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Estrogen Receptor Beta Ligands Exert Pro-Myelinating Effects by Altering Pro-Inflammatory Responses in an Animal Model of Multiple Sclerosis

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Dedication

To my mom and dad.

ABSTRACT OF THE DISSERTATION

Estrogen Receptor Beta Ligands Exert Pro-myelinating Effects by Altering Pro-Inflammatory Responses in an Animal Model of Multiple Sclerosis

by

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Multiple Sclerosis (MS) is a chronic inflammatory demyelinating and neurodegenerative disease with no known etiology nor cure. Current immunomodulatory drugs reduce inflammation or prevent leukocyte egress from lymph nodes, but neither halt disease progression nor promote repair of damaged axons. Since oligodendrocyte (OL) death, neurodegeneration, and axonal pathology are prominent at all stages of MS, therapies that promote remyelination and repair are urgently needed. Estrogens have emerged as attractive candidates that meet those needs. Estrogens, synthetic or natural, exert their effects through two nuclear recpetors, estrogen receptor (ER)α and ERβ. However, while use of estrogens, acting primarily on ERα, produce significant deleterious side effects, including increasing risk of cancer and producing feminizing effects in males, selectively targeting ERβ using synthetic small molecules produces comparable therapeutic benefits. To investigate the mechanisms of ERβ-induced therapeutic benefits, a myelin oligodendrocyte glycoprotein- experimental autoimmune encephalomyelitis (MOG-EAE) animal model was utilized. First, two well studied and characterized ERβ ligands, diarylpropionitrile (DPN), and WAY 202041 (WAY) as well as recently established ERβ ligand, chloroindazole (IndCl) were assessed for their therapeutic efficacy in attenuating clinical EAE disease. All three

ligands ameliorated clinical disease symptoms, increased oligodendrocyte (OL) numbers, and promoted functional remyelination of callosal axons in EAE. As IndCl proved to be the most efficacious and potent ERβ ligand, analogues of IndCl were developed and assayed for their role in immunomodulation and neuroprotection. Two analogues, IndCl-o-Cl and IndCl-o-Me attenuated clinical disease progression over time, increased mature OL numbers and enhanced myelination in the corpus callosum and white matter tracts of the spinal cord, similar to parent compound IndCl. These effects were partly mediated by reduced secretion of highly OL-toxic interferon (IFN)γ and IFNγ-inducible chemokine C-X-C motif 10 (CXCL10) by splenic mononuclear cell and increased chemokine CXCL1 production by both splenic leukocytes and thoracic spinal cord astrocytes. Aside from its role in neutrophil chemoattraction, astrocyte-derived CXCL1 acting through its cognate receptor, CXCR2, on OL lineage cells has been implicated in normal myelination by coordinating developmental OL progenitor cell (OPC) positioning, proliferation, and differentiation. The findings presented here provide novel insight in uncovering how ERβ ligands modulate CXCL1 production to initiate OPC proliferation, differentiation, and myelin repair; and will provide the foundation for future studies to develop novel therapies such as IndCl and analogues with similar immunomodulatory, remyelinating, and neuroprotective qualities that can offer targeted therapies to patients with MS and other demyelinating diseases.

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Chapter 1: Introduction/Background

Subtypes and Diagnosis of MS

Multiple Sclerosis is an autoimmune demyelinating neurodegenerative disease of the central nervous system (CNS) currently affecting 2.5 million people worldwide. The onset of MS typically occurs between ages 20-40 years with a 2:1 female to male ratio[1]. Patients often present with a variety of symptoms including visual disturbances, sensory and motor dysfunction, and cognitive deficits that eventually lead to paralysis and death[1]. About 85% of patients are diagnosed with relapse remitting MS (RRMS) characterized by periods of relapses or new inflammatory exacerbations followed by a remission periods of clinical recovery. RRMS patients develop new multifocal inflammatory demyelinating white matter lesions during relapses, the severity of which can be assessed and monitored by magnetic resonance imaging (MRI) scans[2] . Sixty five percent of RRMS patients often transition to a secondary progressive (SPMS) subtype, characterized by minor remissions with worsening clinical disease over time[3]. Approximately 10-15% of MS patients are diagnosed with primary progressive subtype (PPMS), which is a slow progressive disability from onset[3] (Figure 1).

The diagnosis of MS is often difficult as there isn't a single sufficient diagnostic test that can distinguish MS from other similar neurological diseases[4]. The criteria for obtaining a diagnosis depends on a dissemination of lesions in time, indicating that active lesions have occurred more than once and space, representing lesion activity in more than one area in the CNS[5]. In 2000, the International Panel on the Diagnosis of Multiple Sclerosis led by Dr. W. Ian McDonald met to create and simplify diagnostic criteria of MS. The McDonald criteria combines several laboratory assessments, including MRI scans of the CNS, cerebrospinal fluid (CSF) collection and analysis,

and functional assays to combine with patient history and physical examination[6]. Since then, the criteria have been revised several times in 2005, 2010 and 2017, resulting in increased accuracy and sensitivity in the diagnosis of MS[5,7,8]. The gold standard technique in diagnosing MS accurately is MRI as it can determine the number and the severity of lesions, disease activity, axonal loss, brain atrophy and disease progression over time[9]. These lesions are often detected in periventricular and juxtaxcortical white matter regions, in subcortical areas and the spinal cord[4].

Etiology of MS

The etiology of MS remains unclear, but is thought to be multifactorial as both the environment and genetics play a role in developing MS. Some risk factors include Epstein-Barr virus (EBV) infection, smoking, low vitamin D levels associated with lack of sun exposure, as well as adolescent obesity. Several of these factors interact with human leukocyte antigen (HLA) risk genes, thus increasing the risk of developing MS in predisposed individuals[10–12].

Genetic

Genome-wide association studies have higher improved the identification of more than 100 genetic variants associated with the immune system. Variants in genes encoding HLA class I and II contained within the major histocompatibility complex (MHC) have been found to contribute strongly to disease[13]. MHC molecules bind peptide fragments from foreign invaders and display them on the cell surface of antigen presenting cells for recognition by appropriate T cells, a process vital for adaptive immunity[14]. MHC class I complexes are recognized by cytotoxic CD8+ T cells, while MHC class II molecules can activate CD4+ T cells, leading to downstream effector responses. Allelic variation between the different genes (HLA-A,-B,-C for MHC I and HLA-DR,-DP,-DQ for MHC II) affects the composition and affinity of the peptide binding groove, thus

increasing the diversity of peptides presented on the surface by MHC molecules for cell recognition[14,15]. The greatest risk for developing MS, however, comes from the MHC class II HLA-DRB1 variant, with the haplotype HLA-DRB1*1501 affecting Northern European populations, HLA-DRB1*0301, HLA-DRB1*0405 and HLA-DRB1*1303 affecting Mediterranean basin populations and HLA-DRB1*13 associated risk in Israel, but HLA-DRB1*07 in continental Italy; suggesting every major allotype of HLA-DRB1 is involved in MS risk[16–19]. Other risk factors outside the MHC complex include polymorphisms in the interleukin (IL)-7 receptor (R), IL-2Rα and tumor necrosis factor receptor variant (TNFRSF1A)[20–22] that increase the concentration of the soluble form of the receptor, thereby inhibiting its signaling. Soluble forms of these receptors are found in the sera of healthy individuals but are often elevated in inflammatory diseases[21]. IL-7R is important in T cell differentiation of naïve thymocytes and increases the survival of T cells after positive selection, as well as interacts with the thymic stromal lymphopoeitin (TSLP) receptor, which drives immature B cell development and promotes CD4+ Th2 cell expansion[20,23]. IL-2, interacting with its receptor IL-2R, consists of three distinct subunits, IL-2α, IL-2β and IL-2γ, and is crucial in the proliferation of activated T lymphocytes, and differentiation of B cells[21,24–26]. In addition, a polymorphism in costimulatory cluster of differentiation (CD)58 allele, present on antigen presenting cells, results in decreased FoxP3 expression, resulting in dysfunction of regulatory T cells[27]. CD58, along with CD2 co-stimulates and enhances T cell receptor signaling[27,28]. Of note, a variant of CYP27B1, leads to complete loss of enzyme activity of 1-alpha hydroylase, an enzyme responsible for converting vitamin D to its active form, 1,25-dihydroxyvitamin D3 (calcitriol)[29]. As more genetic data is integrated and analyzed, there is an increased likelihood of revealing crucial mechanisms underlying this complex disease.

