T cells and may have included other Lck-expressing cells such as CD8⁺T cells. Because we found that immobilized T cells interact with neurons in vGM of Type-B EAE mice, these cell interactions may account for neuronal damage via above mechanisms.

It is increasingly accepted that astrocyte and microglial activation are necessary and crucial for host defense and neuron survival, whereas glial over-activation has deleterious effects. In the context of CD4⁺T cell-driven EAE pathology, microglial cells are massively activated in EAE in response to pro-inflammatory cytokines released from T lymphocytes, which, in turn, further potentiate site-specific inflammation ⁵⁸. For example, oxidative burst by activated microglia has a major role in the induction of demyelination and axonal injury in MS

lesions ^{59,60}. Our findings are similar in showing that microglia are in a hyperactivated state, as shown by increased soma roundness and aggregation. Interestingly, our studies using Tcr α ^{-/-} Type-B EAE mice revealed that CD4⁺T cells are required for microglia activation in vGM. Because we do not observed direct interaction between microglia and CD4⁺T cells in the vGM using frozen sections, we suspect that CD4⁺T cells may not directly interact with microglia, but instead influence microglia effector function via secretion of cytokines and other microglia-activating factors. Thus we suspect that activated microglia in vGM likely contributes to neuronal damage observed in vGM of Type-B EAE mice.

Several studies provided data that indicate infiltration of activated peripheral immune cells are associated with astrocytes upregulating chemokines during neuroinflammation or in response to cytokine stimulation ⁶¹. Steelman and colleagues reported that a TAK1 signal is essential to generate a range of chemokine ligands, including high-affinity CXCR2 ligand such as CXCL1 and CXCL5, in astrocytes in vitro ⁴⁵. We found that astrocytes are activated by Type-B EAE induction without T cells and activated microglia. Recently, we reported that Type-B EAE induces upregulation of reactive oxygen species and proinflammatory cytokines in neutrophil ⁶², which can result in astrogliosis ⁴⁵. Therefore, neutrophils may be a critical cell population involved in activating astrocytes in Type-B EAE. We also reported that TAK1 activation in astrocytes is mediated by proinflammatory cytokines ⁴⁵. While genetic deletion of TAK1 is considered to be limited to astrocytes whose hallmark filament protein is GFAP, GFAP expression in adult mouse spinal cord had been observed in neuronal populations, specifically, neural stem cells where it is believed to be vital for neurogenesis ^{63,64}. Thus, we cannot exclude the possibility that the effect of TAK1 deletion on T cell migration is partially dependent on neuronal expression of TAK1. Nonetheless, it is important to note that TAK1 signaling in

neuronal stem cells had not been associated with chemotactic functions whereas TAK1 signaling in astrocytes largely influences production of chemokine ligands⁴⁵. We also showed CXCL1 is not colocalized with neuron in the ventral gray matter of lumbar spinal cord region in Type-B EAE mice. Thus, we attribute the reduction in T cell localization in spinal cord gray matter of GFAP-specific cTak1^{-/-} mice to reduction in astrocyte chemokine ligand production downstream of Tak1 signaling.

Chemokine ligands for CXCR2 are commonly considered to be neutrophil chemo-attraction factors because CXCR2 is abundantly expressed by neutrophils⁶⁵. The Type-B EAE model provides a unique opportunity to study the effects of TAK1-mediated chemokine ligands on migration patterns of CXCR2-expressing CD4⁺T cells. Based on our previous finding of increased CXCL1 levels in serum and the surprising upregulation of CXCR2 on CD4⁺T cells⁶⁵, we speculate that CXCR2 migration plays a role in accumulation of immune cells in the CNS. Indeed, T cell-specific knockout of *Cxcr2* successfully inhibited T cell infiltration into gray matter. To assess whether the presence of Type-B EAE-related intrinsic factors in the inflamed spinal cord were necessary for migration of CD4⁺T cells to gray matter, we performed passive transfer of CD4⁺T cells isolated from Type-B EAE donor mice to Type-A or Type-B EAE-induced recipient *Tcra*^{-/-} mice via an intrathecal route. We chose intrathecal instead of the more commonly used intravenous route to bypass the process of T cell migration from the periphery to the CNS and to avoid the possibility of cell property changes in the periphery. Indeed, while T cells were abundant in gray matter of ventral horns of Type-B EAE recipient mice, they were sparse in Type-A EAE recipient mice. This result indicates that localization of T cells in gray matter is dependent on T cell expression of CXCR2 and signaling factors present in Type-B EAE mice.

We previously demonstrated that serum CXCL1 levels significantly increase during disease development in Type-B but not Type-A EAE mice ^{32,43}. In this current study, we show that CXCL1 and CXCL5 are highly upregulated in gray matter of lumbar ventral horn in Type-B EAE mice, which have interferon-beta insensitive disease ^{32,43}. Incidentally, CXCL1 mRNA was reportedly upregulated in persons with interferon-beta-insensitive MS, whereas CXCL5 was recently reported to be a serum biomarker associated with MS relapse ^{66,67}. In this study, we focused on the migration-inducing capacity of astrocyte-derived chemokine ligands in mediating T cell accumulation in gray matter. While not previously investigated, it is possible that the role of CXCR2 on CD4⁺T cells expands beyond cellular trafficking. For instance, we and others have shown that activation of CXCR2 by recombinant CXCL1 stimulated transcription of genes related to oxidative stress in neutrophils ^{43,68}.

5.5 FIGURES

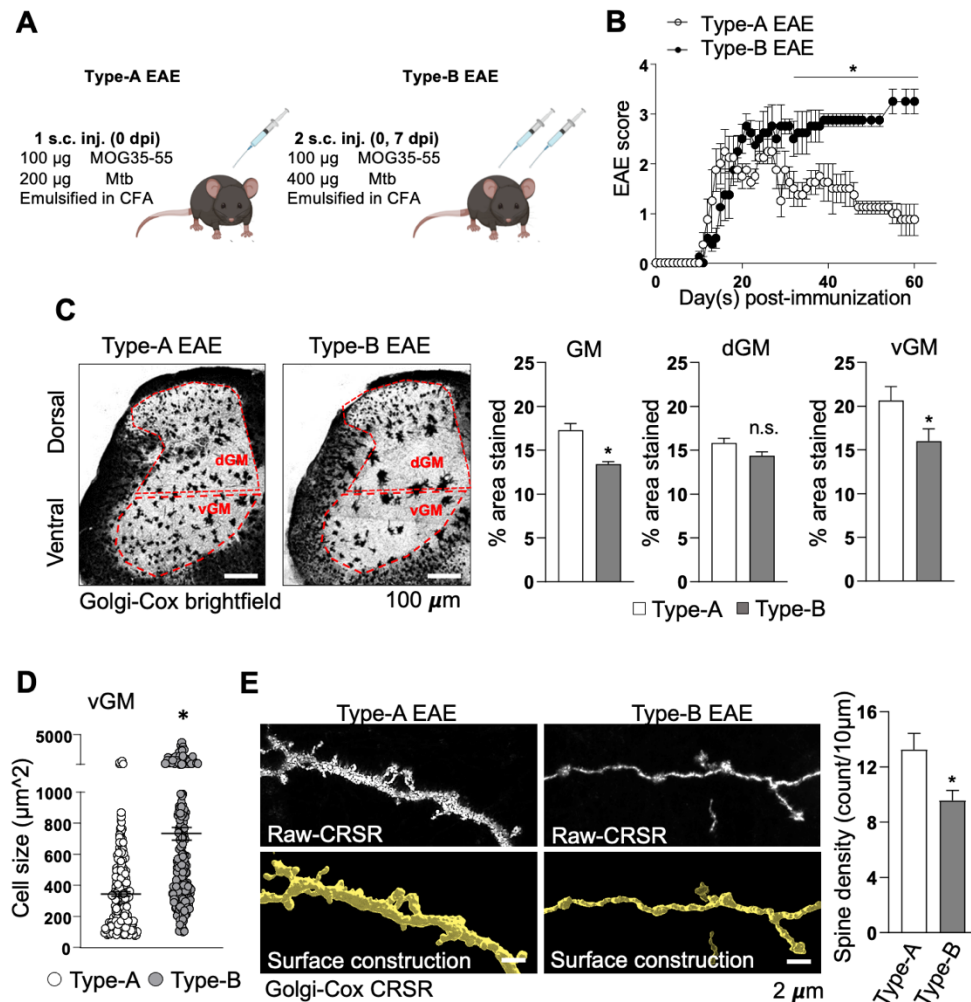


Fig. 5.1. Severe neuronal damage in gray matter of Type-B EAE mice. (A) Schematic for two experimental autoimmune encephalomyelitis (EAE) mouse models. (B) Daily EAE disease scores of Type-A and Type-B EAE mice up to 60 days post-immunization (dpi) (Type-A EAE: n=4; Type-B EAE: n=6). (C) Representative brightfield images of Golgi-Cox-stained lumbar spinal cord hemisections (left) and quantification (right) of percentage stained over region of interest area (GM: gray matter; dGM: dorsal gray matter; vGM: ventral gray matter) at 28 dpi (n = 6 per condition). Data are a compilation of two independent experiments. (D) Size of Golgi-Cox⁺ cells in vGM (n = 3 per condition). (E) Representative 3D images of Golgi-Cox-stained neuron dendrites in vGM (left). Quantitative analysis of dendritic spine density (right) at 28 dpi (n = 3). Statistical analysis: Two-tailed student's t test (B), Mann-Whitney U test (C, D, E). *P < 0.05.

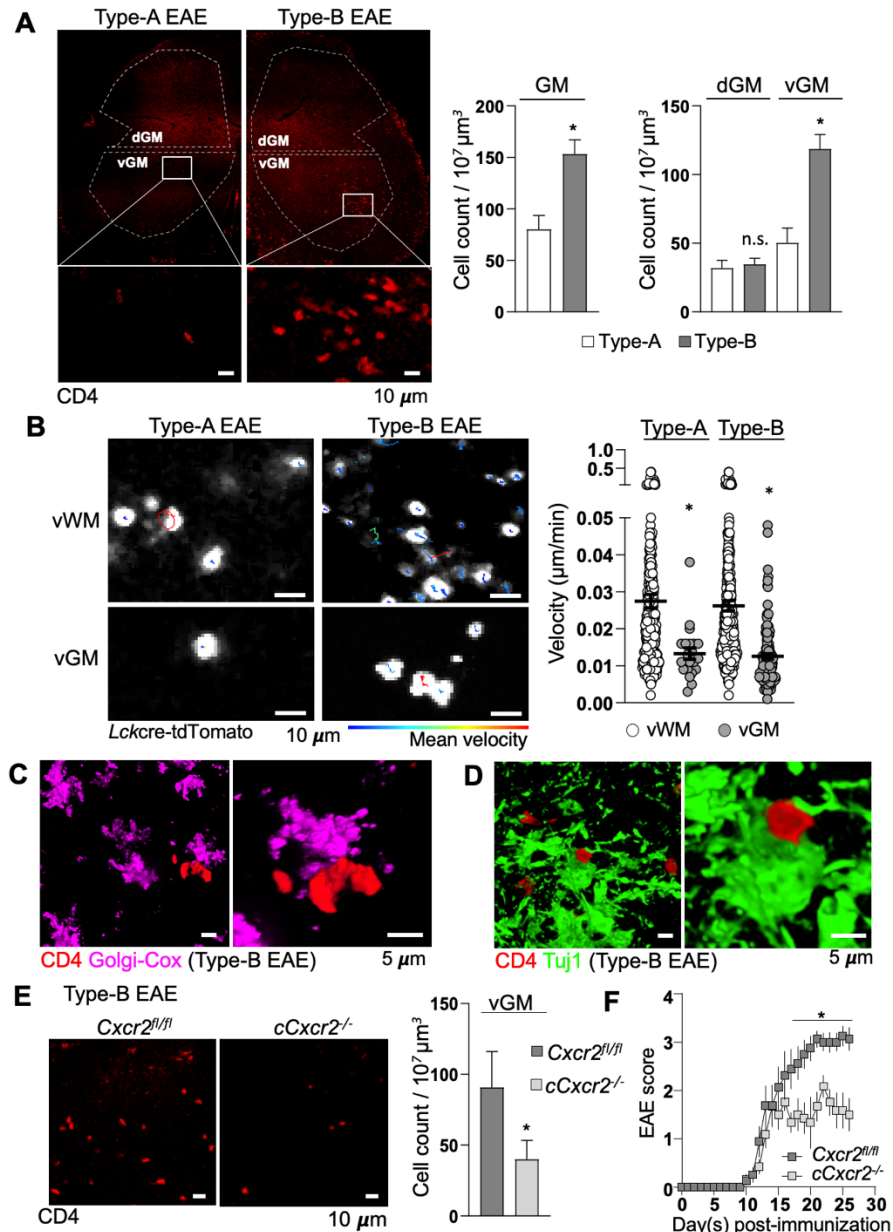


Fig. 5.2. CD4⁺ T migration to gray matter is dependent on CXCR2 chemokine signaling in Type-B EAE mice. (A) Representative micrographs of CD4⁺ T cells in lumbar spinal cord sections labeled with CD4 antibody at 28 days post-immunization (dpi) (left) and quantification of CD4⁺ T cell count in gray matter (GM), dorsal GM (dGM), and vGM (right) (Type-A EAE: n = 5; Type-B EAE: n = 4). (B) Representative confocal images with overlaid T-cell trajectories (corresponding to 40 min of imaging) in vGM at 28 dpi. Average cell velocity ($\mu\text{m}/\text{min}$) is graphed (> 30 cells analyzed per condition). Data are representative of two independent experiments. (C) Representative merged 3D micrograph of CD4⁺ T cell (red) and Golgi-Cox-stained neuron (purple) in vGM of Type-B EAE lumbar spinal cord using conventional and reflection confocal modalities, respectively. (D) Representative merged 3D micrograph of fluorescent CD4⁺ T cell and Tuj1⁺ neuron in ventral gray matter of Type-B EAE lumbar spinal cord. (E) Representative images (left) and quantification (right) of CD4⁺ T cells in vGM of the lumbar spinal cord of *Cxcr2^{fl/fl}* and *cCxcr2^{-/-}* mice at 28 dpi (n = 4 per condition). Statistical analysis: Mann-Whitney U test (A, B, E). *P < 0.05.

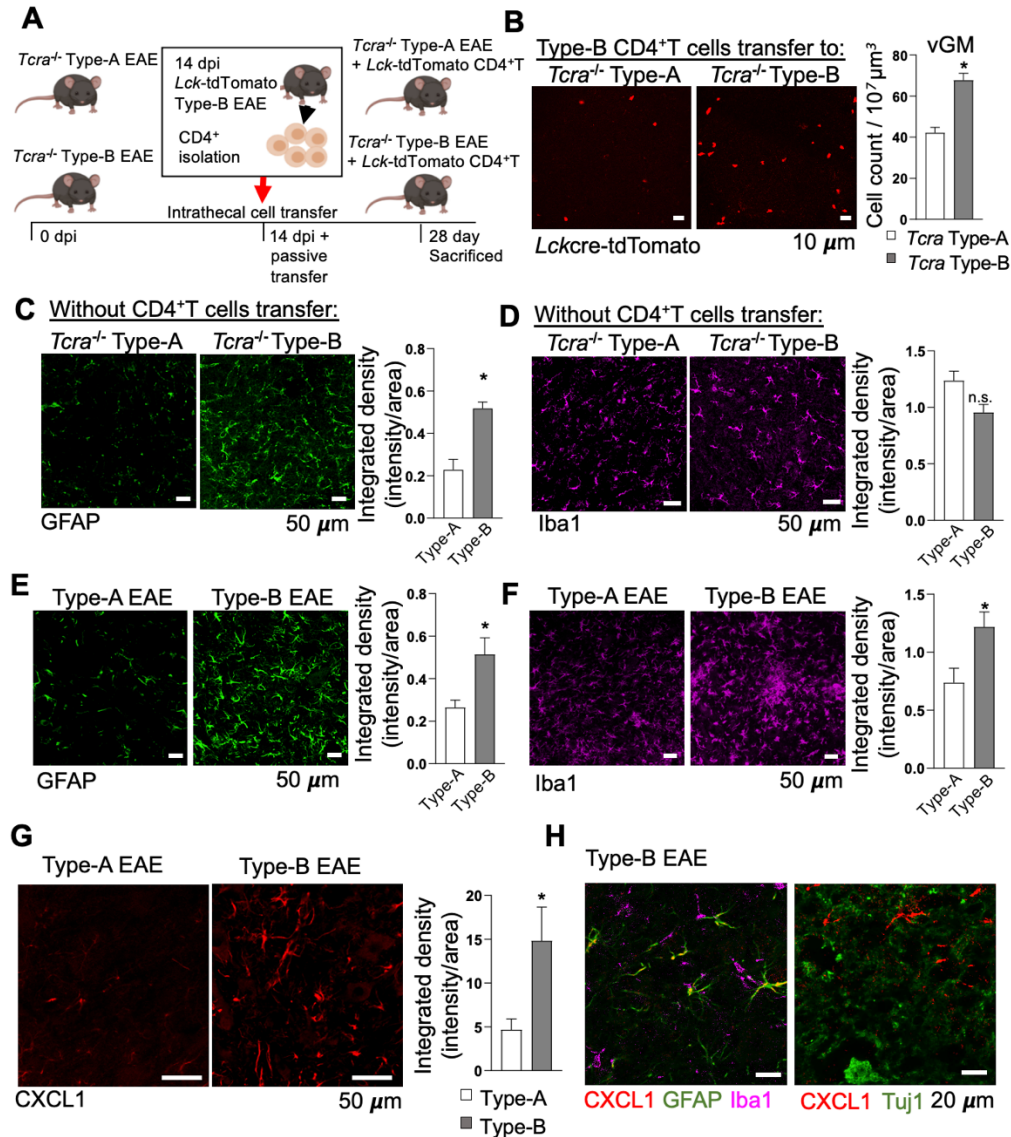


Fig. 5.3. CXCR2 ligands CXCL1 and CXCL5 are upregulated in gray matter astrocytes in spinal cord ventral horns. (A) Schematic for intrathecal passive EAE model. (B) Representative images (left) and quantification (right) of tdTomato CD4⁺T cells in ventral gray matter (vGM) of the spinal cord of Type-A EAE- *Tcra*^{-/-} and Type-B EAE-*Tcra*^{-/-} mice that received passive intrathecal injections of 10⁵ CD4⁺ splenic cells from *Lckcre-tdTomato* Type-B EAE mice at 14 days post-immunization (dpi) (n = 3 per condition). (C, D) Representative micrographs of astrocytes and monocyte/microglia in vGM of lumbar spinal cord sections derived from Type-A EAE- *Tcra*^{-/-} and Type-B EAE-*Tcra*^{-/-} mice before CD4⁺T cell transfer. Bar graphs show glial reactivity measured by integrated density in vGM (Type-A EAE- *Tcra*^{-/-} mice: n = 5; Type-B EAE- *Tcra*^{-/-} mice: n = 4). (E, F) Representative micrographs of astrocytes (E) or microglia/macrophages (F) in vGM of spinal cord sections derived from Type-A EAE and Type-B EAE B6 mice at 28 dpi. Bar graphs show glial reactivity measured by integrated density in vGM (Type-A EAE: n = 5; Type-B EAE: n = 4). Data are representative of two independent experiments. (G) Representative micrographs of CXCL1 in vGM. Bar graph shows chemokine ligand expression levels measured by integrated density in vGM (Type-A EAE: n = 5; Type-B EAE: n = 4). (H) Representative micrographs of CXCL1 co-stained with GFAP and Iba1 or Tuj1 in vGM of Type-B EAE mice at 28 dpi. Statistical analysis: Mann-Whitney U test. *P < 0.05.

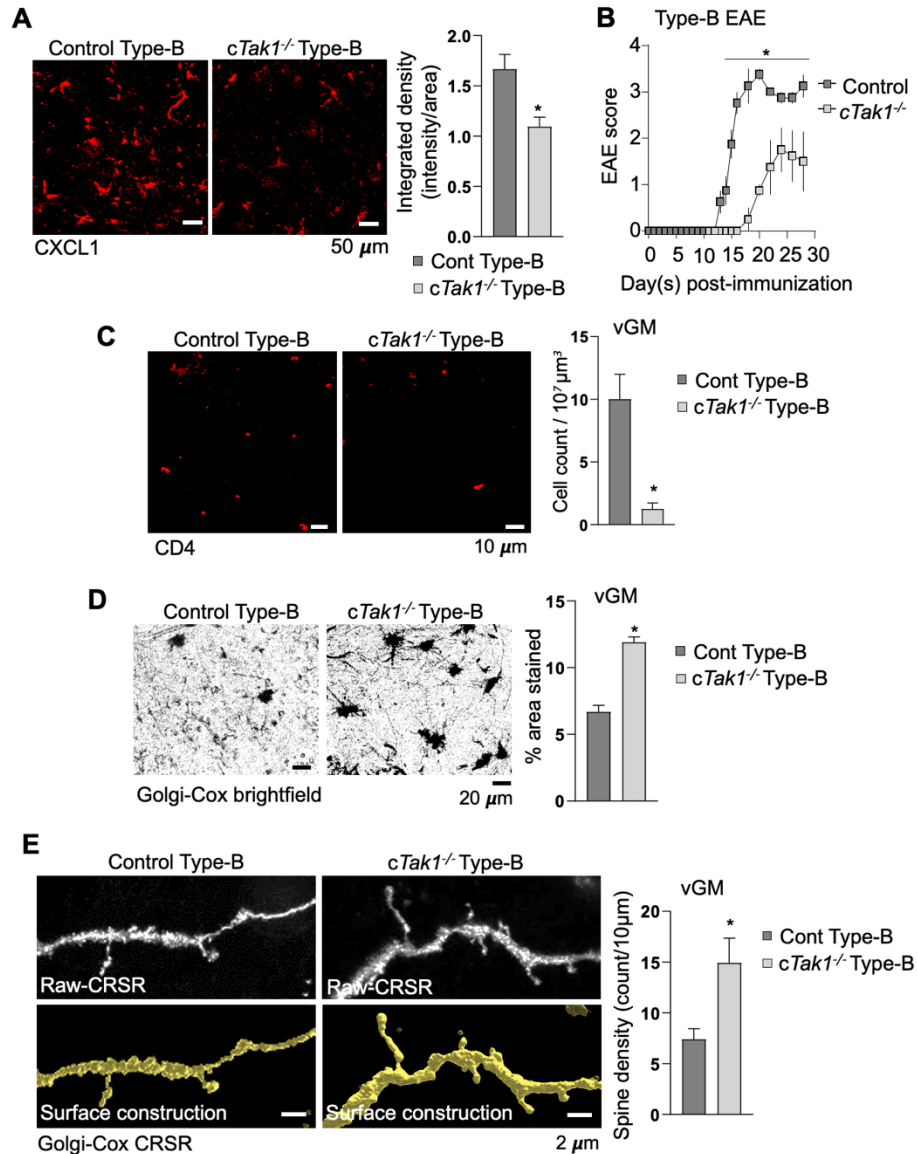


Fig. 5.4. Astrocyte-specific TAK1 depletion prevents CD4⁺ T cell migration to gray matter, motor neuron damage, and motor dysfunction in Type-B EAE mice. (A) Representative micrographs of CXCL1 in vGM. Bar graph shows chemokine ligand expression levels measured by integrated density in vGM. (B) Daily EAE disease scores up to 28 dpi. (C) Representative micrographs (left) and quantification (right) of CD4⁺ T cells in vGM of spinal cord hemisections at 28 dpi. (D) Representative brightfield images of Golgi-Cox-stained vGM (left) and quantification (right) of percentage stained over region of interest area at 28 dpi. (E) Representative 3D images of Golgi-Cox-stained neuron dendrites in vGM (left). Quantitative analysis of dendritic spine density (right). Control Type-B EAE: n = 5; *cTak1*^{-/-} Type-B EAE: n = 4 for all experiments. Statistical analysis: Mann-Whitney U test. *P < 0.05.

5.6 REFERENCES

1. Jasse L, Vukusic S, Durand-Dubief F, et al. Persistent visual impairment in multiple sclerosis: prevalence, mechanisms and resulting disability. *Mult Scler*. Oct 2013;19(12):1618-26. doi:10.1177/1352458513479840
2. Hadjimichael O, Kerns RD, Rizzo MA, Cutter G, Vollmer T. Persistent pain and uncomfortable sensations in persons with multiple sclerosis. *Pain*. Jan 2007;127(1-2):35-41. doi:10.1016/j.pain.2006.07.015
3. Braley TJ, Chervin RD. Fatigue in multiple sclerosis: mechanisms, evaluation, and treatment. *Sleep*. Aug 2010;33(8):1061-7. doi:10.1093/sleep/33.8.1061
4. Souza A, Kelleher A, Cooper R, Cooper RA, Iezzoni LI, Collins DM. Multiple sclerosis and mobility-related assistive technology: systematic review of literature. *J Rehabil Res Dev*. 2010;47(3):213-23. doi:10.1682/jrrd.2009.07.0096
5. Rudick RA. Disease-modifying drugs for relapsing-remitting multiple sclerosis and future directions for multiple sclerosis therapeutics. *Arch Neurol*. Sep 1999;56(9):1079-84. doi:10.1001/archneur.56.9.1079
6. Migliore S, Ghazaryan A, Simonelli I, et al. Validity of the minimal assessment of cognitive function in multiple sclerosis (MACFIMS) in the Italian population. *Neurol Sci*. Aug 2016;37(8):1261-70. doi:10.1007/s10072-016-2578-x
7. Rolak LA. Multiple sclerosis: it's not the disease you thought it was. *Clin Med Res*. Jan 2003;1(1):57-60. doi:10.3121/cmr.1.1.57
8. Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination.

Ann Neurol. Jun 2000;47(6):707-17. doi:10.1002/1531-8249(200006)47:6<707::aid-ana3>3.0.co;2-q

9. Lucchinetti CF, Popescu BF, Bunyan RF, et al. Inflammatory cortical demyelination in early multiple sclerosis. *N Engl J Med.* Dec 8 2011;365(23):2188-97.

doi:10.1056/NEJMoa1100648

10. Trapp BD, Vignos M, Dudman J, et al. Cortical neuronal densities and cerebral white matter demyelination in multiple sclerosis: a retrospective study. *Lancet Neurol.* Oct 2018;17(10):870-884. doi:10.1016/S1474-4422(18)30245-X

11. van der Poel M, Ulas T, Mizee MR, et al. Transcriptional profiling of human microglia reveals grey-white matter heterogeneity and multiple sclerosis-associated changes. *Nat Commun.* Mar 13 2019;10(1):1139. doi:10.1038/s41467-019-08976-7

12. Ganter P, Prince C, Esiri MM. Spinal cord axonal loss in multiple sclerosis: a post-mortem study. *Neuropathol Appl Neurobiol.* Dec 1999;25(6):459-67. doi:10.1046/j.1365-2990.1999.00205.x

13. Holley JE, Gveric D, Newcombe J, Cuzner ML, Gutowski NJ. Astrocyte characterization in the multiple sclerosis glial scar. *Neuropathol Appl Neurobiol.* Oct 2003;29(5):434-44. doi:10.1046/j.1365-2990.2003.00491.x

14. Bjartmar C, Wujek JR, Trapp BD. Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. *J Neurol Sci.* Feb 15 2003;206(2):165-71. doi:10.1016/s0022-510x(02)00069-2

15. Absinta M, Sati P, Masuzzo F, et al. Association of Chronic Active Multiple Sclerosis Lesions With Disability In Vivo. *JAMA Neurol.* Aug 12 2019;doi:10.1001/jamaneurol.2019.2399

16. Bot JC, Blezer EL, Kamphorst W, et al. The spinal cord in multiple sclerosis: relationship of high-spatial-resolution quantitative MR imaging findings to histopathologic results. *Radiology*. Nov 2004;233(2):531-40. doi:10.1148/radiol.2332031572
17. Inglese M, Oesingmann N, Casaccia P, Fleysheer L. Progressive multiple sclerosis and gray matter pathology: an MRI perspective. *Mt Sinai J Med*. Mar-Apr 2011;78(2):258-67. doi:10.1002/msj.20247
18. Agosta F, Pagani E, Caputo D, Filippi M. Associations between cervical cord gray matter damage and disability in patients with multiple sclerosis. *Arch Neurol*. Sep 2007;64(9):1302-5. doi:10.1001/archneur.64.9.1302
19. Popescu BF, Lucchinetti CF. Meningeal and cortical gray matter pathology in multiple sclerosis. *BMC Neurol*. Mar 7 2012;12:11. doi:10.1186/1471-2377-12-11
20. Mony JT, Khorrooshi R, Owens T. Chemokine receptor expression by inflammatory T cells in EAE. *Front Cell Neurosci*. 2014;8:187. doi:10.3389/fncel.2014.00187
21. Whittaker Hawkins RF, Patenaude A, Dumas A, et al. ICAM1+ neutrophils promote chronic inflammation via ASPRV1 in B cell-dependent autoimmune encephalomyelitis. *JCI Insight*. Dec 7 2017;2(23)doi:10.1172/jci.insight.96882
22. Derfuss T, Parikh K, Velhin S, et al. Contactin-2/TAG-1-directed autoimmunity is identified in multiple sclerosis patients and mediates gray matter pathology in animals. *Proc Natl Acad Sci U S A*. May 19 2009;106(20):8302-7. doi:10.1073/pnas.0901496106
23. Lutz SE, Smith JR, Kim DH, et al. Caveolin1 Is Required for Th1 Cell Infiltration, but Not Tight Junction Remodeling, at the Blood-Brain Barrier in Autoimmune Neuroinflammation. *Cell Rep*. Nov 21 2017;21(8):2104-2117. doi:10.1016/j.celrep.2017.10.094