Environmental

Although genetic factors explain familiar clustering of MS, it doesn't explain geographic variations and migration patterns that contribute to MS development. For example, an individual from a low risk country that migrates to a high risk country retains their low risk of developing MS. However, this low risk incidence is not passed down to their children, who develop a higher prevalence of MS[30]. The disease is rare within Asia and tropical environments, such as South America, but widespread in temperate climates, including New Zealand, Europe, The United States and Canada. This may be directly correlate with exposure to ultraviolet sunlight and vitamin D levels, with low exposure to and levels of resulting in increased susceptibility to disease[11,30,31]. In addition, many infectious agents have been suggested to increase the risk of developing MS, with exposure to Epstein Barr virus (EBV) as the leading culprit. Infection with EBV often occurs in childhood and is asymptomatic; however, in adulthood, these individuals develop infectious mononucleosis (IM) which increases the risk of developing MS greater than two-fold[32]. MS patients typically show increased levels of antibody against EBV antigens such as Epstein Barr nuclear antigen 1 (EBNA1) and a particular fragment in the 385-420 amino acid domain[33,34]. Previous studies have shown a high correlation between high EBNA1 IgG levels and increased MS risk among HLA-DRB1*1501 positive individuals[34,35]. Although the exact mechanism remains unclear, it has been suggested that the T cell receptor complex may recognize EBV peptides that mimic myelin proteins, thus increasing MS risk[36]. Other environmental factors, including smoking and adolescent obesity have also been shown to interact with pathways involved in inflammation, thereby further increasing MS risk. Combing genetic and environmental/lifestyle factors have greatly contributed to our understanding of MS risk, although mechanisms remain to be elucidated.

Pathology of MS

Inflammation and Remyelination

MS lesions, found in both white and gray matter is composed of inflammatory lymphocytes (CD8+, CD4+ T cells), macrophages, B and plasma cells, demyelinated axons, and myelin debris[37]. These lesions form due to autoreactive peripheral lymphocytes that mount responses to self-myelin antigens which cross the broken blood brain barrier into the CNS. The pro-inflammatory mediators secreted from these leukocytes induce further inflammation and activation of resident immune cells, resulting in demyelination, axonal loss and neurodegeneration. The frequency of these inflammatory lesions is increased in RRMS patients, but as the disease progresses, new or ongoing inflammatory lesions are reduced. Nonetheless, chronic activation of microglia and astrocytes as well as oxidative stress, and mitochondrial dysfunction leads to amplified axonal loss and neurodegeneration in progressive stages of disease[38–41].

Microglia and Macrophages

Microglia and non-parenchymal macrophages are vital for CNS development, homeostasis and disease progression. Microglia are resident leukocytes representing 5-12% of all cells in the CNS and play an important role in CNS maintenance and innate and adaptive immunity in the CNS[42,43]. Microglia arise from hematopoietic stem cells in the yolk sac during early development and have a capacity for self-renewal and longevity[44,45]. In a healthy adult CNS, microglia are often 'resting' characterized by small cell bodies with ramified processes. Even in their 'resting' state, microglia constantly survey the CNS by extending and retracting their processes[46]. Under CNS damage and inflammatory conditions, these cells become activated and ambeoid in shape where they may contribute to tissue damage by producing pro-inflammatory mediators that activate other immune

cells, but also support tissue repair through production of neurotrophic factors, anti-inflammatory cytokines and chemokines, as well as clearance of myelin debris[47]. Once activated, microglia act as antigen presenting cells, phagocytic cells, and producers of cytokine and chemokines. Microglia share phenotypic markers of macrophage lineage cells including β2 integrins (CD11a, CD11b, CD11c), leukocyte common antigen (CD45), major histocompatibility complex (MHC) class I and II glycoproteins (β2 microglobulin and HLA-DR, respectively), as well as ionized calcium binding adaptor molecule 1 (Iba1)[48–50]. Perivascular, meningeal and choroid plexus CNS macrophages mediate immune responses between the CNS parenchyma and circulation[51–53]. Additionally, there exists a subset of $Ly6C^{hi}$ classical inflammatory monocytes that infiltrate the CNS during inflammatory insult and develop into microglia-like cells[51,53]. Although leukocytes contribute to demyelination in MS, there is increasing evidence that suggests these cells may also make substantial contributions to the repair and remyelinating process. For example, macrophage depletion has been shown to impair remyelination[54–56]. Interestingly, remyelination only fails when these cells are depleted within the first eight days following lysolecithin induced demyelination, suggesting that these cells are important in the early recruitment phases of remyelination[54]. Additionally, when macrophage/microglial activation was inhibited following administration of minocycline within three days of ethidium bromide induced demyelination, the OPC response and thus remyelination were significantly impaired[57]. Studies of microglia/macrophage polarization reveal a switch from an M1 classically activated phenotype associated with pro-inflammatory response to a M2 protective phenotype to drive oligodendrocyte differentiation at the initiation of remyelination day ten post lysolecithin injection, in part due to the production of activin-A by these cells[58]. Supplementing these studies are studies that show enhanced remyelination when microglia/macrophages are stimulated[59,60]. A critical role in the remyelination process is the

removal of myelin debris generated by demyelination by microglia/macrophages as contact with myelin may inhibit OPC differentiation[61,62].

Astrocytes

Astrocytes display distinct morphological, molecular and functional properties based on CNS region [63]. For example, in the retina, radially polarized Muller glia span all retinal layers and facilitate the transmission of light from inside the eye to the photoreceptors as well as protecting the retina from oxidative stress during photoreception[64]. Bergmann glia are also radially polarized astrocytes of the cerebellar cortex with their somas closely associated with Purkinje cell neurons. Their primary function is to regulate glutamate released at synapses[65]. In contrast, astrocytes in the arcuate nucleus of the hypothalamus regulate satiety and energy homeostasis, whereas astrocytes in the dorsal suprachiasmatic nucleus are the main regulators of circadian rhythm[66,67]. The heterogeneity in astrocyte function is an indicator of the critical roles these cells pay in the CNS[68]. Found in white matter tracts, most fibrous astrocytes have a distinct stellate shaped morphology and play many functional roles in the CNS, which include formation and support of blood brain barrier integrity, regulation of neurotransmitters and immune cells, and neuronal support[63,69]. In response to brain injury and inflammation, astrocytes become reactive with increased expression of glial fibrillary acidic protein (GFAP) and possess distinct gene expression profiles with roles including scar formation, and cytokine and chemokine secretion that may play both detrimental and/or beneficial roles[70,63]. Astrocyte scars have long been regarded as major barriers to regeneration of axons after injury. However, ablation of proliferating astrocytes in autoimmune demyelination animal models of MS disrupts the formation of the glial scar, thus increasing leukocyte migration into the CNS[71]. Astrocytes may also reduce the inflammatory response and enhance neuroprotection through the production of anti-inflammatory cytokines: interleukin (IL)-

10, transforming growth factor-B (TGFβ) as well as growth factors: leukemia inhibitory factor, ciliary neurotrophic factor (CNTF), insulin-like growth factor-1 (IGF-1), and fibroblast growth factor-2 (FGF-2)[70,72–75]. Additionally, it has been demonstrated that astrocytes upregulate chemokines such as CXCL12 and CXCL1 that interacts with CXCR4+ and CXCR2+ OPCs, respectively, to promote differentiation into oligodendrocytes (OLs)[76–78].

The activation of microglia and astrocytes initially by demyelination triggers inflammatory events that cause destruction to OLs and myelin in MS. However, these same cells, also regulate a pro-remyelinating and protective environment in which cytokines, chemokines, growth factors and other signaling mechanisms play a role.

Role of cytokines and chemokines

Cytokines are essentials components of the pro-inflammatory cascade and are implicated in oligodendrocyte death, and axonal degeneration, hallmarks of MS pathology[79]. IL-1β is a pro-inflammatory cytokine produced primarily by microglia and macrophages that induces the production of IL-6, TNFα, and nitric acid as well as induces the proliferation of macrophages and astrocytes[80–82]. Some studies have observed that IL-1β and TNFα is cytotoxic to OLs[83], while other studies have shown that IL-1β promotes proliferation and differentiation of OPCS[84]. Additionally, IL-1β may stimulate OL differentiation and survival by activating cells to produce growth factors, such as IGF-1[85,86]. In addition, IL-1β knockout mice failed to remyelinate properly correlating with a lack of IGF-1 production by microglia and astrocytes[87], indicating a role for this cytokine in the repair process.

TNFα is produced by mononuclear phagocytic cells, activated T cells and by astrocytes and microglia and is typically associated with a Th1 response. TNFα is higher in the CSF of progressive MS patients, correlates with lesion activity and contributes to neuronal cell death[88,89]. Transgenic expression of TNF leads to demyelinating disease and oligodendrocyte death[90,91]. This led to the view that TNFα inhibitors might be effective in treating MS. However, clinical trials showed that MS patients treated with recombinant TNF receptor lenercept (sTNFR-IgG p55) experienced exacerbated disease pathology[92] indicating TNFα may have multiple roles in MS, some of which may be neuroprotective[93,94].

Interferon (IFN)γ, a type II IFN family is secreted by activated T and natural killer cells and has pleiotropic functions[95,96]. It is traditionally viewed as a pro-inflammatory mediator, as its levels correlate with MS and animal models of autoimmune demyelination exacerbation[97,98]. IFNγ stimulates the expression of MHC class I and II molecules and is involved in the activation of macrophages and other pro-inflammatory mediators[99]. However, several studies have demonstrated a protective role for IFNγ in autoimmune demyelination animal models. Anti-IFNγ therapy exacerbated autoimmune demyelination of animal models of MS, while treatment with IFNγ reduced disease severity, and mortality[100,101]. Moreover, deletion of IFNγ or its receptor makes resistant mice more susceptible to autoimmune demyelination[102]. Furthermore, IFNγ transgenic mice confer direct protection on mature OLs in an mouse model of autoimmune demyelination by activation of the pancreateic ER kinase (PERK)–phosphorylated eukaryotic translation initiation factor 2 (eIF2α) integrated stress response pathway in OLs[103,104].

Chemokine role in inflammation and neurodegenerative diseases

In MS, inflammatory immune cells are recruited into the CNS from the periphery where they secrete pro-inflammatory cytokines, resulting in demyelination, oligodendrocyte apoptosis, axonal damage and death[105–108]. Chemokines, or chemotactic cytokines, are often responsible for recruiting activated leukocytes to the site of tissue damage and exert their biological effects by binding to promiscuous chemokine receptors, members of the seven transmembrane G protein coupled receptors to activate signal transduction pathways[109]. Chemokines receptors are not only found on leukocytes but are also present on astrocytes, microglia, oligodendrocytes and neurons and this expression up-regulated after induction with pro-inflammatory mediators. Chemokines have important roles in both homeostasis and disease. Originally thought to be involved in inflammation, it is now known that chemokines are also crucial in development and tissue repair, indicating a pleiotropic role for these small proteins. The approximately 50+ chemokines are classified into four subgroups depending on the position of their terminal cysteine residues: C, CC, CXC, and CX3C chemokine[110–113]. The largest subgroup are the CC chemokines, such as CCL2, CCL3, CCL4 and CCL5, that attract a wide variety of mononuclear cells including monocytes, dendritic cells, natural killer cells, B cells, memory T cells and basophils to the site of injury. The second largest are the CXC chemokines, which have a single amino acid residue between the cysteine groups. A subfamily of CXC chemokines that contain glutamate-leucine-arginine (ELR) motif near the N terminal, such as CXCL1-CXCL8, and attract polymorphonuclear leukocytes such as neutrophils and induce respiratory burst. Additionally, ELR- motif chemokines such as the IFNγ inducible chemokines, CXCL9, 10 and 11 attract CXCR3+ effector T cells to the site of injury.[114,115].

Many studies have investigated the role of chemokines in the pathogenesis of MS and animal models of MS. In both MS and animal models of MS, the recruitment and infiltration of immune

cells into the CNS by chemokines is crucial in disease pathogenesis. During inflammation, chemokines recruit leukocytes by inducing signaling pathways resulting in conformational changes and redistribution of leukocytes across the endothelium. These alternations facilitate firm adhesion of leukocyte integrins such as very late antigen (VLA-4), and lymphocyte function-associated antigen (LFA-1) to endothelial cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), respectively, to facilitate tethering and rolling across the endothelium to the site of injury. Thus, targeting chemokines/chemokine receptors is emerging as a potential therapeutic strategy. However, chemokines and their respective receptors have been shown protective or repair roles for chemokines and chemokine receptors.

CXCL12 and its receptor CXCR4 regulate patterning and function of the immune system[116]. CXCL12 was identified as a bone marrow stromal cell derived chemoattractant for B cell precursors[117]. In the CNS, CXCR4 is expressed by neurons and OLs[118]. Stimulation of in vitro OPCs with CXCL12 augmented their differentiation into mature OLs[119]. In the corpus callosum of cuprizone induced demyelination, CXCL12 was significantly up-regulated within activated astrocytes and microglia, as were numbers of CXCR4+ OPCS[76], suggesting that CXCL12/CXCR4 promotes the differentiation of OPCs into OLs and is critical for repair and remyelination.

CXCL1 is secreted by microglia and astrocytes[120–122] and has been shown to act synergistically with PDGFα to induce proliferation of OPCs in vitro[120,123]. Elevated levels of CXCL1 in *jimpy* mice correlate with increased proliferation of NG2+ OPCs in vivo[124]. CXCL1 upregulation is observed around demyelinating lesions[125,126]. The receptor for CXCL1, CXCR2, is constitutively expressed by OL lineage cells and modulates downstream signaling pathways, suggesting a mechanism for recruitment and regulation of OLs to areas of insult, allowing for repair to occur[121,122]. Treatment of human OPC cultures by CXCL1 promoted proliferation and differentiation of OPCs in part

through the ERK1/2 pathway[127] (Figure 2). Defective CXCR2 signaling led to ectopic positioning and reduced OL number in neonatal rodents[123], while CXCR2 knockout mice demonstrated alterations in OPC density, hypomyelination as well as altered expression of mature OLs proteins MBP, and PLP[128]. Addition of IFNγ and CXCL10, induces OPC and OL apoptosis in vitro. CXCL10 is also elevated in tissues from MS patients and models of demyelination[126]. Signaling through CXCL1/CXCR2, however, inhibited OPC apoptosis by limiting caspase 3 cleavage and augmenting anti-apoptotic Bcl2 marker[129,130]. These studies suggest the importance of the CXCL1/CXCR2 signaling pathway in the normal development and regulation of OL lineage cells, and myelination in the neonatal and adult CNS. However, it is important to note that although cytokines and chemokines might not be directly involved in oligodendrocyte regulation and maintenance during the remyelination process, they are, however, necessary to create the appropriate environment for this process to occur.

Oligodendrocytes and Remyelination Failure

Oligodendrocytes

Oligodendrocytes (OLs) are the myelinating cells of the CNS. Myelination of CNS axons is a tightly regulated complex process involving numerous signaling pathways to elicit an efficient and rapid transmission of nerve signals, as well as appropriate trophic support to axons[131,132]. Compact myelin provides insulation for a neuron and is essential for salutatory conduction. Myelin contains about 70-80% long chain fatty acids along with glycosphingolipids and cholesterol with a small percentage of proteins such as myelin basic protein (MBP) and proteolipid protein (PLP) that make up the majority of compact myelin[133,134]. Individual myelin segments are termed internodes while the unmyelinated space between internodes forms the node of Ranvier, an area rich in sodium

channels. The lateral edge of myelin consists of cytoplasm heavy paranodal regions which outline the myelin-nodal border and harbor nutrients such as lactate for axonal energy and metabolism[135]. Adjacent to the paranode is the juxtaparanodal region with is a small area rich in potassium channels, important for propagating signals with the sodium channels located at the nodes of Ranvier[132,136].

OL development in the rodent CNS

In the spinal cord, after the initial wave of OPCs from the ventral progenitor doman (pMN) that make up about 80% of all OPCs, a second wave arises from the dorsal spinal cord and ultimately makes up about 20% of all OPCs in the mouse spinal cord[137]. In the mouse forebrain, OPCs first originate around embryonic day 12, from the ventral regions of the medial ganglion eminence and anterior entopeduncular area[138], followed by a second wave of OPCs originating from the lateral and caudal ganglionic eminences. At birth, a third wave of OPCs arises from the dorsal subventricular zone into the cortex[139].

OPCs are often identified through expression of platelet-derived growth factor receptor alpha (PDGFRα), chondroitin sulfate proteoglycan (NG2), and A2B5, along with the transcription factors basic helix-loop-helix oligodendrocyte lineage transcription factor, Olig2 and SRY-related HMG-box, Sox10. As OPCS differentiate into mature OLs, PDGFRα, NG2 and A2B5 are downregulated, while Olig2 and Sox10 continue to be expressed throughout the OL lineage[140–142]. Pre-oligodendrocyte/immature oligodendrocytes are categorized as O4+ with multipolar morphology[143]. Various mediators such as Sox10, Olig2, Zinc-finger protein 191 (Zfp191) as well as growth factors including IGF-1, CNTF, FGF-2 and thyroid hormone (T3), are involved in promoting OPC differentiation to OLs along the OL lineage[144–147]. Differentiation of OPCs into

mature myelinating OLs often peaks in the second week after birth, continuing until about 8 months postnatally. As OLs mature, they contact neuronal axons with a diameter over 0.2 μm[148], and express mature myelin lipids such as galactocerebroside (GalC) and myelin proteins, including MBP, and PLP to begin myelin sheath assembly and axonal enwrapping[149], upwards of 50 or more myelin sheaths in certain CNS regions[150] (Figure 3). Throughout adulthood, many PDGFRα+ NG2+ OPCs persist in the neuroepithelial zones surrounding the ventricles, while some adult OPCs are dispersed throughout the adult brain and make up about 4%-5% of all cells in the rodent CNS[151,152].

Remyelination

Remyelination is an endogenous process by which new myelin is laid down onto demyelinated axons, thus enabling them to restore lost function and carry out action potentials[153,154]. Remyelination occurs when adult parenchymal or SVZ derived-OPCs proliferate, migrate to the site of injury and differentiate into mature OLs which extend their cell processes to remyelinate axons[155–158]. Remyelination might fail due to inadequate OPC recruitment to the lesion site or because a failure of OPC differentiation into mature OLs capable of remyelinating unmyelinated axons[155]. Furthermore, although a pool of OPCs may be present in or around lesions areas and have the capacity to self-renew[159], repeated demyelinating insults may exhaust the existing pool of OPCs leading to less efficient remyelination or failure to remyelinate[160]. Failure of differentiation is evidenced in MS patients that show copious amounts of undifferentiated OPCs that fail to remyelinate in many chronic MS lesions[161].