24. Engelhardt B. Molecular mechanisms involved in T cell migration across the blood-brain barrier. *J Neural Transm (Vienna)*. Apr 2006;113(4):477-85. doi:10.1007/s00702-005-0409-y
25. Takeshita Y, Ransohoff RM. Inflammatory cell trafficking across the blood-brain barrier: chemokine regulation and in vitro models. *Immunol Rev*. Jul 2012;248(1):228-39. doi:10.1111/j.1600-065X.2012.01127.x
26. Babcock AA, Kuziel WA, Rivest S, Owens T. Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS. *J Neurosci*. Aug 27 2003;23(21):7922-30.
27. Shrestha B, Ge S, Pachter JS. Resolution of central nervous system astrocytic and endothelial sources of CCL2 gene expression during evolving neuroinflammation. *Fluids Barriers CNS*. Mar 4 2014;11(1):6. doi:10.1186/2045-8118-11-6
28. Davis BM, Salinas-Navarro M, Cordeiro MF, Moons L, De Groef L. Characterizing microglia activation: a spatial statistics approach to maximize information extraction. *Sci Rep*. May 8 2017;7(1):1576. doi:10.1038/s41598-017-01747-8
29. Chini M, Popplau JA, Lindemann C, et al. Resolving and Rescuing Developmental Miswiring in a Mouse Model of Cognitive Impairment. *Neuron*. Jan 8 2020;105(1):60-74 e7. doi:10.1016/j.neuron.2019.09.042
30. Tinevez JY, Perry N, Schindelin J, et al. TrackMate: An open and extensible platform for single-particle tracking. *Methods*. Feb 15 2017;115:80-90. doi:10.1016/j.ymeth.2016.09.016
31. Dombrowski Y, O'Hagan T, Dittmer M, et al. Regulatory T cells promote myelin regeneration in the central nervous system. *Nat Neurosci*. May 2017;20(5):674-680. doi:10.1038/nn.4528

32. Inoue M, Chen PH, Siecinski S, et al. An interferon-beta-resistant and NLRP3 inflammasome-independent subtype of EAE with neuronal damage. *Nat Neurosci*. Dec 2016;19(12):1599-1609. doi:10.1038/nn.4421
33. Tripathi RB, Rivers LE, Young KM, Jamen F, Richardson WD. NG2 glia generate new oligodendrocytes but few astrocytes in a murine experimental autoimmune encephalomyelitis model of demyelinating disease. *J Neurosci*. Dec 1 2010;30(48):16383-90. doi:10.1523/JNEUROSCI.3411-10.2010
34. Lu HC, Kim S, Steelman AJ, et al. STAT3 signaling in myeloid cells promotes pathogenic myelin-specific T cell differentiation and autoimmune demyelination. *Proc Natl Acad Sci U S A*. Mar 10 2020;117(10):5430-5441. doi:10.1073/pnas.1913997117
35. Yan SS, Wu ZY, Zhang HP, et al. Suppression of experimental autoimmune encephalomyelitis by selective blockade of encephalitogenic T-cell infiltration of the central nervous system. *Nat Med*. Mar 2003;9(3):287-93. doi:10.1038/nm831
36. Lees JR, Golumbek PT, Sim J, Dorsey D, Russell JH. Regional CNS responses to IFN-gamma determine lesion localization patterns during EAE pathogenesis. *J Exp Med*. Oct 27 2008;205(11):2633-42. doi:10.1084/jem.20080155
37. Sivaguru M, Khaw YM, Inoue M. A Confocal Reflection Super-Resolution Technique to Image Golgi-Cox Stained Neurons. *J Microsc*. Aug 2019;275(2):115-130. doi:10.1111/jmi.12821
38. Hammond JW, Bellizzi MJ, Ware C, et al. Complement-dependent synapse loss and microgliosis in a mouse model of multiple sclerosis. *Brain Behav Immun*. Jul 2020;87:739-750. doi:10.1016/j.bbi.2020.03.004

39. Rossi S, Furlan R, De Chiara V, et al. Exercise attenuates the clinical, synaptic and dendritic abnormalities of experimental autoimmune encephalomyelitis. *Neurobiol Dis*. Oct 2009;36(1):51-9. doi:10.1016/j.nbd.2009.06.013
40. Siffrin V, Radbruch H, Glumm R, et al. In vivo imaging of partially reversible th17 cell-induced neuronal dysfunction in the course of encephalomyelitis. *Immunity*. Sep 24 2010;33(3):424-36. doi:10.1016/j.immuni.2010.08.018
41. Chu CQ, Wittmer S, Dalton DK. Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med*. Jul 3 2000;192(1):123-8. doi:10.1084/jem.192.1.123
42. Garrod KR, Moreau HD, Garcia Z, et al. Dissecting T cell contraction in vivo using a genetically encoded reporter of apoptosis. *Cell Rep*. Nov 29 2012;2(5):1438-47. doi:10.1016/j.celrep.2012.10.015
43. Khaw YM, Cunningham C, Tierney A, Sivaguru M, Inoue M. Neutrophil-selective deletion of Cxcr2 protects against CNS neurodegeneration in a mouse model of multiple sclerosis. *J Neuroinflammation*. Feb 4 2020;17(1):49. doi:10.1186/s12974-020-1730-y
44. Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. *Acta Neuropathol*. Jan 2010;119(1):7-35. doi:10.1007/s00401-009-0619-8
45. Soto-Diaz K, Juda MB, Blackmore S, Walsh C, Steelman AJ. TAK1 inhibition in mouse astrocyte cultures ameliorates cytokine-induced chemokine production and neutrophil migration. *J Neurochem*. Mar 2020;152(6):697-709. doi:10.1111/jnc.14930
46. Kang Z, Altuntas CZ, Gulen MF, et al. Astrocyte-restricted ablation of interleukin-17-induced Act1-mediated signaling ameliorates autoimmune encephalomyelitis. *Immunity*. Mar 26 2010;32(3):414-25. doi:10.1016/j.immuni.2010.03.004

47. Cai W, Xue C, Sakaguchi M, et al. Insulin regulates astrocyte gliotransmission and modulates behavior. *J Clin Invest*. Jul 2 2018;128(7):2914-2926. doi:10.1172/JCI99366
48. Haroon F, Drogemuller K, Handel U, et al. Gp130-dependent astrocytic survival is critical for the control of autoimmune central nervous system inflammation. *J Immunol*. Jun 1 2011;186(11):6521-31. doi:10.4049/jimmunol.1001135
49. Gibson-Corley KN, Boyden AW, Leidinger MR, et al. A method for histopathological study of the multifocal nature of spinal cord lesions in murine experimental autoimmune encephalomyelitis. *PeerJ*. 2016;4:e1600. doi:10.7717/peerj.1600
50. Mori Y, Murakami M, Arima Y, et al. Early pathological alterations of lower lumbar cords detected by ultrahigh-field MRI in a mouse multiple sclerosis model. *Int Immunol*. Feb 2014;26(2):93-101. doi:10.1093/intimm/dxt044
51. Othy S, Jairaman A, Dynes JL, et al. Regulatory T cells suppress Th17 cell Ca(2+) signaling in the spinal cord during murine autoimmune neuroinflammation. *Proc Natl Acad Sci U S A*. Aug 18 2020;117(33):20088-20099. doi:10.1073/pnas.2006895117
52. Pasantes-Morales H, Tuz K. Volume changes in neurons: hyperexcitability and neuronal death. *Contrib Nephrol*. 2006;152:221-240. doi:10.1159/000096326
53. Jun DJ, Kim J, Jung SY, et al. Extracellular ATP mediates necrotic cell swelling in SN4741 dopaminergic neurons through P2X7 receptors. *J Biol Chem*. Dec 28 2007;282(52):37350-8. doi:10.1074/jbc.M707915200
54. Freund P, Weiskopf N, Ward NS, et al. Disability, atrophy and cortical reorganization following spinal cord injury. *Brain*. Jun 2011;134(Pt 6):1610-22. doi:10.1093/brain/awr093
55. Magliozzi R, Howell OW, Reeves C, et al. A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis. *Ann Neurol*. Oct 2010;68(4):477-93. doi:10.1002/ana.22230

56. Peterson JW, Bo L, Mork S, Chang A, Trapp BD. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. *Ann Neurol*. Sep 2001;50(3):389-400. doi:10.1002/ana.1123
57. Okuno T, Nakatsuji Y, Moriya M, et al. Roles of Sema4D-plexin-B1 interactions in the central nervous system for pathogenesis of experimental autoimmune encephalomyelitis. *J Immunol*. Feb 1 2010;184(3):1499-506. doi:10.4049/jimmunol.0903302
58. Lively S, Schlichter LC. Microglia Responses to Pro-inflammatory Stimuli (LPS, IFN γ +TNF α) and Reprogramming by Resolving Cytokines (IL-4, IL-10). *Front Cell Neurosci*. 2018;12:215. doi:10.3389/fncel.2018.00215
59. Walter L, Neumann H. Role of microglia in neuronal degeneration and regeneration. *Semin Immunopathol*. Nov 2009;31(4):513-25. doi:10.1007/s00281-009-0180-5
60. Lassmann H, van Horssen J, Mahad D. Progressive multiple sclerosis: pathology and pathogenesis. *Nat Rev Neurol*. Nov 5 2012;8(11):647-56. doi:10.1038/nrneurol.2012.168
61. O'Sullivan SA, O'Sullivan C, Healy LM, Dev KK, Sheridan GK. Sphingosine 1-phosphate receptors regulate TLR4-induced CXCL5 release from astrocytes and microglia. *J Neurochem*. Mar 2018;144(6):736-747. doi:10.1111/jnc.14313
62. Ishii T, Takanashi Y, Sugita K, et al. Endogenous reactive oxygen species cause astrocyte defects and neuronal dysfunctions in the hippocampus: a new model for aging brain. *Aging Cell*. Feb 2017;16(1):39-51. doi:10.1111/accel.12523
63. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell*. Jun 11 1999;97(6):703-16. doi:10.1016/s0092-8674(00)80783-7

64. Liu C, Sun R, Huang J, et al. The BAF45D Protein Is Preferentially Expressed in Adult Neurogenic Zones and in Neurons and May Be Required for Retinoid Acid Induced PAX6 Expression. *Front Neuroanat*. 2017;11:94. doi:10.3389/fnana.2017.00094
65. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest*. Jul 2010;120(7):2423-31. doi:10.1172/JCI41649
66. Rumble JM, Huber AK, Krishnamoorthy G, et al. Neutrophil-related factors as biomarkers in EAE and MS. *J Exp Med*. Jan 12 2015;212(1):23-35. doi:10.1084/jem.20141015
67. Blackmore S, Hernandez J, Juda M, et al. Influenza infection triggers disease in a genetic model of experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A*. Jul 25 2017;114(30):E6107-E6116. doi:10.1073/pnas.1620415114
68. Jin L, Batra S, Doua DN, Palaniyar N, Jeyaseelan S. CXCL1 contributes to host defense in polymicrobial sepsis via modulating T cell and neutrophil functions. *J Immunol*. Oct 1 2014;193(7):3549-58. doi:10.4049/jimmunol.1401138

CHAPTER 6: NEUTROPHIL-SELECTIVE DELETION OF CXCR2 PROTECTS AGAINST CNS NEURODEGENERATION⁵

6.1 INTRODUCTION

Multiple sclerosis (MS) is a chronic debilitating immune-mediated disease of the central nervous system (CNS) that affects nearly 1 million adults >18 years of age in the United States ¹. MS symptoms range from common (e.g., pain, spasms, fatigue ^{2,3}, and muscle weakness ⁴) to rarer and more severe (e.g., vision loss ^{5,6}, physical paralysis, and neurologic disabilities ^{7,8}).

Immunomodulatory drugs are very effective at shortening the duration of acute flares, decreasing relapse frequencies, and providing symptomatic relief, but there is no cure for MS.

MS has long been considered a white matter disease based on observations of immune-mediated demyelinating lesions in the CNS, but gray matter neurodegeneration is now also appreciated as a major contributor to worsening and permanent disability ^{9,10}. Gray matter neuronal pathologies including neuronal apoptosis ¹¹, axonal injury ¹², and dendritic spine loss ¹³ have been observed in the CNS of MS patients. Similarly, gray matter abnormalities have been recapitulated in various animal models of MS, particularly experimental autoimmune encephalitis (EAE) ¹⁴, cuprizone toxic demyelination ¹⁵, and Theiler's murine encephalitis virus-mediated demyelination ¹⁶ models.

MS disease is believed to be autoimmune in origin, arising when myelin-specific T cells initiate an inflammatory cascade resulting in demyelination and axonal damage ^{17,18}. While T

⁵ This chapter is published in its entirety in Journal of Neuroinflammation. This article is available from <https://jneuroinflammation.biomedcentral.com/articles/10.1186/s12974-020-1730-y>

cells are recognized as the main driver of MS, elevated numbers of other peripheral leukocytes have been observed in the CNS, suggesting they may also have effector functions in MS. Specifically, a recent report demonstrated that MS patients have a higher circulating neutrophil-to-lymphocyte ratio compared to healthy controls, and the ratio increases with relapse and aggravated disability ^{19,20}.

Rodent studies have revealed several mechanisms by which neutrophils contribute to disease development. Neutrophils are key regulators of blood brain barrier permeability, allowing further infiltration of leukocytes into the CNS parenchyma ^{21,22}. Neutrophils found in the CNS at the onset of EAE also produce proinflammatory mediators, including TNF- α and IL-1 β , which are thought to contribute to the inflammatory cascade within the CNS by stimulating endothelial cell cytokine production and antigen-presenting cell (APC) maturation ^{23,24}. Additionally, neutrophils can function as APCs themselves, thereby directly regulating antigen-specific T cell responses ²⁵. However, their role in neuronal damage has not been rigorously assessed in EAE disease.

CXCR2 is thought to be the main receptor in regulating neutrophil chemotaxis ²⁶ and effector function ²⁷ during inflammation. CXCR2 signaling can be activated by receptor ligand CXCL1 which has been shown to be increased in MS patients ²⁸. Genetic deletion of CXCR2, a chemokine receptor predominantly expressed by neutrophils, prevents development of hindlimb paresis or demyelination in animals subjected to EAE ²⁹ or cuprizone-induced toxic demyelination ³⁰, respectively. The same studies demonstrated that passive transfer of CXCR2-expressing neutrophils to *Cxcr2*^{-/-} mice is sufficient to restore susceptibility to EAE and cuprizone demyelination. Further, we and others have reported that administering a CXCR2 antagonist ameliorates EAE symptoms ^{31,32}. In this study, we used neutrophil-specific *Cxcr2*

conditional knockout (Cxcr2 cKO) mice to demonstrate for the first time that CXCR2 signaling in neutrophils is critical for ongoing EAE disease via CNS neuronal damage.