In response to demyelination, OPCs are recruited and promoted to proliferate and differentiate by growth factors PDGFα and FGF, which also induce activation of cell cycle regulatory proteins[162–165], cytokines, chemokines (such as CXCL1), and semaphorins. Several signaling pathways,

including the Notch, Wnt, RxRy, PI3K and ERK pathways are also implicated in OPC migration, and differentiation[166–169]. Importantly, the phosphatidyl inositol-3-phosphate kinase (PI3K)/Akt/mTOR pathway and the extracellular signal-regulated kinases-1 and -2 (ERK1/2), downstream mediators of mitogen-activated protein kinases (MAPKs) pathway play critical roles in OL differentiation and myelination in the CNS[170–173]. Conditional ablation of mTOR component, a major downstream mediator of PI3K/AKT demonstrates impairment of OL differentiation and thinner myelin sheaths in mouse spinal cords[174,175], whereas adult mice with ablated ERK1/2 exhibited decreased myelin thickness and axonal integrity[176,177].

Experimental models of MS

Many experimental animal models were developed to study MS pathogenesis. These include demyelination models such as cuprizone, ethidium bromide, or lysolecithin administration;[178,179]; and viral models, such as infection with Theiler murine encephalomyelitis virus or murine hepatitis virus[180,181]. However, the oldest, most studied and most widely used animal model of MS is experimental autoimmune encephalomyelitis (EAE), which has been shown to recapitulate MS pathology, including inflammation, and demyelination[182–185]. EAE can be induced in rodents by active or passive immunization with specific myelin antigens, such as MBP, PLP, and myelin oligodendrocyte glycoprotein (MOG), which, when emulsified with an adjuvant initiates an autoimmune response similar to what is observed in MS. Several types of EAE can be induced depending on the specific myelin peptide used along with a particular strain of rodent. For example, immunization of SJL mice with PLP139-151 peptide yields a RR like disease with acute monophasic disease[186] whereas immunization of C57BL/6 mice with MOG35-55 produces a chronic progressive disease course[187]. The ease and reproducibility of mice makes them an attractive model for use. The EAE model has played critical roles in testing the efficacy of many

therapies currently on the market, including glatiramer acetate and natlizumab as well as identifying mechanisms of MS pathology[188–190].

Active and Passive EAE

In active EAE, susceptible strains of rodents are subcutaneously immunized with a myelin antigen emulsified in an adjuvant supplemented with heat killed mycobacterium tuberculosis to induce an immune response[191,192]. An intraperitoneal injection of pertussis toxin further facilitates an immune response and compromises the blood brain barrier. The ratio of antigen and adjuvant as well as concentration of pertussis toxin is vital for EAE development and disease progression[185,193–195]. In passive immunization, encephalitogenic myelin reactive T cells are isolated from lymphoid tissues of a previously immunized animal, re-stimulated in vitro with myelin antigen and then transferred into irradiated naïve recipient[196,197]. Both active and passive immunization initiate priming of myelin specific responses in secondary lymphoid tissues. Neutrophils, natural killer cells, macrophages, and myelin specific CD4+ T cells cross the compromised blood brain barrier (BBB), gaining access to the CNS[198]. Once in the CNS, antigen presenting cells present MHC class II myelin peptides, reactivating T cells, resulting in a cascade of inflammatory events, including the secretion and production of cytokines and chemokines such as TNFα, IL-1β, IFNγ, CCL19, CCL21, CXCL9 and CXCL10 that amplify inflammation and consequently induce CNS tissue damage[199,200] (Figure 4).

Understanding demyelination and subsequent remyelination is crucial for identifying therapies for attenuating disease severity, however, this is harder to parse out using EAE, due to the heavy adaptive immune aspect. Thus, toxin induced models are often used: 2% cuprizone (CPZ) diet and microinjection of lysolecithin. CPZ diet induces demyelination without the adaptive immunity by

causing mitochondrial complex IV dysfunction, leading to a disruption in energy metabolism that results in apoptosis of oligodendrocytes, causing demyelination after three weeks of treatment[201,202]. As lesions develop and progress, spontaneous remyelination often coincides with demyelination, an asset, as damage and repair occurs concomitantly in MS lesions. Microinjection of lysolecithin into white matter tracts results in rapid demyelination, followed by rapid remyelination[154,203]. This model has been vastly utilized for pre-clinical testing of candidate therapies due to its prompt repair following damage[203,204]. Both CPZ and lysolecithin models have been effective in examining the cellular and molecular mechanisms of remyelination.

Limitations of mouse models

Despite the similarities between some animal models and MS, it is important to note the differences. First, many animal models require external immunization steps in order to develop, as well as the use of chemicals to elicit an immune response. Second, the induction step of animal models is almost always known, whereas in MS, the cause of autoimmune activation remains unclear[205,206]. Furthermore, CD8+ T cells are found in higher frequency than CD4+ T cells in white and gray matter lesions, whereas in EAE, pathology is mediated by CD4+ T cells[185,206,207]. In addition, animal models sometimes fail to uncover the contribution of B cells in disease, even though these cells are highly implicated in MS pathology[208,209]. However, despite these differences, animal models, in particular, EAE, has allowed researchers to better understand the mechanism and pathogenesis of this complex and heterogeneous disease.

Treatment of MS

During the last few decades, there have been several advancements regarding MS treatments. Various immunosuppressive injectable therapies such as interferon beta, glaterimer acetate, and

natalizumab were the main treatment options[210–213], until the approval in 2010 of the first oral treatment, fingolimod, a sphingosine receptor modulator[214,215]. Other oral MS treatments include dimethyl fumarate, an antioxidant and immunomodulatory agent[216,217], teriflunomide, which inhibits proliferation of activated T and B cells[218,219], cladribine, which induces T and B cell death[220–222] and siponimod, a sphingosine receptor modulator and the first oral drug approved for SPMS[223]. Additional monoclonal antibodies, besides natalizuamb, indicated for the treatment of RRMS include, Alemtuzumab which is a monoclonal antibody against CD52, a cell surface molecule expressed on B and T cells, NK cells and neutrophils[224,225] and daclizumanb, which is a monoclonal antibody against CD25, expressed on surface of T cells[226,227], which was recently pulled from the market citing safety concerns. Ocrelizumab, another monoclonal antibody against CD20, which functions to deplete pre-B cells and mature B cells is the first drug approved for PPMS[228,229].

Importantly, most of these approved therapies act on immune system with severe side effects. These treatments postpone/delay clinical disease but do little to prevent it. Remyelination in the CNS is a spontaneous and important process. Yet, remyelination often fails in MS patients. Thus, several remyelination agents are currently under investigation including: Opicinumab, a monoclonal antibody against LINGO1, a negative regulator of OPC differentiation[230,231]; Clemastine, an over the counter histamine and muscarinic receptor antagonist, which permits efficient remyelination[232]; Quetiapine, a antipsychotic drug that also is a muscarinic receptor antagonist, similar to clemastine[233,234] ; Olesoxime, a small cholesterol resembling molecule with remyelinating potential[235,236]; GSK239512, an oral H3 histamine antagonist that promotes OPC differentiation[237,238]; miconazole, an antifungal and the steroid clobetasol, were effective in increasing OL numbers and promoting myelination[234].

MS is a complex and heterogeneous disease with unknown etiology. There is a large number of MS immunomodulatory and immunosuppressive therapies available that do not directly support remyelination. Furthermore, as inflammation is needed for effective repair/remyelination, treatments that shut down inflammatory response might be detrimental to the repair process. Therefore, it is essential to investigate treatments that trigger myelin repair and promote neuroprotection in addition to controlling inflammation.

Estrogens and MS

Estrogens, in addition to their actions on the reproductive system, have been documented as potential neuroprotective agents in the adult brain with neurodegenerative diseases such as with Parkinson's disease, Alzheimer's and cerebrovascular stroke[239]. Genomic and non-genomic steroid signaling mediate the defensive actions of estrogens including modulation of synaptogenesis, protection from apoptosis by stimulation of anti-apoptotic factors, counteracting oxidative stress, and dampening pro-inflammatory processes[240]. These effects are mediated primarily through the activation of intracellular estrogen receptors (ER), ERα and ERβ, which exhibit distinct transcriptional properties[241]. The classical estrogen signaling pathway involves ligand binding to receptor, activation of ERα and ERβ and subsequent activation of estrogen target genes. Additionally, estrogens interact with plasma membrane ERs that activate downstream signaling pathways, including the PI3K/AKT/mTOR and ERK/MAPK pathway[242] (Figure 5). The use of estrogens as a potential therapy for MS is based on notable effects seen in the third trimester of pregnancy in patients with MS and other autoimmune diseases, including rheumatoid arthritis and psoriasis [243–245]. These patients experience a reduction in relapse frequency in the third trimester of pregnancy followed by a comparable increase in relapse risk postpartum. One of the important

reasons for this reduction in relapse may be due to a shift from Th1 dominance, correlated with increased inflammatory response in MS to Th2 dominance that confers protection in autoimmune disease[246,247]. Circulating levels of pregnancy related factors, including endogenous estrogens-estradiol (E2) and estriol (E3), have been shown to exert immunomodulatory effects[248,249]. Pregnancy also confers protection in animal models as disease course is attenuated in response to administration of estriol equivalent to pregnancy concentrations[250]. Furthermore, lymphocytes from pregnancy mice in a mouse model of MS showed a decrease in pro-inflammatory cytokines TNFα and IL-17 with an increase in anti-inflammatory cytokine IL-10[251]. Additionally, estrogens can suppress CNS immune activation such as lipopolysaccharide induced microglial activation[252] and can inhibit NF-κB localization, preventing downstream activation of pro-inflammatory genes[253]. Estrogens and various growth factors such as brain-derived neurotrophic factor (BDNF) and IGF-1 may have complementary or coupled signaling pathways, which lead to OL differentiation and myelination[254,255,256]. Given the potent effects of endogenous estrogens on pregnancy in MS patients and in animal models of MS, studies have investigated the effect of exogenous and synthetic estrogens on disease. Clinical protection from EAE by estradiol is primarily mediated through ERα signaling as ERα also mediates anti-inflammatory effects[241], in addition to neuroprotective benefits. However, the use of estrogens as a therapy must be carefully considered as estrogens, acting primary on ERα, increase the risk of breast and endometrial cancer in females and feminizing effects in males. ERβ and respective ligands do not seem to have these deleterious effects[257,258].