6.2 METHODS

Animals

MRP8Cre (021614) and Cxcr2^{fl/fl} mice (024638) were purchased from The Jackson Laboratory. MRP8Cre-Cxcr2^{fl/fl} (Cxcr2 cKO) mice were bred in our animal facility. Healthy 6–8-week-old male Cxcr2 cKO and Cxcr2^{fl/fl} (control wild type) mice were randomly selected and used in this study. All mice were group-housed (2–5 mice per cage) in a specific pathogen-free facility with a 12-h light–dark cycle and were fed regular chow ad-libitum. This study was approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (protocol #19171).

EAE induction

To induce EAE disease, complete Freund's adjuvant, CFA (#F5881-10X10ML, Sigma) containing 400 µg Mycobacterium tuberculosis, Mtb (#DF3114-33-8, Fisher) and 100 µg MOG_{35–55} peptide (MOG35-55, United Peptides) were subcutaneously administered at 0 and 7 days post induction (dpi). Pertussis toxin (200 ng/mouse) (#181, List Biological Laboratories Inc) was administered on day 0, 2, and 7 dpi. Clinical signs of EAE were scored daily for 40 days in a blinded fashion as follows: 0.5, partial tail limpness; 1, tail limpness; 1.5, reversible impaired righting reflex; 2, impaired righting reflex; 2.5, paralysis of one hindlimb; 3, paralysis of both hindlimbs; 3.5, paralysis of both hindlimbs and one forelimb; 4, hindlimb and forelimb paralysis; 5, death. We provided water gel and powdered food when the score reached 2 to avoid

body weight reduction due to the inability to reach food and water. Disease scoring was performed at midday (during the light cycle).

Elisa measurement of serum CXCL1

Blood was collected via submandibular bleeding from control mice and EAE-induced diseased mice at 9, 21, and 40 dpi. These time points were selected to represent disease onset, disease peak time, and disease late phase, respectively. Serum was isolated and stored at -80°C until CXCL1 measurement with a mouse CXCL1/KC Duo set ELISA kit (#DY453, R&D Systems).

Golgi-Cox neuron staining

At 63dpi, mice were fixed by 4% paraformaldehyde/PBS infusion, and spinal cords were harvested. Tissue samples were processed using a FD Rapid Stain kit (#NC0292960, FD Neurotechnologies) following the manufacturer's instructions, embedded in Tissue-tek OCT compound (#23-730-571, Sakura Finetek), and stored at -80°C until sectioning. Spinal cords were transversely cut into 50-μm sections using a cryostat (Reichert Jung Cryocut 1800 Cryostat) and mounted onto poly-L-lysine-coated glass slides. After overnight drying, at least 8 sections were developed for Golgi-Cox neuronal staining performed according to the manufacturer's protocol. After drying, slides were covered with resinous promount and 0.17-μm coverslips.

Confocal reflection super-resolution acquisition and analysis

Samples of Golgi-Cox-stained spinal cords were imaged using a Nikon A1 confocal scanning microscope under the confocal modality and CRSR modality (with minimized pinhole at 0.3

AU) using 20x/0.8 NA objective and 100x/1.49 NA oil objective, respectively³³. A 405-nm continuous wave laser was used, and the reflectance mirror (BS 20/80) was applied. Images were acquired using 100x/1.49 NA Oil (for dendritic spine analysis) and Plan-Apochromat 20x/0.8 NA (for soma volume analysis) objectives. For dendritic spine analysis, z-stacks of at least 150 intervals were acquired. Pixel dimensions were: x: 0.0628 μm ; y: 0.0628 μm ; z: 0.075 μm . 4-8 z-stacks of spinal cord ventral roots from 4-8 individual 50 μm -thick spinal cord sections per animal were visualized. A total of 220 dendrites (in 3-4 animals per condition) were included in our analyses (naïve, 60 dendrites; control EAE, 80 dendrites; Cxcr2 cKO EAE, 80 dendrites) using the filament tracer autopath function (IMARIS), as previously described^{33,34}. Importantly, Gaussian filter and background subtraction were applied to z-stacks of cropped individual dendrites prior to tracing filaments. For neuron soma size analysis, neuron soma sizes were determined by individual analysis of soma volumes based on 40- μm z-stacks of Golgi-Cox-stained slices from the ventral horn of the lumbar spinal cord using the IMARIS software surface application. Six z-stacks of spinal cord ventral roots from six individual 50 μm -thick spinal cord sections per animal were visualized. A total of 628 neuron somas (in 3-4 animals per condition) were included in our analyses (naïve, 133 neurons; control EAE, 254 neurons; Cxcr2 cKO EAE, 241 neurons) using the surface rendering function (IMARIS).

Mononuclear cell isolation

Brains, spinal cords, spleens, and draining lymph nodes (inguinal and axillary lymph nodes) were harvested from mice at 26–29 dpi. Brains and spinal cords were individually transferred into 5-mL collagenase D (1 mg/mL) (#11088866001, Sigma) solution in 6-inch petri dishes, chopped into small pieces using a metal blade, and incubated at 37°C for 30 min. Tissue slurries

were filtered through 70- μ m cell strainers. Cells were pelleted by centrifugation at 1500 rpm for 5 min at 4°C and then suspended in PBS containing 2% FBS. To isolate mononuclear cells from the brains and spinal cords, 70%/30% Percoll gradients were used as previously reported ³⁵. Spleens and lymph nodes were mashed using frosted glass slides in 5 mL PBS containing 2% FBS, filtered through fine mesh, and pelleted by centrifugation at 1500 rpm/1685 g for 5 min at 4°C. Cells were washed with hemolysis buffer, pelleted again by centrifugation, and resuspended in PBS containing 2% FBS. Cells were then counted using trypan blue and a hemocytometer.

Iba1 immunohistochemistry and analysis

Spinal cords were harvested from PBS-perfused and 4% paraformaldehyde-fixed mice at chronic disease (33 dpi). Spinal cords were post-fixed in 4% paraformaldehyde overnight and then cryoprotected by immersion in 30% sucrose solution for 24 h. Samples were frozen in OCT compound and stored at -80°C until cryostat sectioning. Transverse sections (30- μ m) of spinal cords were mounted on poly-L-lysine-coated glass slides. Mounted samples were permeabilized with 0.05% Triton-X for 15 min at room temperature, blocked with 2% BSA for 2 h at room temperature, incubated overnight at 4°C with goat polyclonal AIF-1/Iba1 primary antibody (#NB100-1028, Novus Biologicals) diluted in PBS, and incubated with chicken anti-goat Alexa 647 secondary antibody (#A21469, Invitrogen) for 2 h. Labeled samples were dried, covered with mounting media (Prolong Gold Antifade Mountant, #P36930, Invitrogen), and sealed with a coverslip. Tissue sections (3 images of ventral roots from individual L4–L6 lumbar spinal cord sections per animal) were visualized using a Nikon A1 confocal scanning microscope at 20x magnification. A total of 2116 Iba1+ cells (in 3 animals per condition) were included in our

analyses of soma size and roundness (control EAE, 1236 Iba1+ cells; Cxcr2 cKO EAE, 880 Iba1+ cells) using the ImageJ morpholibj plugin, as previously reported ³⁶.

Flow cytometry

To stain immune cells for flow cytometry, cells were incubated with Fc-Blocker (purified anti-mouse CD16/32 antibody, #101302, Biolegend) in 96-well plates for 7 minutes and then incubated with fluorochrome-conjugated antibodies for 20 min on ice. Data acquisition was performed on a flow cytometer (Cytex Aurora) and analyzed with Fcs Expression software 6 (De Novo Software). Information from 30 000 gated mononuclear cells was acquired for analysis.

Neutrophil isolation

Neutrophils from lymphoid organs (spleen and lymph nodes) were isolated by first removing T, B, and DC populations using biotin-labeled anti-CD4 (#100404, Biolegend), anti-CD8 (#100704, Biolegend), anti-CD19 (#115504, Biolegend), and anti-CD11c antibodies (#117304, Biolegend) with streptavidin-beads (#19860, Stemcell Technologies), followed by neutrophil positive selection using a biotin-labeled anti-Ly6G antibody (#127604, Biolegend) and dextran-coated magnetic particles (#18556, Stemcell Technologies). Isolated neutrophils were used for reactive oxygen species (ROS) quantitation, co-culture, and qPCR studies.

Neuronal cell death detection in n2a cells

N2a cells were a gift from Dr. Keith Kelly (UIUC). N2a cells (1×10^3 cells/well) were cultured on cover slips in 24-well plates in 1% FBS/DMEM before initiating co-culture to initiate neuron differentiation ³⁷. After 3 days, neutrophils were added to N2a neuron culture triplicates at a 2:1

cell ratio and incubated for 18 h at 37°C with 5% carbon dioxide circulation in a sterile incubator. After 18 h, cells were stained for apoptosis using the FITC Annexin V Apoptosis Detection kit with 7-aad (#640922, Biolegend). Cells were post-fixed in 4% paraformaldehyde. Coverslips were mounted on glass slides with Prolong Gold and stored at 4°C until image acquisition. A total of 15007 N2a cells were included in our analyses of 7-aad+ N2a cells (control naïve, 2965 N2a cells examined; control EAE, 4259 N2a cells examined; Cxcr2 cKO EAE, 7852 N2a cells examined) by manual counting from a blinded experimenter. To evaluate neutrophil-mediated neuronal cell death, value of cell death signal in neuron culture alone was subtracted from that of cell death signal in co-culture of neuron with neutrophil.

Neutrophil ROS detection

Isolated neutrophils were stained with neutrophil markers (Ly6G, CD11b) and a ROS marker in duplicates to detect oxidative stress (CellROX deep red reagent, #C10422, Invitrogen) according to the manufacturer's protocol. Data acquisition was performed on a flow cytometer (Cytex Aurora) and analyzed with Fcs Expression software 6 (De Novo Software).

Recombinant CXCL1 treatment to neutrophil in vitro

Wild type mice were treated with thioglycolate solution (3%, 2 ml/mouse) via i.p. injection. At 24 hr after injection, we isolated cells from peritoneal lavage, and isolated neutrophils from them by beads-selection, as mentioned above. Then, neutrophils were seeded in 96-well-plate at 3×10^5 cells/wells. Neutrophils were treated with vehicle (negative control), rCXCL1 (10 or 30 ng/ml, #573702, Biolegend), and LPS (100 ng/ml, positive control, #L4391, Sigma) for 2 hours prior to

staining with CellROX deep red reagent (Invitrogen). Data acquisition was performed on a flow cytometer (Cytek Aurora) and analyzed with Fcs Expression software 6 (De Novo Software).

RNA and cDNA preparation for qPCR analysis

Neutrophil total RNA was extracted with a RNeasy Kit (#74106, Qiagen). cDNA synthesis was performed with qScript cDNA SuperMix (#101414-106, VWR). qPCR was performed using KiCqStart SYBR Green qPCR ReadyMix (#250RXN, Sigma Millipore) with an initial denaturing step of 95°C for 2 min, followed by 40 cycles of denaturation at 94°C for 3 s and annealing and extension at 60°C for 30 s. Relative amounts of qPCR triplicates were determined with the $\Delta\Delta C_t$ method to compare relative expression of target genes and housekeeping genes. Expression of the gene encoding β -actin was used as an internal control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. All results were evaluated with two-tailed unpaired Student's t tests and p-values. Data is expressed as mean $\pm$ standard error of mean (SEM). A p-value < 0.05 was considered significant. Animals were randomly used for experiments. All behavior experiments were performed in a blinded fashion. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field^{31,38}.

6.3 RESULTS

Neutrophil-specific Cxcr2 knockout ameliorates EAE disease

We performed repeated induction of EAE in control wild type (cont) and neutrophil-specific Cxcr2 cKO mice, a procedure shown to cause prolonged disease with severe neuronal damage involving CXCR2³¹. Experimental design is shown in Fig. 6.1 A. Disease onset and severity of motor disturbances were similar in Cxcr2 cKO mice and control mice at an early disease phase (12–14 dpi) (Fig. 6.1 B). In contrast, student t-test revealed that control mice showed significantly severe motor disturbances (*p < 0.05) from 15 dpi up to 40 dpi when compared with Cxcr2 cKO mice that showed weak disease at peak and late phases (Fig. 6.1 B). Repeated induction of EAE induced significant increase in serum CXCL1 levels in EAE-induced mice when compared with non-induced control mice as indicated by data point at 0 dpi (before EAE induction). Notably, at 21 dpi (around peak time), serum levels of CXCR2 ligand CXCL1 were significantly higher than at 9 dpi (onset) and were higher still at 40 dpi in control mice (Fig. 6.1 C). These results suggest that CXCR2 in neutrophils is crucial for EAE disease maintenance and that its function is possibly attributable to high circulating CXCL1 at peak and late phases.

CXCR2⁺ neutrophils mediate spinal cord neuronal abnormalities at late phase of EAE

We assessed neutrophil Cxcr2-dependent morphological changes in Golgi-Cox stained neurons within the ventral horn of lumbar spinal cord samples in control and Cxcr2-cKO mice. At low magnification, we observed gross neuronal anatomical differences between control and Cxcr2 cKO mice (Fig. 6.2 A). Using our recently developed confocal reflection super-resolution (CRSR) technique³³, student t-test revealed that control EAE mice exhibited significantly larger (*p < 0.05) neuronal somas than Cxcr2 cKO EAE mice during late phase disease (Fig. 6.2 B-D).

Consistent with abnormalities reflective of neuronal damage and inflammation, neurons from control EAE mice exhibited fewer dendritic spines than control naïve and Cxcr2 cKO EAE mice (Fig. 6.2 E, F). This suggests that CXCR2 in neutrophils is crucial for CNS pathology during EAE.

CXCR2⁺ neutrophils are not required for CNS infiltration of immune cells at late phase of EAE.

Student t-tests were performed to reveal that total immune cell counts and counts of various immune cell types in the brain and spinal cord were not significantly different ($p > 0.05$) between control and Cxcr2 cKO mice at late phase disease (Fig. 6.3 A, B). We also found no significant differences in immune cell counts in spleens and lymph nodes from control and Cxcr2 cKO EAE mice (Fig. 6.3 C, D). Thus, the contribution of CXCR2-expressing neutrophils to EAE disease maintenance was not dependent upon neutrophil CXCR2-mediated migration of immune cells into the CNS.