ERα and ERβ ligands: Differential Effects

Both ERα and ERβ are expressed on various cell types, including the immune system and CNS. Exogenous ERα selective ligand, propylpyrazoletriol (PPT) treatment ameliorated clinical disease, and decreasedimmune cell infiltration in EAE[259,260]. Additionally, pre-treatment with ERα ligand

suppressed clinical disease, whereas treatment in the effector phase of disease reduced clinical severity but does not offer complete clinical disease protection[261]. ERα ligand treatment protected against demyelination and axon loss and was able to decrease pro-inflammatory mediators such as IFNγ, TNFα and matrix metalloproteases (MMP-9), while increasing anti-inflammatory cytokines, such as IL-5 and IL-10 [259,260,262]. ERβ ligands also offer neuroprotection, but have differential effects on inflammation. Treatment of EAE mice with ERβ ligand did not have a significant effect early in disease but EAE mice exhibited significantly lower clinical severity over time[260]. However, the mechanism underlying disease protection is not dependent on inflammation as ERβ ligands do not alter significantly CNS inflammation, nonetheless, these compounds protect against oligodendrocyte apoptosis, demyelination and axon loss and alter the pro-inflammatory cytokine milieu in EAE mice[260,263,264]. For example, ERβ ligand treatment on EAE mice decreased the levels of TNFα produced by dendritic cells, as well as suppressed inflammatory cytokine responses of microglia and astrocytes[265,266].

An ideal selective estrogen receptor modulator (SERM) will possess agonist activity in target tissue, such as the CNS, while possessing antagonist activity in the mammary gland and uterus[267]. Several ERβ SERMs, both synthetic and plant derived, with different affinities and selectivities for ERβ have been developed [268,269]. Diarylpropionitrile (DPN) is an ERβ ligand with 70 fold relative binding affinity for ERβ than ERα. Prophylactic or therapeutic treatment with DPN reduces EAE clinical severity over time[263,270–272]. DPN induced EAE improvement of clinical disease was associated with enhanced myelination, increased mature OL numbers, and improved corpus callosal axon conduction, all in the presence of an active immune response[270,271]. DPN induces myelination and neuroprotection by increasing growth factors such as BDNF and activating the PI3K/Akt/mTOR pathway, essential for OL survival and differentiation[163,272]. Another class of

SERMs includes WAY-202041 (WAY), which has a 200 fold binding affinity for ERβ over ERα [259]. WAY has demonstrated anti-inflammatory responses in animal models for arthritis, inflammatory bowel disease and acute inflammatory pain[273,274]. Treatment of WAY in an acute, monophasic PLP-EAE disease model did not attenuate clinical disease severity and only had minimal effects on inflammation[259]. A third class of SERMs includes Chloroindazole (IndCl), a potent ERβ selective agonist based on a halogen substituted phenyl-2H-indazole core with a 100 fold selectivity for ERβ over ERα[275]. Prophylactic and therapeutic treatment with IndCl in chronic progressive MOG-EAE attenuated clinical severity over time[276,277]. IndCl also induced functional remyelination and corpus callosum conduction by augmenting the survival, and differentiation of OPCs[277]. Similar to DPN, IndCl treatment increased BDNF levels and activated the PI3K/Akt/mTOR pathway. In contrast to DPN, however, IndCl reduced pro-inflammatory cytokine production such as IFNγ, IL-6, TNFα, and IL-17 in spleens ex vivo while suppressing the cytokine profile of microglia and astrocytes in vitro[276,277]. Although DPN, WAY and IndCl are all ERβ-selective ligands, the mechanisms for immune modulation appear to be distinct. For example, IndCl repressed induction of inducible nitric oxide synthase mRNA in LPS-stimulated microglial BV2 cells, while DPN and WAY did not[276], suggesting that altering immune responses by ERβ may be cell type specific. Thus, the development of an ERβ-selective ligand capable of improving myelination and altering pro-inflammatory responses may be of therapeutic benefit.

Hypothesis

Estrogens and selective estrogen receptor modulators have been studied by several groups and documented to exert remyelinating effects with differential effects on inflammation in animal models of MS. Roles for these ligands and their receptors in the brain and periphery are complex. Ongoing studies by the Tiwari-Woodruff lab and others have shown that ERβ ligands ameliorate

clinical disease symptoms, increase OLs numbers and promote remyelination. However, the mechanisms underlying ERβ ligand mediated improvement in symptoms and remyelination remains to be elucidated. Thus, the work presented in this dissertation sought to investigate the mechanisms of ERβ ligand mediated repair and remyelination.

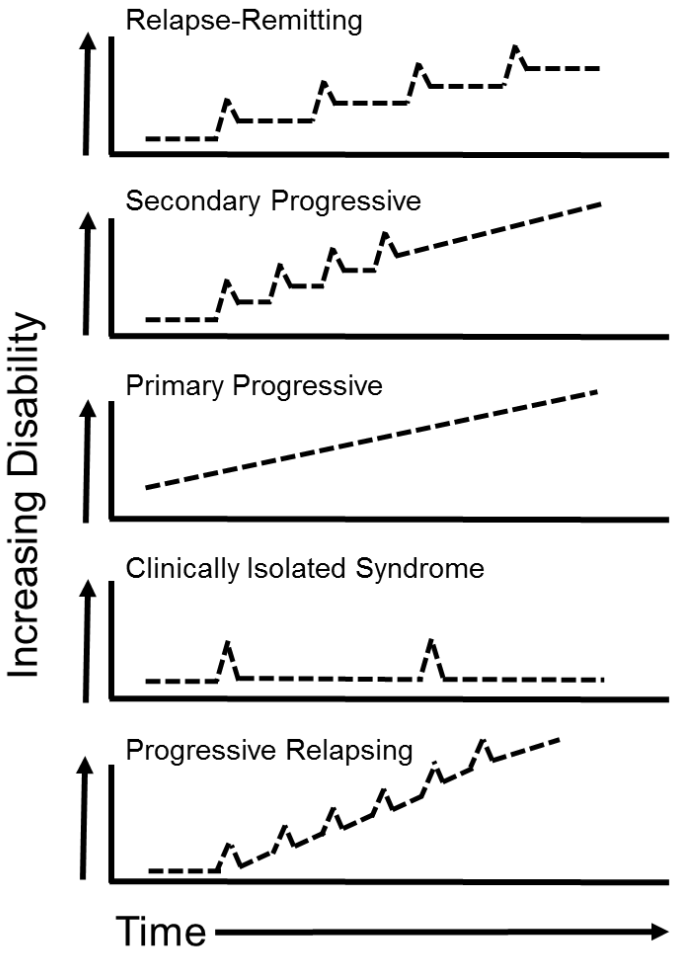

Figure 1. Clinical subtypes of multiple sclerosis (MS). The clinical course of MS is classified into different classes according to occurrence of active lesions and accumulation of disability. The most common subtype is relapse remitting MS (RRMS), which affects about 85% of MS patients. This disease subtype is characterized by periods of relapses followed by periods of remission. RRMS often progresses to a secondary progressive (SPMS), characterized by relapses with no clear remissions. About 15% of patients present with a primary progressive, characterized by accumulation of disability from the onset of disease, with no remissions. Clinically isolated syndrome (CIS) is defined by a 24 hour inflammatory episode but does not yet meet the criteria for a diagnosis of MS, whereas progressive relapsing MS (PRMS) is described as steadily worsening neurological symptoms from onset of disease with occasional relapse. Adapted from[278–280].

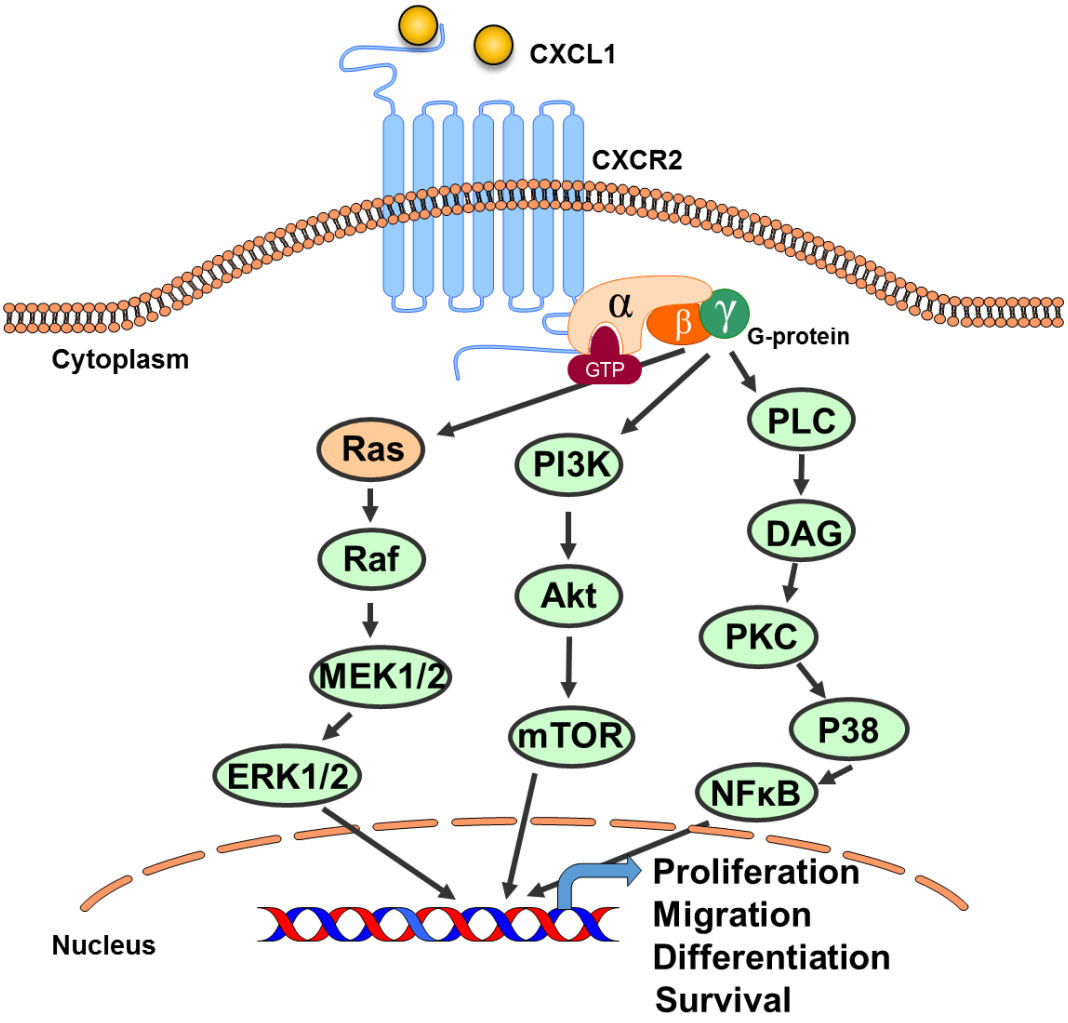

Figure 2. CXCL1/CXCR2 signaling pathway. The chemokine receptor CXCR2 is a member of the G-protein coupled receptor (GPCR) family. CXCR2 binds CXCL1, a neutrophil chemoattractant of the ELR+ family of CXC chemokines. Upon ligand (CXCL1) binding, CXCR2 G protein complex α and βγ portions dissociate and activate intracellular signaling processes, including phospholipase C (PLC) leading to phosphatidylinositol (PI) and calcium increase. PI3K, catalyzes the phosphorylation of phosphatidylinositol-3,4-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) leading to downstream signaling and is associated with cell survival, angiogenesis and motility. Binding also leads to activation of the MAPK pathway, including the phosphorylation of ERK1/2, associated with cell survival and proliferation. Moreover, CXCR2 activation leads to increased transcriptional activity of NF-κB. Adapted from[281,282].

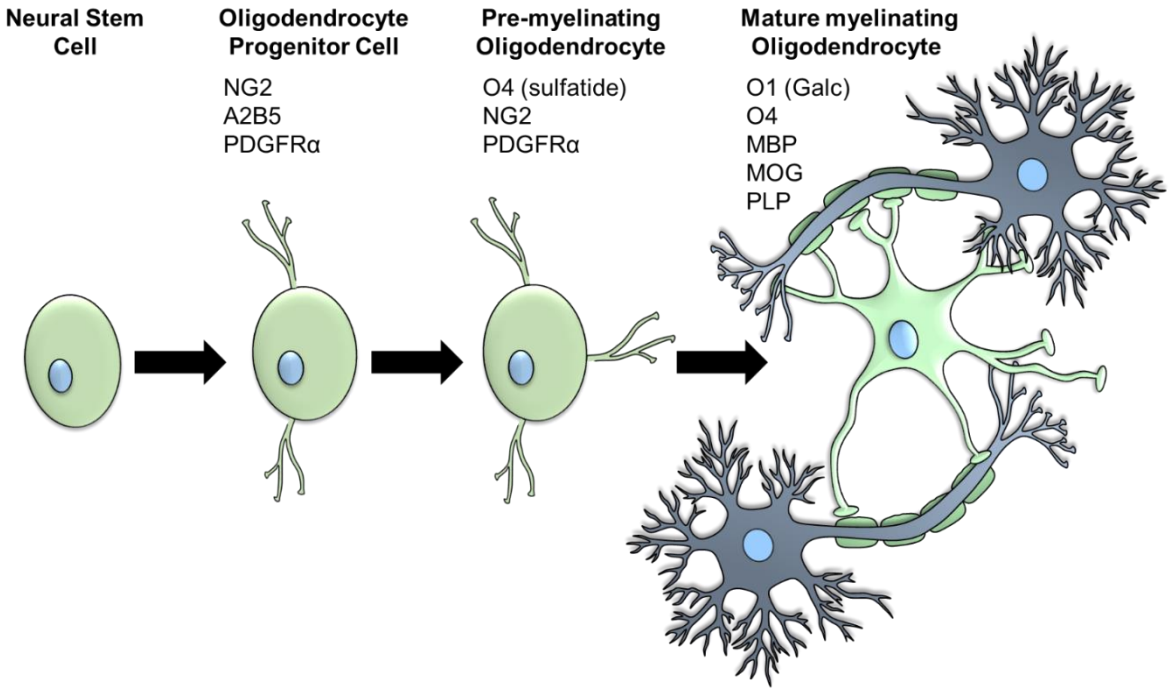

Figure 3. Oligodendrocyte development and lineage in the CNS. Oligodendrocyte progenitor cells (OPCs), are initially specified from neural stem cells by their expression of NG2, A2B5 and PDGFRα. Pre-myelinating immature OPCs are identified by the marker O4 (sulfatide), which continues to be expressed throughout subsequent differentiation stages. As OPCs differentiate into mature myelinating oligodendrocytes (OLs), they acquire a more branched morphology and they make contact with axons. They can be identified by lipid markers galactocerebroside (O1; GalC) as well as myelin proteins such as myelin basic protein (MBP); proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) that continue to be expressed by all mature myelinating OLs. Adapted from[139,283,284].

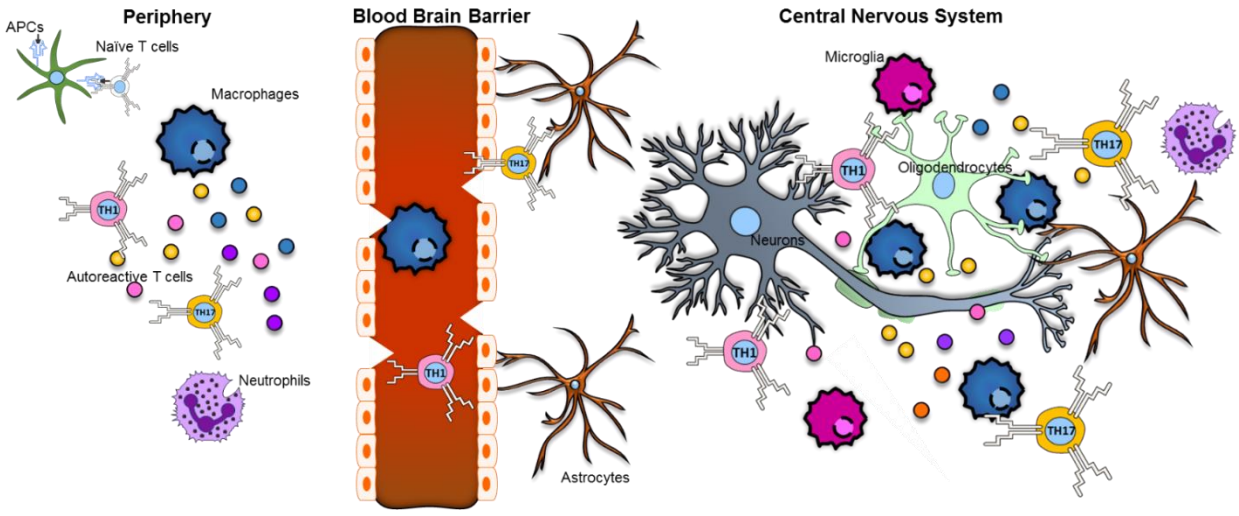

Figure 4. Immunopathology of experimental autoimmune encephalomyelitis (EAE). Schematic diagram highlighting key features of EAE pathogenesis. T helper (Th) 1 and Th17 cells are the primary drivers of EAE disease. They are primed in the periphery by antigen presenting cells (APCs) such as dendritic cells, and along with other activated leukocytes, such as macrophages and neutrophils, cross the disrupted blood brain barrier, were they are re-activated by central nervous system (CNS) APCs. Once in the CNS, leukocytes produce pro-inflammatory mediators such as IFNγ, TNFα, IL-17, and IL-1β. These pro-inflammatory mediators also attract additional inflammatory cells into the CNS that leads to myelin and oligodendrocyte damage resulting in demyelination, axon damage and eventual neurodegeneration that is irreversible in chronic stages.