CXCR2⁺ neutrophils are not required for microglial activation at late phase of EAE

We asked if depletion of neutrophil CXCR2 influences microglia activation during EAE.

Student t-test was performed to reveal that Iba1⁺ cells in the ventral horn of lumbar spinal cords from control and Cxcr2 cKO mice at late phase disease were not significantly different ($p > 0.05$) in soma size (Fig. 6.4 A, 6.4 B). Thus, neutrophil CXCR2 does not affect microglia activation during EAE, indicating that amelioration of disease severity and spinal cord pathology in Cxcr2 cKO mice is independent of this process.

CXCR2⁺ neutrophils are required for neuronal damage in vitro

We carried out co-culture of neuronal cell line N2a with neutrophils isolated from control and Cxcr2 cKO mice at late phase disease. Student t-test revealed that co-cultures with control neutrophils exhibited significantly higher levels of 7-aminoactinomycin D (7-aad) (*p < 0.05), a marker of neuronal cell death, than co-cultures with Cxcr2 cKO neutrophils (Fig. 6.5 A, B), implying neutrophil expression of CXCR2 is necessary to induce neuronal cell death.

CXCR2⁺ neutrophils contribute to the proinflammatory phenotype during EAE

We measured ROS, a well-defined trigger of neuronal damage and cell death³⁹⁻⁴². At late phase disease, student t-test revealed that there was a significantly higher percentage of splenic ROS-expressing neutrophils (*p < 0.05) in control EAE samples relative to control naïve mice, control EAE mice, and Cxcr2 cKO EAE mice (Fig. 6.6 A). To confirm CXCR2 activation signal in neutrophil induces ROS, we isolated thioglycolate-activated neutrophil from naïve WT mice, and stimulated with recombinant CXCL1. As expected, CXCL1 treatment increased percentage of ROS producing neutrophils (Fig. 6.6 B). Further, we measured expression of neutrophil cytosolic factor 1 (Ncf1) and myeloperoxidase (Mpo), both involved in ROS generation⁴³, in neutrophils isolated from spleens and lymph nodes of naïve control, control EAE and Cxcr2 cKO EAE mice at EAE. Neutrophil treatment of liposaccharide (LPS) was a positive control condition. Consistent with ROS results, neutrophils from control EAE mice exhibited significantly higher expression of Ncf1 mRNA than neutrophils from control naïve mice (Fig. 6.6 C). However, we observed no differences in Mpo mRNA levels (Fig. 6.6 C). Neutrophils from control EAE mice significantly exhibited higher expression Il1b than neutrophils from control naïve mice and Cxcr2 cKO EAE mice. Tnfa gene expression levels was not significant

different among all three conditions (Fig. 6.6 C). These results demonstrate that CXCR2 in neutrophils is necessary and sufficient for EAE-induced ROS production, revealing a potential mechanism for the observed neuronal damage.

6.4 DISCUSSION

We investigated the role of neutrophil-specific CXCR2 signal in EAE development. Our initial speculation was that CXCR2 signaling in neutrophils is important for initiating disease because neutrophils, which express abundant CXCR2, are early responders during EAE-associated neuroinflammation^{23, 44, 45}. However, neutrophil-specific ablation of *Cxcr2* did not suppress early-phase disease development but did affect peak and late phases of EAE. Importantly, we found a large increase in plasma levels of CXCL1, a CXCR2 ligand, during peak and late-phase disease, suggesting that CXCR2 signaling in neutrophils is important at those stages instead of early phase in this neurotoxic EAE model. Because CXCL1 was upregulated in the serum and cerebrospinal fluid of a subset of MS patients²⁸, CXCR2 signals in neutrophils likely contribute to MS disease.

We previously reported that severe CNS neuronal damage and elevated serum CXCL1³¹ are induced during EAE resulting from repeated immunization, the EAE induction method used in this study, hinting that CNS pathology might be affected by neutrophil CXCR2. To test this, we examined the lumbar spinal cord ventral horn because it is the residential address of lower motor neurons and interneurons, both indispensable for normal functioning of hindlimbs^{46, 47}. Further, neutrophils are more abundant in the ventral spinal cord than in lateral areas⁴⁸. Neutrophil-specific *Cxcr2* ablation markedly improved spinal cord neuron morphology, as measured by neuron soma size and dendritic density, at late-phase EAE. Changes in neuron

sizes are thought to reflect their inflammation state. Increased soma sizes, sometimes also referred to as neuronal swelling ⁴⁹, have been observed in disease-vulnerable motor neurons during ALS progression ⁵⁰. These significant alterations in neuron morphology correlate with the prolonged motor impairments observed in control mice exposed to repeated EAE, which occur up to 40 dpi. Because dendritic spine loss was also reported in MS patients and EAE models ^{14, 51, 52}, we also quantified spine density of dendrites that reside in the ventral horn. Similar to the phenotype of soma enlargement, loss of spines induced by EAE was blunted by neutrophil-specific ablation of *Cxcr2*. Therefore, *CXCR2*⁺ neutrophils contribute to CNS neuronal damage.

To address the mechanism underlying the role of *CXCR2* in neutrophils during EAE, we focused on immune cell infiltration because neutrophils influence migration of other immune cells into the CNS in this model ^{53, 54}. However, deletion of neutrophil *Cxcr2* did not affect immune cell migration to the CNS. This result is consistent with a previous study demonstrating no deficit in neutrophil recruitment to the CNS in cuprizone-fed mice with global ablation of *Cxcr2* ³⁰. Additionally, another study demonstrated no change in the number of infiltrating neutrophils to sites of inflammation after administration of a *CXCL1* inhibitor ⁵⁵. Therefore, *CXCR2*-mediated neutrophil migration is not implicated in EAE.

Neutrophils are reported to mediate neurotoxic effects by activating CNS resident microglia in cell contact-dependent and -independent manners ⁵⁶. For example, depleting CNS neutrophils significantly decreases in vivo maturation of microglia and infiltrating monocytes, resulting in impaired leukocyte trafficking to the CNS ²³ and reduced levels of microglial activation marker *CD68* ⁵⁷. However, neutrophil-specific loss of *Cxcr2* did not affect microglia activation as evaluated by increases in soma size. Thus, amelioration of spinal cord pathology in *Cxcr2* cKO mice cannot be attributed to changes in microglia effector function.

Neutrophils can be direct inducers of neuron damage via enhanced secretion of neurotoxic elastases ⁵⁸, ROS ⁵⁹, and extracellular traps ²³. Understanding the range of effector functions that neutrophils can exert upon neurons is important because neutrophils invade CNS parenchyma in multiple contexts of neuroinflammation, including MS, Alzheimer's disease, and ischemic CNS damage ⁶⁰. We found that neutrophils isolated from control EAE mice induced severe neuronal cell death in vitro and deleting neutrophil Cxcr2 rescued this effect. Our results suggest that neutrophils may have a direct effect on neuronal damage, and CXCR2 signal is a key regulator of their neurotoxicity.

Neutrophils generate large amounts of ROS, which can trigger neuronal cell death ⁶¹. Exogenous ROS-induced neuronal cell death was shown to be induced via mitochondria-dependent oxidative burst ⁶². In this study, deletion of Cxcr2 in neutrophils suppressed ROS production in neutrophils during EAE. We also found that CXCR2 signal activated by rCXCL1 is sufficient to induce ROS production, which agrees with a previous finding that identified CXCL1 as being a mediator for ROS production in vivo ²⁷. We also showed that Ncf1 is upregulated in neutrophils of control EAE mice and suppressed in neutrophils of Cxcr2 cKO mice. NCF-1 is a crucial in the production of ROS ^{63,64}. In addition, Il1b mRNA is also upregulated in neutrophil of control EAE mice, but not Cxcr2 cKO EAE mice. IL-1 β are known to drive direct neuronal damage by activating neuronal apoptosis signaling ⁶⁵, inducing glutamate excitotoxicity ⁶⁶. IL-1 β also orchestrates neuron damage by promoting T cell pathogenicity ^{67,68} and endothelial cell inflammatory cytokine secretion ⁶⁹. Therefore, CXCR2⁺ neutrophil-derived IL-1 β may be also mediated CNS neuronal damage in EAE. Our study provides evidence of neutrophil-driven neuronal swelling and synaptic loss via CXCR2 signaling which is a key regulator in ROS production.

6.5 FIGURES

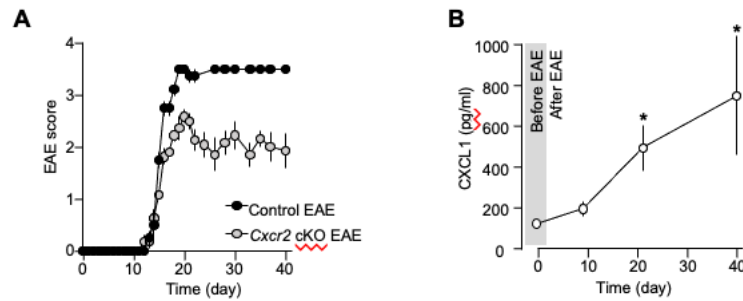


Fig. 6.1. Neutrophil-specific *Cxcr2* knockout ameliorates EAE disease. (A) Schema for experimental design and time schedule. (B) Daily EAE disease scores of wild-type control and neutrophil-specific *Cxcr2* knockout mice (*Cxcr2* cKO), up to 40 days post-induction (dpi). Control EAE, $n = 10$; *Cxcr2* cKO EAE, $n = 10$. (C) Serum CXCL1 levels in control non-induced mice (as indicated by 0 dpi) and EAE-induced mice at 9, 21, and 40 dpi. Note: error bar is present for control group (0 dpi). However, error bar is not visible due to small variation in CXCL1 amount in control groups. Significance was calculated by comparing CXCL1 levels at different time points with 0 dpi. $n = 8$. * $p < 0.05$, two-tailed unpaired Student's t test.

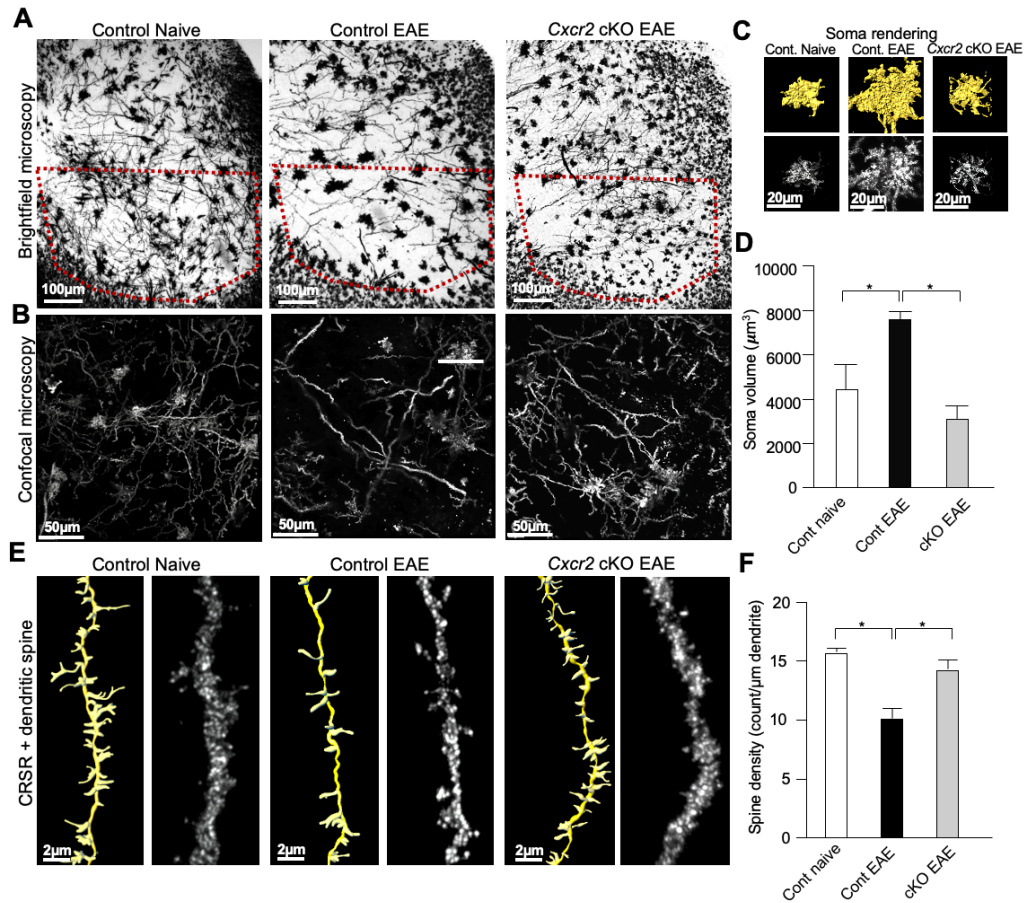


Fig. 6.2. CXCR2⁺ neutrophils mediate spinal cord neuronal abnormalities. (A) Representative brightfield images of Golgi-Cox staining in the lumbar spinal cord (L4–L6) from control naïve mice, control mice induced with high-dose EAE, and *Cxcr2* cKO mice induced with high-dose EAE. Ventral horn region of interest is identified by red dotted lines. (B) Representative low magnification confocal reflection images of Golgi-Cox stained spinal cord ventral horn. (C) Representative images of volume-rendered neuron soma. (D) Quantitative analysis of neuron soma volume derived from surface rendering of Golgi-Cox-stained neurons, as shown in (C). A total of 628 neuron somas were included in our analyses (naïve, 133 neurons; control EAE, 254 neurons; *Cxcr2* cKO EAE, 241 neurons) using the surface rendering function (IMARIS). (E) Representative raw and rendered CRSR images of Golgi-Cox-stained neuron dendrites. (F) Quantitative analysis of dendritic spine density. A total of 220 dendrites were included in our analyses (naïve, 60 dendrites; control EAE, 80 dendrites; *Cxcr2* cKO EAE, 80 dendrites). Control naïve, n = 3; control EAE, n = 4; *Cxcr2* cKO EAE, n = 4. *p < 0.05, two-tailed unpaired Student's t test.