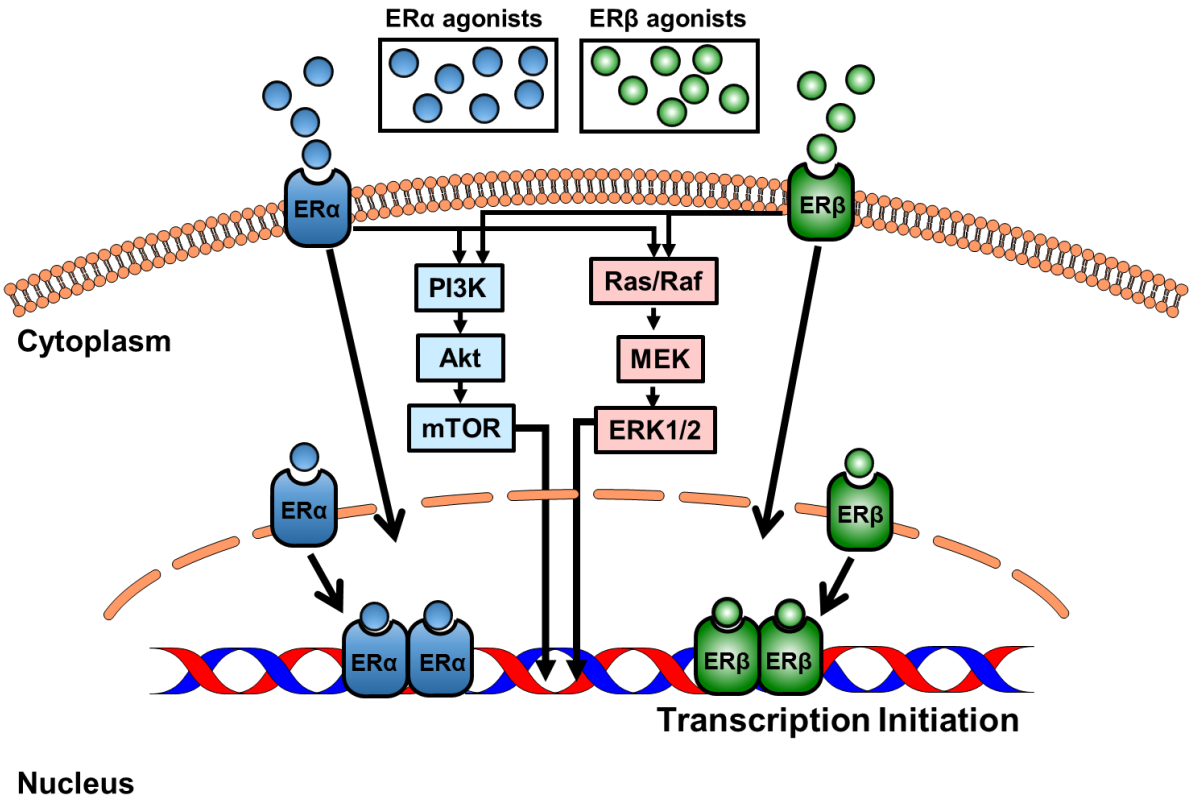

Figure 5. General estrogen signaling pathway. Endogenous estrogens and estrogen receptor (ER) agonists can activate cell membrane and nuclear ERs. ERα and ERβ activation induces dimerization and translocation of ERs to the nucleus, where they exert transcriptional effects. Binding can also affect intracellular signaling pathways, including PI3K/Akt/mTOR, and Ras/Raf/MEK/ERK, ultimately influencing gene expression. Adapted from[285,286].

Chapter 2: General methodology

A version of this chapter is published in Proceedings of the National Academy of Sciences of the United States of America; Scientific Reports from Nature Research and The Journal of Neuroscience Methods.

Karim, H. *et al.* Increase in chemokine CXCL1 by ER beta ligand treatment is a key mediator in promoting axon myelination. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 6291–6296 (2018).

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Hasselmann, J. P. C., Karim, H., Khalaj, A. J., Ghosh, S. & Tiwari-Woodruff, S. K. Consistent induction of chronic experimental autoimmune encephalomyelitis in C57BL/6 mice for the longitudinal study of pathology and repair. J. Neurosci. Methods (2017).

Experimental Autoimmune Encephalomyelitis (EAE)

Active EAE was induced in eight-week-old female C57BL/6 mice as previously described[287] (Figure 6) with representative clinical EAE score and immunohistochemical pathology in Figure 7. Briefly, mice received subcutaneous (s.c) injections of MOG_{35-55} peptide (Mimotopes, Clayton, Victoria, Australia) emulsified in complete Freund's adjuvant with *M. butyricum* (BD Difco, Franklin Lakes, NJ) supplemented with *M. tuberculosis* (BD Difco) followed by intraperitoneal injection of *Bordetella pertussis* toxin (List Biological Laboratories, Campbell, CA) at days 0 and 7 post-induction. Mice were monitored daily in accordance with the standard EAE clinical disease scoring scale as detailed in Hassemann et al., 2017 that was modified from Pettinelli and McFarlin[288] (Table 1). Animals were maintained in accordance with guidelines set by the National Institute of Health and as mandated by the University of California Riverside Office of Research Integrity and the Institutional Animal Care and Use Committee in compliance with the American Association for Laboratory Animal Science.

Treatment

Drugs were dissolved in vehicle solution (10% ethanol and 90% Miglyol 812N (Cremer; Sasol, Germany)) at the following concentrations and were then administered daily by s.c. injection; 17β estradiol (E2): 2.5 mg/kg (Sigma-Aldrich; St. Louis, MO), chloroindazole: 5 mg/kg (De Angelis et al 2005), WAY-202041: 1 mg/kg or 10 mg/kg (Sigma Aldrich), and diarylpropionitrile: 8 mg/kg (Tocris; Avonmouth, Bristol, UK). As a positive control, prophylactic E2 (preE2) was initiated at the time of immunization and proceeded throughout experiments. Therapeutic treatment with vehicle or ERβ ligands (postIndCl, postWAY, or postDPN) began at the onset of clinical symptoms (postEAE; ~8 days post-induction) and continued through peak disease at day 21 post-induction[289]. Normal and 6 treatment groups comprised of n=10 animals/group. Prophylactic 17β-estradiol (E2)

(Sigma-Aldrich; St. Louis, MO), therapeutic E2, therapeutic chloroindazole (IndCl), chloroindazole-*o*-chloro (IndCl-*o*-Cl) and chloroindazole-*o*-methyl (IndCl-*o*-Me) were dissolved in 10% ethanol and 90% Miglyol 812N (vehicle) (Cremer; Sasol, Germany). Positive control groups received a 0.1 ml subcutaneous (s.c.) injection at 0.05 mg/kg/day E2 at EAE day 0 (preEAE). Therapeutic treatment (s.c) with vehicle and various ERβ ligands at 5 mg/kg/day was initiated at EAE postinduction day 8 (postEAE; onset of clinical symptoms) and continued until day 30. Another subset of EAE mice were treated at day 17 postEAE and continued until day 35. Animals were euthanized according to the 2013 AVMA Guidelines on Euthanasia and after induction of disease were sacrificed on either on day 20-21 for flow cytometry, and luminex analysis with immunohistochemistry performed on day 21 and day 35 postEAE, and day 30 for electrophysiology[290]. Vehicle (10% ethanol and 90% Miglyol 812N) and IndCl (5 mg/kg in vehicle) treatment was administered at day 16 postEAE and continued until day 35/36 for all groups (GFAP:ERβ-cKO) and littermate controls (Karim et al 2019, unpublished).

Splenocyte Isolation

Prior to intracardiac perfusion, whole spleens were dissected from mice under deep anesthesia. Splenocytes were processed to a single cell suspension by disaggregating tissue through a 100 μm nylon mesh cell strainer (VWR, Radnor, PA) in RPMI 1640 (Thermo Fisher Scientifc, Chino, CA) supplemented with sodium pyruvate (Sigma-Aldrich), L-glutamine (Thermo Fisher Scientific), and heat-inactivated 10% fetal bovine serum (Thermo Fisher Scientific)(complete RPMI). Red blood cells were lysed in ACK lysing buffer (VWR, Radnor, PA), then splenocytes were washed, counted using a hemocytometer (Hausser Scientific, Horsham, PA), and resuspended at concentrations indicated in downstream applications.

CNS Mononuclear Cell Isolation

Mononuclear cells were isolated from brains and spinal cords by digesting tissue with complete RPMI containing DNase I (Sigma-Aldrich) and collagenase from *Clostridium histolyticum* (Sigma-Aldrich). Dissociated CNS tissue was resuspended in 30% Percoll then overlayed onto 70% Percoll (GE Healthcare, Pittsburgh, PA) and subject to density centrifugation following a previously published protocol[291]. Mononuclear cells were collected from the 30:70% interface, washed with RPMI (Thermo Fisher Scientific), counted, and resuspended for further assay.