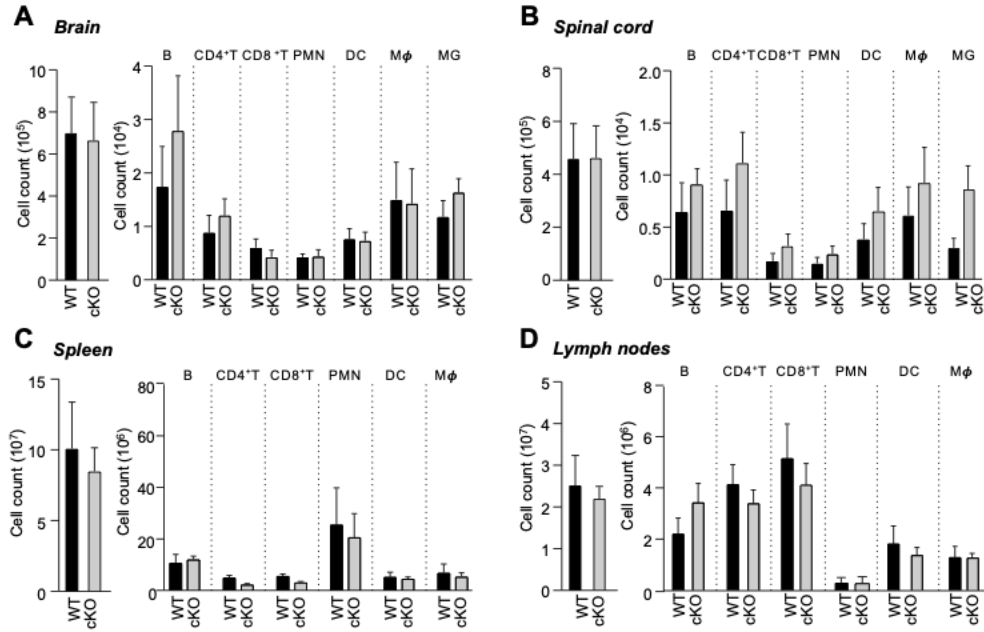


Fig. 6.3. CXCR2⁺ neutrophils are not required for CNS infiltration of immune cells. Numbers of total mononuclear cells, adaptive immune cells (B cells: CD45⁺CD19⁺; CD4⁺T cells: CD45⁺CD3⁺CD4⁺; CD8⁺T cells: CD45⁺CD3⁺CD8⁺), innate immune cells (neutrophils: CD45⁺Ly6G^{high}; dendritic cells: CD45⁺CD11c⁺; macrophages: CD45⁺CD11b⁺Ly6G⁻CD11c⁻), and CNS resident glia (microglia: CD45^{low}CD11b⁺) in the (A) brain and (B) spinal cord of control or Cxcr2 cKO mice. Numbers of total mononuclear cells, adaptive immune cells (B cells: CD19⁺; CD4⁺T cells: CD3⁺CD4⁺; CD8⁺T cells: CD3⁺CD8⁺), and innate immune cells (neutrophils: CD11b⁺Ly6G^{high}; dendritic cells: CD11c⁺; macrophages: CD11b⁺Ly6G⁻CD11c⁻) in the (C) spleen and (D) lymph nodes of control or Cxcr2 cKO mice. Control EAE, n = 5; Cxcr2 cKO EAE, n = 7. *p < 0.05, two-tailed unpaired Student's t test.

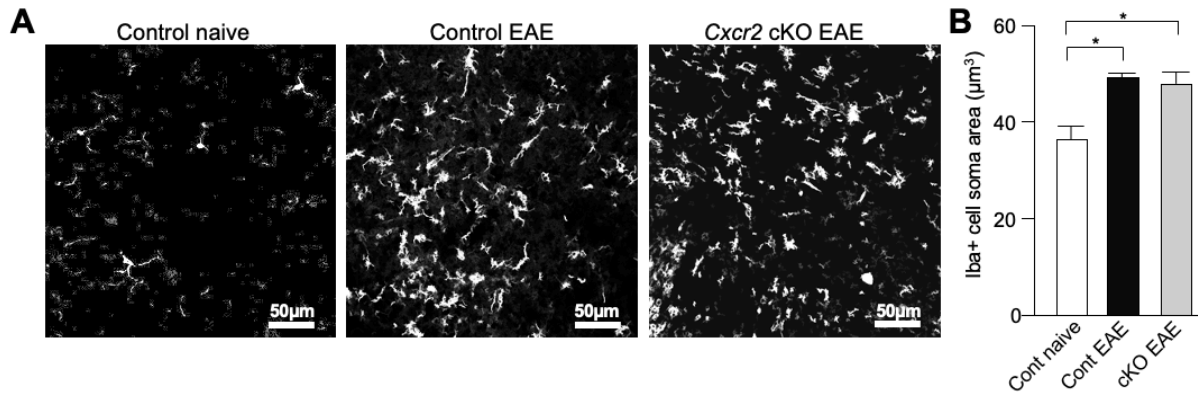


Fig. 6.4. CXCR2⁺neutrophils are not required for microglial activation. (A) Representative images of Iba1 immunohistochemistry staining in lumbar spinal cord ventral horns of naïve control, control EAE, and *Cxcr2* cKO EAE mice. Quantitative analysis of Iba1⁺ cell (B) soma area as shown in (A). A total of 2338 Iba1⁺ cells were included in our analyses of soma size (cont naïve, 222 Iba1 cells; control EAE, 1236 Iba1⁺ cells; *Cxcr2* cKO EAE, 880 Iba1⁺ cells). Control naïve, n = 3; control EAE, n = 3; *Cxcr2* cKO EAE, n = 3. *p < 0.05, two-tailed unpaired Student's t test.

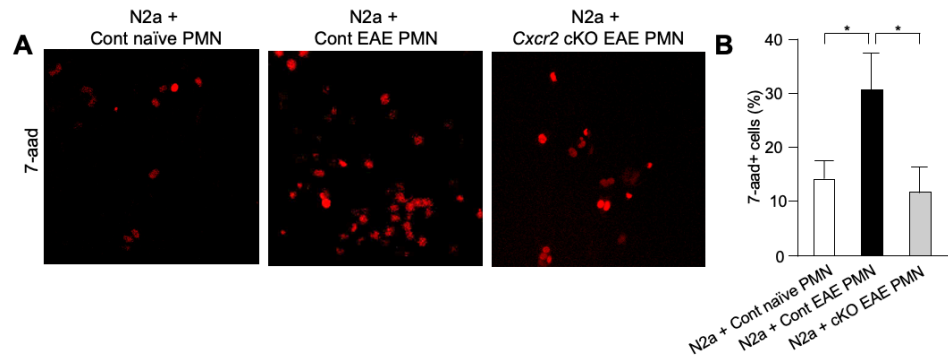


Fig. 6.5. CXCR2 in neutrophils is required for neutrophil-induced neuronal damage in vitro. (A) Representative images of N2a neurons co-cultured with neutrophils isolated from control naïve mice, control EAE mice, or *Cxcr2* cKO EAE mice, showing 7-aad neuronal cell death fluorescent signals. (B) Quantification of 7-aad⁺ neurons 18-h post-co-culture with ex vivo neutrophils. A total of 15,007 N2a cells were included in our analyses of 7-aad⁺N2a cells (control naïve, 2965 N2a cells examined; control EAE, 4259 N2a cells examined; *Cxcr2* cKO EAE, 7852 N2a cells examined). * $p < 0.05$, two-tailed unpaired Student's *t* test.

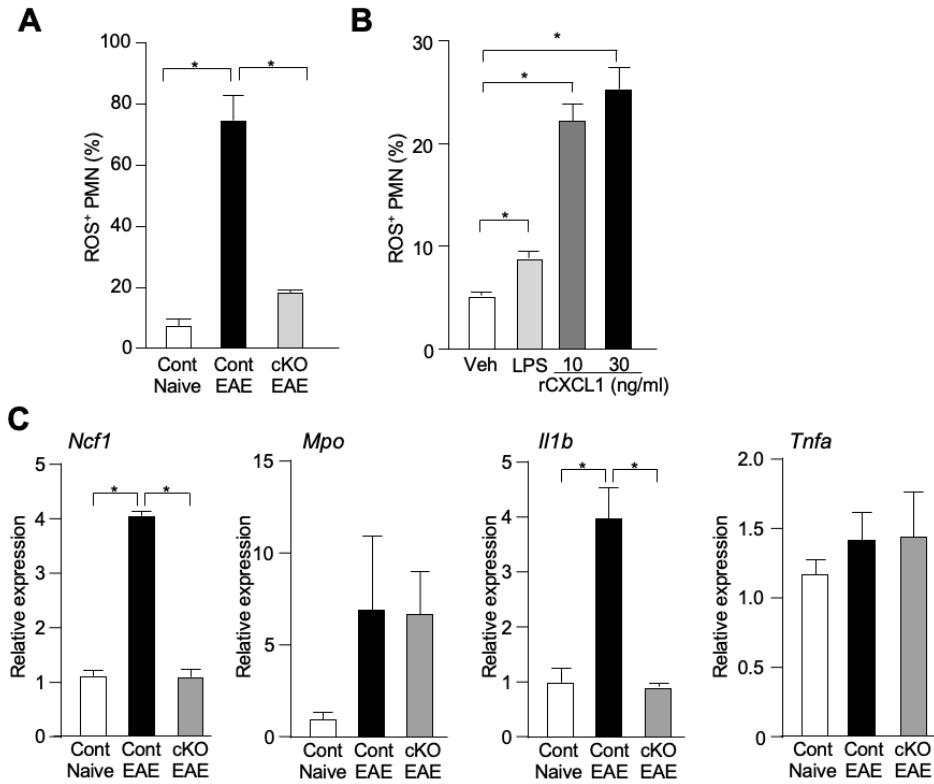


Fig. 6.6. CXCR2 in neutrophils contributes to the proinflammatory phenotype during EAE. (A) Percentage of ROS⁺ neutrophils isolated from spleens and lymph nodes of control naïve mice, control EAE mice, and *Cxcr2* cKO mice. (B) Percentage of ROS⁺ neutrophils isolated from peritoneal space of thioglycolate-injected wild-type mice post-treatment with rCXCL1 at 10 or 30 ng/ml or LPS at 100 ng/ml as positive control. (C) Gene transcription levels of *Ncf1*, *Mpo*, *Il1b* and *Tnfa* in neutrophils isolated from spleens and lymph nodes. * $p < 0.05$, two-tailed unpaired Student's *t* test.

6.6 REFERENCES

1. Rosati G. The prevalence of multiple sclerosis in the world: an update. *Neurol Sci.* Apr 2001;22(2):117-39.
2. Krupp LB, Alvarez LA, LaRocca NG, Scheinberg LC. Fatigue in multiple sclerosis. *Arch Neurol.* Apr 1988;45(4):435-7. doi:10.1001/archneur.1988.00520280085020
3. Fisk JD, Pontefract A, Ritvo PG, Archibald CJ, Murray TJ. The impact of fatigue on patients with multiple sclerosis. *Can J Neurol Sci.* Feb 1994;21(1):9-14.
4. Rice CL, Vollmer TL, Bigland-Ritchie B. Neuromuscular responses of patients with multiple sclerosis. *Muscle Nerve.* Oct 1992;15(10):1123-32. doi:10.1002/mus.880151011
5. Talman LS, Bisker ER, Sackel DJ, et al. Longitudinal study of vision and retinal nerve fiber layer thickness in multiple sclerosis. *Ann Neurol.* Jun 2010;67(6):749-60. doi:10.1002/ana.22005
6. Sakai RE, Feller DJ, Galetta KM, Galetta SL, Balcer LJ. Vision in multiple sclerosis: the story, structure-function correlations, and models for neuroprotection. *J Neuroophthalmol.* Dec 2011;31(4):362-73. doi:10.1097/WNO.0b013e318238937f
7. Whitlock FA, Siskind MM. Depression as a major symptom of multiple sclerosis. *J Neurol Neurosurg Psychiatry.* Oct 1980;43(10):861-5. doi:10.1136/jnnp.43.10.861
8. Krupp LB, Elkins LE. Fatigue and declines in cognitive functioning in multiple sclerosis. *Neurology.* Oct 10 2000;55(7):934-9. doi:10.1212/wnl.55.7.934
9. Dalton CM, Chard DT, Davies GR, et al. Early development of multiple sclerosis is associated with progressive gray matter atrophy in patients presenting with clinically isolated syndromes. *Brain.* May 2004;127(Pt 5):1101-7. doi:10.1093/brain/awh126

10. Sanfilipo MP, Benedict RH, Weinstock-Guttman B, Bakshi R. Gray and white matter brain atrophy and neuropsychological impairment in multiple sclerosis. *Neurology*. Mar 14 2006;66(5):685-92. doi:10.1212/01.wnl.0000201238.93586.d9
11. Peterson JW, Bo L, Mork S, Chang A, Trapp BD. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. *Ann Neurol*. Sep 2001;50(3):389-400.
12. De Stefano N, Guidi L, Stromillo ML, Bartolozzi ML, Federico A. Imaging neuronal and axonal degeneration in multiple sclerosis. *Neurol Sci*. Dec 2003;24 Suppl 5:S283-6. doi:10.1007/s10072-003-0175-2
13. Jurgens T, Jafari M, Kreutzfeldt M, et al. Reconstruction of single cortical projection neurons reveals primary spine loss in multiple sclerosis. *Brain*. Jan 2016;139(Pt 1):39-46. doi:10.1093/brain/awv353
14. Zhu B, Luo L, Moore GR, Paty DW, Cynader MS. Dendritic and synaptic pathology in experimental autoimmune encephalomyelitis. *Am J Pathol*. May 2003;162(5):1639-50. doi:10.1016/S0002-9440(10)64298-8
15. Buschmann JP, Berger K, Awad H, Clarner T, Beyer C, Kipp M. Inflammatory response and chemokine expression in the white matter corpus callosum and gray matter cortex region during cuprizone-induced demyelination. *J Mol Neurosci*. Sep 2012;48(1):66-76. doi:10.1007/s12031-012-9773-x
16. Tsunoda I, Kuang LQ, Libbey JE, Fujinami RS. Axonal injury heralds virus-induced demyelination. *Am J Pathol*. Apr 2003;162(4):1259-69. doi:10.1016/S0002-9440(10)63922-3

17. Huseby ES, Liggitt D, Brabb T, Schnabel B, Ohlen C, Goverman J. A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. *J Exp Med.* Sep 3 2001;194(5):669-76. doi:10.1084/jem.194.5.669
18. Axtell RC, de Jong BA, Boniface K, et al. T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis. *Nat Med.* Apr 2010;16(4):406-12. doi:10.1038/nm.2110
19. Demirci S, Demirci S, Kutluhan S, Koyuncuoglu HR, Yurekli VA. The clinical significance of the neutrophil-to-lymphocyte ratio in multiple sclerosis. *Int J Neurosci.* Aug 2016;126(8):700-6. doi:10.3109/00207454.2015.1050492
20. D'Amico E, Zanghi A, Romano A, Sciandra M, Palumbo GAM, Patti F. The Neutrophil-to-Lymphocyte Ratio is Related to Disease Activity in Relapsing Remitting Multiple Sclerosis. *Cells.* Sep 20 2019;8(10)doi:10.3390/cells8101114
21. Bolton SJ, Anthony DC, Perry VH. Loss of the tight junction proteins occludin and zonula occludens-1 from cerebral vascular endothelium during neutrophil-induced blood-brain barrier breakdown in vivo. *Neuroscience.* Oct 1998;86(4):1245-57. doi:10.1016/s0306-4522(98)00058-x
22. Veldhuis WB, Floris S, van der Meide PH, et al. Interferon-beta prevents cytokine-induced neutrophil infiltration and attenuates blood-brain barrier disruption. *J Cereb Blood Flow Metab.* Sep 2003;23(9):1060-9. doi:10.1097/01.WCB.0000080701.47016.24
23. Steinbach K, Piedavent M, Bauer S, Neumann JT, Friese MA. Neutrophils amplify autoimmune central nervous system infiltrates by maturing local APCs. *J Immunol.* Nov 1 2013;191(9):4531-9. doi:10.4049/jimmunol.1202613

24. Levesque SA, Pare A, Mailhot B, et al. Myeloid cell transmigration across the CNS vasculature triggers IL-1 β -driven neuroinflammation during autoimmune encephalomyelitis in mice. *J Exp Med*. May 30 2016;213(6):929-49. doi:10.1084/jem.20151437
25. Vono M, Lin A, Norrby-Teglund A, Koup RA, Liang F, Lore K. Neutrophils acquire the capacity for antigen presentation to memory CD4(+) T cells in vitro and ex vivo. *Blood*. Apr 6 2017;129(14):1991-2001. doi:10.1182/blood-2016-10-744441
26. Li L, Xu L, Yan J, et al. CXCR2-CXCL1 axis is correlated with neutrophil infiltration and predicts a poor prognosis in hepatocellular carcinoma. *J Exp Clin Cancer Res*. Oct 26 2015;34:129. doi:10.1186/s13046-015-0247-1
27. Jin L, Batra S, Douda DN, Palaniyar N, Jeyaseelan S. CXCL1 contributes to host defense in polymicrobial sepsis via modulating T cell and neutrophil functions. *J Immunol*. Oct 1 2014;193(7):3549-58. doi:10.4049/jimmunol.1401138
28. Campbell SJ, Meier U, Mardiguan S, et al. Sickness behaviour is induced by a peripheral CXC-chemokine also expressed in multiple sclerosis and EAE. *Brain Behav Immun*. Jul 2010;24(5):738-46. doi:10.1016/j.bbi.2010.01.011
29. Carlson T, Kroenke M, Rao P, Lane TE, Segal B. The Th17-ELR+ CXC chemokine pathway is essential for the development of central nervous system autoimmune disease. *J Exp Med*. Apr 14 2008;205(4):811-23. doi:10.1084/jem.20072404
30. Liu L, Belkadi A, Darnall L, et al. CXCR2-positive neutrophils are essential for cuprizone-induced demyelination: relevance to multiple sclerosis. *Nat Neurosci*. Mar 2010;13(3):319-26. doi:10.1038/nn.2491

31. Inoue M, Chen PH, Siecinski S, et al. An interferon-beta-resistant and NLRP3 inflammasome-independent subtype of EAE with neuronal damage. *Nat Neurosci.* Dec 2016;19(12):1599-1609. doi:10.1038/nn.4421
32. Kerstetter AE, Padovani-Claudio DA, Bai L, Miller RH. Inhibition of CXCR2 signaling promotes recovery in models of multiple sclerosis. *Exp Neurol.* Nov 2009;220(1):44-56. doi:10.1016/j.expneurol.2009.07.010
33. Sivaguru M, Khaw YM, Inoue M. A Confocal Reflection Super-Resolution Technique to Image Golgi-Cox Stained Neurons. *J Microsc.* Aug 2019;275(2):115-130. doi:10.1111/jmi.12821
34. Staffend NA, Meisel RL. DiOlistic labeling in fixed brain slices: phenotype, morphology, and dendritic spines. *Curr Protoc Neurosci.* Apr 2011;Chapter 2:Unit 2 13. doi:10.1002/0471142301.ns0213s55
35. Lin YY, Jones-Mason ME, Inoue M, et al. Transcriptional regulator Id2 is required for the CD4 T cell immune response in the development of experimental autoimmune encephalomyelitis. *J Immunol.* Aug 1 2012;189(3):1400-5. doi:10.4049/jimmunol.1200491
36. Davis BM, Salinas-Navarro M, Cordeiro MF, Moons L, De Groef L. Characterizing microglia activation: a spatial statistics approach to maximize information extraction. *Sci Rep.* May 8 2017;7(1):1576. doi:10.1038/s41598-017-01747-8
37. Tsai SY, Chuang JY, Tsai MS, et al. Sigma-1 receptor mediates cocaine-induced transcriptional regulation by recruiting chromatin-remodeling factors at the nuclear envelope. *Proc Natl Acad Sci U S A.* Nov 24 2015;112(47):E6562-70. doi:10.1073/pnas.1518894112

38. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest*. Jul 2010;120(7):2423-31. doi:10.1172/JCI41649
39. Padureanu R, Albu CV, Mititelu RR, et al. Oxidative Stress and Inflammation Interdependence in Multiple Sclerosis. *J Clin Med*. Nov 1 2019;8(11)doi:10.3390/jcm8111815
40. Long TC, Tajuba J, Sama P, et al. Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons in vitro. *Environ Health Perspect*. Nov 2007;115(11):1631-7. doi:10.1289/ehp.10216
41. Halliwell B. Reactive oxygen species and the central nervous system. *J Neurochem*. Nov 1992;59(5):1609-23. doi:10.1111/j.1471-4159.1992.tb10990.x
42. Valencia A, Moran J. Reactive oxygen species induce different cell death mechanisms in cultured neurons. *Free Radic Biol Med*. May 1 2004;36(9):1112-25. doi:10.1016/j.freeradbiomed.2004.02.013
43. Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScues: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Front Cell Infect Microbiol*. 2017;7:373. doi:10.3389/fcimb.2017.00373
44. Soulika AM, Lee E, McCauley E, Miers L, Bannerman P, Pleasure D. Initiation and progression of axonopathy in experimental autoimmune encephalomyelitis. *J Neurosci*. Nov 25 2009;29(47):14965-79. doi:10.1523/JNEUROSCI.3794-09.2009
45. Christy AL, Walker ME, Hessner MJ, Brown MA. Mast cell activation and neutrophil recruitment promotes early and robust inflammation in the meninges in EAE. *J Autoimmun*. May 2013;42:50-61. doi:10.1016/j.jaut.2012.11.003

46. Ueno M, Nakamura Y, Li J, et al. Corticospinal Circuits from the Sensory and Motor Cortices Differentially Regulate Skilled Movements through Distinct Spinal Interneurons. *Cell Rep.* May 1 2018;23(5):1286-1300 e7. doi:10.1016/j.celrep.2018.03.137
47. Vallstedt A, Muhr J, Pattyn A, et al. Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. *Neuron.* Sep 13 2001;31(5):743-55. doi:10.1016/s0896-6273(01)00412-3
48. Caravagna C, Jaouen A, Desplat-Jego S, et al. Diversity of innate immune cell subsets across spatial and temporal scales in an EAE mouse model. *Sci Rep.* Mar 23 2018;8(1):5146. doi:10.1038/s41598-018-22872-y
49. Centonze D, Prosperetti C, Barone I, et al. NR2B-containing NMDA receptors promote the neurotoxic effects of 3-nitropropionic acid but not of rotenone in the striatum. *Exp Neurol.* Dec 2006;202(2):470-9. doi:10.1016/j.expneurol.2006.07.009
50. Dukkupati SS, Garrett TL, Elbasiouny SM. The vulnerability of spinal motoneurons and soma size plasticity in a mouse model of amyotrophic lateral sclerosis. *J Physiol.* May 1 2018;596(9):1723-1745. doi:10.1113/JP275498
51. Ziehn MO, Avedisian AA, Tiwari-Woodruff S, Voskuhl RR. Hippocampal CA1 atrophy and synaptic loss during experimental autoimmune encephalomyelitis, EAE. *Lab Invest.* May 2010;90(5):774-86. doi:10.1038/labinvest.2010.6
52. Mandolesi G, Gentile A, Musella A, et al. Synaptopathy connects inflammation and neurodegeneration in multiple sclerosis. *Nat Rev Neurol.* Dec 2015;11(12):711-24. doi:10.1038/nrneurol.2015.222
53. Pierson ER, Wagner CA, Goverman JM. The contribution of neutrophils to CNS autoimmunity. *Clin Immunol.* Apr 2018;189:23-28. doi:10.1016/j.clim.2016.06.017

54. Eberle M, Ebel P, Mayer CA, et al. Exacerbation of experimental autoimmune encephalomyelitis in ceramide synthase 6 knockout mice is associated with enhanced activation/migration of neutrophils. *Immunol Cell Biol.* Oct 2015;93(9):825-36. doi:10.1038/icb.2015.47
55. Oral H, Kanzler I, Tuchscheerer N, et al. CXC chemokine KC fails to induce neutrophil infiltration and neoangiogenesis in a mouse model of myocardial infarction. *J Mol Cell Cardiol.* Jul 2013;60:1-7. doi:10.1016/j.yjmcc.2013.04.006
56. Megiovanni AM, Sanchez F, Robledo-Sarmiento M, Morel C, Gluckman JC, Boudaly S. Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J Leukoc Biol.* May 2006;79(5):977-88. doi:10.1189/jlb.0905526
57. Moxon-Emre I, Schlichter LC. Neutrophil depletion reduces blood-brain barrier breakdown, axon injury, and inflammation after intracerebral hemorrhage. *J Neuropathol Exp Neurol.* Mar 2011;70(3):218-35. doi:10.1097/NEN.0b013e31820d94a5
58. Zhang J, Harada Y, Hayashi Y. A TLR-CXCL1 pathway in DRG neurons induces neutrophil accumulation in the DRG and mechanical allodynia in EAE mice. *Sci Rep.* Aug 19 2019;9(1):12003. doi:10.1038/s41598-019-48558-7
59. Yan Z, Yang W, Parkitny L, et al. Deficiency of Socs3 leads to brain-targeted EAE via enhanced neutrophil activation and ROS production. *JCI Insight.* Apr 2 2019;5doi:10.1172/jci.insight.126520
60. Neumann J, Sauerzweig S, Ronicke R, et al. Microglia cells protect neurons by direct engulfment of invading neutrophil granulocytes: a new mechanism of CNS immune privilege. *J Neurosci.* Jun 4 2008;28(23):5965-75. doi:10.1523/JNEUROSCI.0060-08.2008

61. Sengpiel B, Preis E, Krieglstein J, Prehn JH. NMDA-induced superoxide production and neurotoxicity in cultured rat hippocampal neurons: role of mitochondria. *Eur J Neurosci*. May 1998;10(5):1903-10. doi:10.1046/j.1460-9568.1998.00202.x
62. Choi K, Kim J, Kim GW, Choi C. Oxidative stress-induced necrotic cell death via mitochondria-dependent burst of reactive oxygen species. *Curr Neurovasc Res*. Nov 2009;6(4):213-22. doi:10.2174/156720209789630375
63. Pizzolla A, Hultqvist M, Nilsson B, et al. Reactive oxygen species produced by the NADPH oxidase 2 complex in monocytes protect mice from bacterial infections. *J Immunol*. May 15 2012;188(10):5003-11. doi:10.4049/jimmunol.1103430
64. Urbonaviciute V, Luo H, Sjowall C, Bengtsson A, Holmdahl R. Low Production of Reactive Oxygen Species Drives Systemic Lupus Erythematosus. *Trends Mol Med*. Oct 2019;25(10):826-835. doi:10.1016/j.molmed.2019.06.001
65. Rossi S, Motta C, Studer V, et al. Interleukin-1beta causes excitotoxic neurodegeneration and multiple sclerosis disease progression by activating the apoptotic protein p53. *Mol Neurodegener*. Dec 12 2014;9:56. doi:10.1186/1750-1326-9-56
66. Rossi S, Furlan R, De Chiara V, et al. Interleukin-1beta causes synaptic hyperexcitability in multiple sclerosis. *Ann Neurol*. Jan 2012;71(1):76-83. doi:10.1002/ana.22512
67. Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med*. Jul 10 2006;203(7):1685-91. doi:10.1084/jem.20060285
68. Hirota K, Duarte JH, Veldhoen M, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol*. Mar 2011;12(3):255-63. doi:10.1038/ni.1993

69. Li Q, Powell N, Zhang H, et al. Endothelial IL-1R1 is a critical mediator of EAE pathogenesis. *Brain Behav Immun*. Jan 2011;25(1):160-7. doi:10.1016/j.bbi.2010.09.009

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

The current theses provide insight into the relationship between environmental factors and disease pathogenesis in a mouse model of MS and identified several immunological pathways with potential implications in disease-related neuronal atrophy and drug sensitivity. Through work conducted under Aim 1 (shown in Chapter 3 and 4), I identified the stress factor of early life trauma and the phenomenon of diminished estrogen receptor alpha activity in lymph nodes as environmental/non-genetic factors that significantly shape EAE disease pathogenesis.

Interestingly, both conditions generated an altered EAE disease that share some key overlapping phenotypes. In brief, mice subjected to early life trauma (ELT-EAE mice) and mice with ablated endogenous estrogen signaling ($ER\alpha^{-/-}$ mice) suffered $IFN\beta$ -resistant disease with severe spinal cord neuron atrophy, in spite of differential disease modifying mechanisms in play. In the ELT work, I show that ELT modifies EAE pathogenesis via desensitization of $\beta 1$ adrenergic receptor in DCs and T cells; in the work related to endogenous estrogen expression, I show that reduction in endogenous estrogen modifies EAE pathogenesis likely via reduced DC expression of $ER\alpha$.

Like the previously reported Type-B EAE condition that induces EAE disease via severe activation of the innate immune cells, ELT-EAE mice and $ER\alpha^{-/-}$ mice (as well as WT mice with inhibited estrogen production in LNs specifically via A-NP aromatase inhibitor delivery) develop EAE that is resistant to $IFN\beta$ treatment, suggesting similarities in disease pathogenesis. A central pathogenic protein in both EAE conditions appeared to be membrane lymphotoxin (mLT) which is expressed by a significantly larger portion of dendritic cells (DCs) compared to control conditions in both studies. Importantly, treatment of recombinant LT in EAE mice was shown to be sufficient to “convert” disease responsiveness to $IFN\beta$ treatment. Additionally, mLT was previously shown to be intimately related to elevations in CXCL1-CXCR2 signaling which has

been proposed to have neurotoxic functions. Through work conducted under Aim 2 (shown in Chapter 5 and 6), I explored the function of CXCL1-CXCR2 signaling with respect to its impact on neuronal morphology and viability. This section will briefly discuss findings resultant of this dissertation as a whole and identify limitations as well as future directions of the current studies.

The role of membrane lymphotoxin in EAE heterogeneity

mLT is composed of heterotrimer of $LT\alpha_1$ and $LT\beta_2$. $LT\alpha$ is also known as TNF- β due its amino acid sequence similarity with other members of the TNF family. Unlike mLT that consists of $LT\beta$ which is exclusively a transmembrane protein, homotrimer of $LT\alpha$ can be secreted as a soluble factor. Soluble $LT\alpha_3$ and membrane bound $LT\alpha_2\beta_1$ are ligands of TNFR1 or TNFR2, whereas $LT\alpha_1\beta_2$ is a ligand for $LT\beta R$ ^{1,2}. Another ligand for $LT\beta R$ is LIGHT (TNFSF14)³. Unlike our observations in EAE mice with severe pathology, under physiological conditions, $LT\beta R$ are expressed by stromal cells, myeloid cells, but never in lymphocytes; whereas the ligand of mLT is expressed by lymphocytes such as T, B, and NK cells. In normal physiology, $LT\beta R$ signaling serves pleiotropic functions including the control of lymphoid organ development and support of efficient immune responses against pathogens. As such, mice with genetic ablation of $LT\beta R$ lack all lymph nodes, develop splenic structure without marginal zones, and have impaired antigen-specific response.

In the current work, mice subjected to early life trauma (ELT-EAE mice) and mice with ablated endogenous estrogen signaling ($ER\alpha^{-/-}$ mice) both had a significantly greater proportion of mLT expressing DCs when compared to control. Despite the use of different environmental triggers, upregulation of mLT coincides with EAE phenotypes of IFN β resistance and severe neuron damage, suggesting that upregulation of mLT may have a causal relationship with these

disease features. The implication of lymphotoxin signaling in MS heterogeneity is not a new notion. In the clinical setting, *ltbr* (which encodes for the mLT receptor of LT β R) was shown to be upregulated in MS patients who are non-responders to IFN β , compared to patients who respond to IFN β treatment⁴.

In Chapter 3 and 4, we demonstrate through in vitro experiments that the increased transcription of mLT components in DCs can be induced by TLR4 activation via TRIF-TRAF3 signaling pathway in the absence of estrogen receptor alpha-mediated or β 1 adrenergic receptor mediated TRIF-TRAF3 inhibition. Interestingly, the activation of the TLR4-mediated TRIF-TRAF3 signaling pathway also induce transcription of endogenous IFN β . In agreement with in vitro evidence, we show that endogenous IFN β levels are significantly upregulated in global and DC-specific ER $\alpha^{-/-}$ EAE mice with heightened mLT expression (Chapter 4). Thus, we propose that the absence of TRIF-TRAF3 limiting factor (such as ER α or β 1AR activation) increases endogenous IFN β production, hence blunting response to exogenous IFN β treatment. In support of this hypothesis, MS patients with active virus infections, that triggers endogenous IFN β production, have less response to IFN β therapy⁵. Furthermore, genome-wide expression profiling in peripheral blood mononuclear cells of MS patients demonstrated that endogenous IFN β level is associated with poor response to IFN β therapy^{6,7}. Because treatment of recombinant LT to EAE mice was previously shown to be sufficient to “convert” IFN β responsiveness, this indicates that upregulated LT β R signaling alone can confer IFN β resistance.

Several possible mechanisms exist for the impact of mLT-mediated LT β R signaling in neuronal atrophy. In the current work, we focus on the direct and indirect impact of mLT-mediated LT β R signaling in shaping CD4⁺ T cell neurotoxicity. The rationale behind this choice are two-fold: first, a 2015 *Immunity* report showed that LT β R, which is never expressed by

lymphocytes under physiological conditions, are upregulated in MS patient derived T cell⁸; second, the mLT-LT β R pathway is necessary for the development of EAE with altered phenotypes in ELT-EAE mice and mice with diminished physiological ER α signaling. Using an in vitro co-culture system, we show that LT β R activation in T cells resulted in degenerative outcomes in neurons, suggesting that LT β R signaling can enhance T cell neurotoxicity. Because LT β R can be activated by the alternative ligand of LIGHT, it is possible that soluble factor LIGHT, in addition to mLT, is responsible for triggering T cell neurotoxic function via LT β R. This possibility remains to be tested. Second, because LT β R is expressed in abundance by stromal cells in the CNS, it is likely that neuron atrophy can be mediated by LT β R activation in stromal cells by CNS-infiltrating DCs that express mLT under in vivo condition. Indeed, a recent study revealed that mLT-mediated LT β R activation in meningeal stromal cells is necessary for the propagation of Th17-mediated interleukin-17 responses⁸. A third possibility for the induction of neuron atrophy by mLT-mediated LT β R signaling is that ectopic LT expression suffices to generate lymphoid-like structures^{9, 10}, also termed tertiary lymphoid organs or tissues, which has been observed in post-mortem samples derived from patients with multiple sclerosis and in preclinical animal models^{11, 12}. Although I did observe the appearance of tertiary lymphoid structure in EAE mice with more severe clinical outcomes, this was not formally quantitated due to it being out of the scope of the current dissertation.

In summary, the current work progressed our current understanding underlying interferon beta insensitivity in the context of lymphotoxin signaling. Further work in larger animals or humans are needed to validate the use of membrane lymphotoxin expression as a potential biomarker for interferon-beta insensitive disease with progressive neuronal atrophy.

The role of CXCL1-CXCR2 signaling as a neurotoxic event

Previous and current work implicate the involvement in enhanced CXCL1-CXCR2 in driving EAE disease with a more progressive disease course and severe neurodegeneration. In fact, CXCR2 was uniquely expressed at high levels in Type-B EAE animals with severe neurodegeneration but not in animal subjected to the less neurodegenerative EAE paradigm of Type-A EAE. Thus in Chapter 5, I explore the role of CXCR2 signaling in T cells specifically in the context of spinal cord neurodegeneration. In this work, we show that CD4⁺ T cell specific knockout of CXCR2 was sufficient to prevent T cell localization in the gray matter and the source of T cell attracting CXCL1 was attributed to astrocytes that reside in the region of T cell migration. CD4⁺ T cells can be seen directly interacting with neurons, but not microglia or astrocytes, in the spinal cord gray matter. Indeed, CD4⁺ T cells has been proposed to induce neuron atrophy via direct interactions such as IFN γ -mediated induction of calcium-permeable complexes between IFN γ receptor and glutamate receptor 1 expressed on neurons; induction of cellular apoptosis by Fas–FasL interactions; and initiation of neurite retraction by semaphorin glycoproteins. Considering the gray matter is a compact microenvironmental that is made up of many cell types, it is possible that CD4⁺ T interact with stromal cell types such as endothelial cells and pericytes. Based on the current discovery that T cells are massively accumulated in the gray matter, future studies can take advantage of the Type-B EAE induction method to understand the interactions of T cells within the CNS microenvironment to understand GM pathology in MS that is currently understudied when compared with WM pathology.

Because CXCR2 is abundantly expressed by neutrophils under physiological conditions and this expression is further upregulated under Type-B EAE condition compared with Type-A EAE condition, I next explore the role of neutrophil CXCR2 in Type-B EAE disease

pathogenesis using neutrophil-specific CXCR2 knockout mice. Because CXCR2 is a well-known chemotactic receptor, we initially sought to understand if the outcomes of severe neuronal degeneration under Type-B condition is attributable to increased CXCL1-mediated migration of neutrophils to the CNS. However, surprisingly, number of neutrophils that infiltrate the CNS was different between WT and transgenic mice under Type-B EAE. A search of available literature yielded the possibility that CXCR2-CXCL1 signaling in neutrophils may yield effector cell function independent of cellular migration. In fact, CXCL1 signaling in neutrophils can stimulate expression of proinflammatory mediators and the production of reactive oxygen species (ROS). ROS represents a class of molecules that, when not in balance with endogenous antioxidant molecules, generates oxidative stress which is highly toxic to the CNS microenvironment. Indeed, when treated with recombinant CXCL1 neutrophils secrete significantly more ROS and are capable of inducing apoptosis in neuron cell line to a greater extent. This study confirms previous findings that the function of neutrophil CXCR2 activation is beyond its traditional chemotaxis function and provides a mechanism by which neutrophils may induce neurodegeneration under MS and EAE condition.

A limitation in our investigation into the cell-specific function of CXCR2 is the lack of assessment of CXCL8 levels. Because the predominant ligand for CXCR2 is CXCL8¹³, future studies should implore the levels of CXCL8 in EAE animals that develop CXCR2-dependent disease. Additionally, the discovery of elevated CXCL1 secretion by astrocytes in the CNS gray matter indicate elevated levels of CXCR2 signaling events in the CNS microenvironment. Because CXCR2 are highly expressed by CNS endothelial cells as well as neurons¹⁴, it is highly likely that CXCR2 signaling in CNS-resident contribute to our observation of severe neurodegeneration in Type-B EAE mice.

Golgi-Cox imaging and the CRSR technique

One of the best histologic methods to reveal the cytoarchitecture of the brain and detailed morphology of neurons with unsurpassed clarity has been the Golgi staining, or the simplified version of Golgi-Cox staining. It is based on the principle of metallic impregnation of neurons, allowing visualization in their entirety including cell soma, axons, dendrites, and spines. To a significantly lesser extent compared to neuronal structures, Golgi-Cox also stains glial cells with clear distinctions in their structures when compared against neurons ¹⁵. In the current work, a key approach used to assess neuron atrophy in all studies is the morphological analysis of neuron and glia through confocal 3D visualization of Golgi-Cox-stained cellular morphology.

As shown in Chapter 2, in collaboration with IGB core facilitator Dr. Sivaguru, we report that super-resolution (defined by resolution capacity of <200nm in optical systems) can be achieved in Golgi-Cox samples which can significantly impact spine density quantitation outcomes using the IMARIS software ¹⁶. The technique was developed to allow spine density detected with greater accuracy. In studies that adopt the CRSR approach, we detected dendritic spine loss in neurons that reside in the ventral horns of L4-6 spinal cord segments, however, we did not trace dendrite origin to neurons. In other words, while we established that spine density loss can be found on dendrites found in the region where lower motor neurons reside, it is unclear which neuron population(s) are affected. This limiting factor is the result of intentional and purposeful design with consideration to affordable time. As such, because the resolution of Golgi-Cox stained cellular structures can be significantly diminished in a thicker tissue section due to the interruption of metallic reflection, spinal cord slices of 50 microns were used. While complete view of dendrites can be obtained, it is impossible to track the soma source of each dendrite due to limited depth, unless one goes to the length of image stitching techniques which

allows multiple continuous tissue sections to be reconstructed. Another practical limitation that prevented the tracking of dendrites to its neuronal soma source is that images (in z-stack form) need to be acquired at high magnifications with significantly limits field view for the assessment of dendrite morphology.

While cell morphology is a respected field where insights into cellular functions can be gleaned, our evidence will benefit from further confirmation through transcriptomic or proteomic approach in examination of relevant hypotheses. This effort is reflected in our approach taken in Chapter 3 whereby we conducted RNA-seq analysis in T cells to understand its transcriptomics in relation to CNS pathology.

7.1 REFERENCES

1. Browning JL, Ngam-ek A, Lawton P, et al. Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell*. Mar 26 1993;72(6):847-56. doi:10.1016/0092-8674(93)90574-a
2. Force WR, Walter BN, Hession C, et al. Mouse lymphotoxin-beta receptor. Molecular genetics, ligand binding, and expression. *J Immunol*. Dec 1 1995;155(11):5280-8.
3. Schneider K, Potter KG, Ware CF. Lymphotoxin and LIGHT signaling pathways and target genes. *Immunol Rev*. Dec 2004;202:49-66. doi:10.1111/j.0105-2896.2004.00206.x
4. Inoue M, Chen PH, Siecinski S, et al. An interferon-beta-resistant and NLRP3 inflammasome-independent subtype of EAE with neuronal damage. *Nat Neurosci*. Dec 2016;19(12):1599-1609. doi:10.1038/nn.4421
5. Petersen T, Moller-Larsen A, Ellermann-Eriksen S, Thiel S, Christensen T. Effects of interferon-beta therapy on elements in the antiviral immune response towards the human herpesviruses EBV, HSV, and VZV, and to the human endogenous retroviruses HERV-H and HERV-W in multiple sclerosis. *J Neuroimmunol*. Aug 15 2012;249(1-2):105-8. doi:10.1016/j.jneuroim.2012.04.013
6. Comabella M, Lunemann JD, Rio J, et al. A type I interferon signature in monocytes is associated with poor response to interferon-beta in multiple sclerosis. *Brain*. Dec 2009;132(Pt 12):3353-65. doi:10.1093/brain/awp228
7. Killestein J, Polman CH. Determinants of interferon beta efficacy in patients with multiple sclerosis. *Nat Rev Neurol*. Apr 2011;7(4):221-8. doi:10.1038/nrneurol.2011.22

8. Pikor NB, Astarita JL, Summers-Deluca L, et al. Integration of Th17- and Lymphotoxin-Derived Signals Initiates Meningeal-Resident Stromal Cell Remodeling to Propagate Neuroinflammation. *Immunity*. Dec 15 2015;43(6):1160-73. doi:10.1016/j.immuni.2015.11.010
9. Haybaeck J, Zeller N, Wolf MJ, et al. A lymphotoxin-driven pathway to hepatocellular carcinoma. *Cancer Cell*. Oct 6 2009;16(4):295-308. doi:10.1016/j.ccr.2009.08.021
10. Gommerman JL, Browning JL. Lymphotoxin/light, lymphoid microenvironments and autoimmune disease. *Nat Rev Immunol*. Aug 2003;3(8):642-55. doi:10.1038/nri1151
11. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol*. Apr 2004;14(2):164-74. doi:10.1111/j.1750-3639.2004.tb00049.x
12. Prineas JW. Multiple sclerosis: presence of lymphatic capillaries and lymphoid tissue in the brain and spinal cord. *Science*. Mar 16 1979;203(4385):1123-5. doi:10.1126/science.424741
13. Nasser MW, Raghuwanshi SK, Grant DJ, Jala VR, Rajarathnam K, Richardson RM. Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer. *J Immunol*. Sep 1 2009;183(5):3425-32. doi:10.4049/jimmunol.0900305
14. Veenstra M, Ransohoff RM. Chemokine receptor CXCR2: physiology regulator and neuroinflammation controller? *J Neuroimmunol*. May 15 2012;246(1-2):1-9. doi:10.1016/j.jneuroim.2012.02.016
15. Yuste R, Hawrylycz M, Aalling N, et al. A community-based transcriptomics classification and nomenclature of neocortical cell types. *Nat Neurosci*. Dec 2020;23(12):1456-1468. doi:10.1038/s41593-020-0685-8

16. Sivaguru M, Khaw YM, Inoue M. A Confocal Reflection Super-Resolution Technique to Image Golgi-Cox Stained Neurons. *J Microsc.* Aug 2019;275(2):115-130.
doi:10.1111/jmi.12821