Cytokine Analysis

Splenocytes were plated in complete RPMI and stimulated with 25 µg/mL MOG_{35-55} peptide. Supernatants were collected after 48 hours and concentrations of the following cytokines were determined by Cytokine Mouse Magnetic 20-plex Panel for Luminex (Thermo Fisher Scientific; Waltham, MA) run on a Luminex xMAP MAGPIX 100TM instrument (Luminex, Austin, TX) according to manufacturer's instructions; pro-inflammatory cytokines: GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-6, IL-12, IL-17, TNFα, and VEGF; anti-inflammatory cytokines: IL-4, IL-5, IL-10, IL-13, and FGF; and chemokines: CCL2, CCL3, CXCL1, CXCL9, and CXCL10.

Flow Cytometry

2×10^5 cells or 10^6 CNS mononuclear cells and splenocytes, respectively, were washed in FACS buffer (PBS with 1% bovine serum albumin (Sigma-Aldrich) and 0.1% NaN_3 (Fisher Scientific)). Fc receptors on mononuclear cells were blocked by anti-CD16/32 antibody (BD Biosciences, San Jose, CA), then stained with antibodies raised against the following extracellular epitopes; anti-CD11b, anti-CD45, anti-CD4 (all from BD Biosciences). For staining of intracellular epitopes, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL, Sigma-Aldrich), ionomycin

(500 ng/mL, Sigma-Aldrich) and 1 μL/mL GolgiPlug (BD Biosciences) protein transport inhibitor in complete RPMI for 6 hours at 37 °C and 5% CO_2. Extracellular targets were stained, then cells were fixed in 10% formalin, permeabilized, and stained with anti-IFNγ Alexa Fluor 647 (Biolegend, San Deigo, CA) and anti-IL-17A Alexa Fluor 647 (Thermo Fisher Scientific) antibodies for FACS analysis. Flow cytometry data were acquired on a FACsDiva BD LSR II system (BD Biosciences) and analyzed using FlowJo Software (FlowJo, LLC, Ashland, OR).

Histological Preparation of Tissues

Mice were deeply anesthetized by isoflurane (Piramal Healthcare) inhalation and perfused transcardially with 1x PBS followed by 10% formalin (Thermo Fisher Scientific) to fix tissues. Brains and spinal cords were dissected and post-fixed in 10% formalin (Thermo Fisher Scientific) for 24 hours, then cryoprotected in 30% sucrose (EMD Millipore, Darmstadt, Germany) for 48 hours and embedded in gelatin for sectioning. Embedded brains and spinal cords were then cut into 40-μm coronal sections using an HM525 NX cryostat (Thermo Fisher Scientific). Sections were collected serially and stored in PBS with 1% sodium azide at 4°C until staining by immunohistochemistry, following a previously described protocol[277,292].

Immunohistochemistry

Immunohistochemistry (IHC) was performed following a previously described protocol[263]. Markers of myelination, gliosis and immune cells were stained using the following primary antibodies at a concentration of 1:500 unless otherwise noted: chicken anti-myelin basic protein (MBP; EMD Millipore, Darmstadt, Germany), rabbit anti-neurofilament heavy chain (NF200; Sigma-Aldrich), chicken anti-glial fibrillary acidic protein (GFAP; EMD Millipore), rat anti-cluster of differentiation 45 (CD45; clone 30-F11, BD Biosciences, San Diego, CA), mouse anti-ionized

calcium-binding adapter molecule 1/ allograft inflammatory factor-1 (Iba1/AIF1; clone 20A12.1, EMD Millipore), rabbit anti-Olig2 (Thermo Fisher Scientific), goat anti-CXCL1 (R&D systems, Minneapolis, MN; 1:250), rabbit anti-CXCR2 (Abcam; Cambridge, MA; 1:250) and mouse anti-adenomatous polyposis coli (CC-1; clone CC-1, Genetex and EMD Millipore, Irvine and Temecula, CA). Secondary staining was performed using the following fluorophore-conjugated polyclonal antibodies from Thermo Fisher Scientific, at a concentration of 1:500 unless otherwise specified: goat anti-chicken Alexa Fluor 555, goat anti-rabbit Alexa Fluor 647, donkey anti-chicken IgY Cy3 (EMD Millipore), goat anti-rat IgG Alexa Fluor 647, goat anti-mouse IgG Alexa Fluor 647, goat anti-mouse IgG2a Alexa Fluor 647, goat anti-rabbit IgG Cy3 (EMD Millipore), rabbit anti-goat Alexa Fluor 647, and biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in combination with streptavidin Alexa Fluor 647 (Thermo Fisher Scientific). Nuclei were counter stained with 4′,6-Diamidino-2-phenylindole (DAPI, 2 ng/ml; Molecular Probes) for 10 minutes after incubation with secondary antibodies and sections were mounted on glass slides, allowed to dry, and coverslipped with Fluoromount G mounting medium (Southern Biotech, Birmingham, AL) for imaging. Details of antibodies used for IHC and flow cytometry analyses are in Table 2.

Primary OPC Cultures

Primary OPCs, isolated from postnatal day P1 C57BL/6 male and female mouse cortices as described earlier[293]. Primary OPCs were cultured into 8-well chamber slides (three wells per condition, $2.5x10^5$ cells/well) for three days to attach and five days in differentiating media (consisting of DMEM-F12 with triiodothyronine- and thyroxine-containing Sato media as well as penicillin, streptomycin, insulin, N-acetyl-L-cysteine, forskolin, ciliary neurotrophic factor, neurotrophin-3, and platelet-derived growth factor receptor α[293]. A positive control (IndCl), a negative control (vehicle, consisting of the media+EtOH mixture used to dissolve IndCl), and a

normal control (differentiating media alone) were used for comparison. At the end of the treatment period, cells were fixed, stained by immunocytochemistry (primary antibody polyclonal chicken myelin basic protein (MBP, Millipore AB9348), shown in green and co-stained with nuclear stain-DAPI shown in blue), and imaged with an Olympus BX61 confocal microscope (Olympus America Inc., Center Valley, PA) at 10X magnification (3 images per well). Cells were counted using the ImageJ multipoint tool, and counts were then divided by the image area (mm^2). Average cell density for each condition was then divided by the normal condition cell density. Analysis of OL differentiation consisted of counting the number of MBP+ cells and process extensions that were longer than the respective cell-body diameter and tracking the number of highly branched MBP+ cells (with three or more processes) [8]. Statistics were performed using GraphPad Prism 6 Software (La Jolla, CA). One-way ANOVA with Tukey's posthoc test for multiple comparisons was used to generate p-values, and data are presented as mean ± SEM (with $\alpha \leq 0.05$).

Primary Astrocyte Cultures and OPC/OL Astrocyte Conditioned Media Treatment

Astrocyte cultures were prepared by chemical dissociation of the cerebral cortex from p0-p4 C57BL/6 pups[294]. Cells were plated onto cell culture flasks and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin–streptomycin under 5% CO2 atmosphere at 37 °C. Cultures were enriched for astrocytes after five days in vitro by the removal microglia and oligodendrocytes by shaking overnight at 37°C at 200 rpm in a table top shaker. Purified astrocyte cultures were then treated with 13 ng/ml IL-1β, 10 nM vehicle, IL-1β+IndCl-*o*-Cl, IL-1β+IndCl-*o*-Me or just media for 48 hours. Astrocyte conditioned media (ACM) from the various conditions was used for primary oligodendrocyte culture treatment and ELISA. Purified oligodendrocyte cultures (40,000 cells/12 mm coverslip) were plated onto poly-L-lysine coated glass coverslips and maintained in N2B3 medium supplemented with 10 ng/ml PDGF-AA

(Sigma) for 48 hours. After which, ACM from the various conditions was added to primary oligodendrocyte cultures with and without 100 nM CXCR2 antagonist, SB225002 (Tocris, Minneapolis, MN)) for 48 hours. Cells were then fixed and analyzed.

Enzyme-linked Immunosorbent Assay (ELISA)

CXCL1 concentrations (pg/ml) in astrocyte culture supernatant were measured usingenzyme-linked immunosorbent assay (ELISA) murine CXCL1 kit (PeproTech US, Rocky Hill, NJ) according to the manufacturer's instructions. Samples were incubated in a 96 well plate precoated with mouse monoclonal antibody against CXCL1 overnight at room temperature. After washing, plates were incubated with CXCL1 conjugate for two hours at RT. Following washing and addition of substrate solution, enzymatic reaction was stopped, and absorbance was read in a microplate reader (Bio-Rad) set to 405 nm with 605 nm wavelength corrections.

Rotarod behavioral assay

Motor behavior was tested up to two times per week for each mouse using a rotarod apparatus (Med Associates, Inc., St. Albans, VT). Briefly, animals were placed on a rotating horizontal cylinder for a maximum of 200 seconds. The amount of time the mouse remained walking on the cylinder without falling was recorded. Each mouse was tested on a speed of 3-30 rpm and given three trials for any given day. The three trials were averaged to report a single value for an individual mouse, and averages were then calculated for all animals within a given treatment group[277]. The first two trial days prior to immunization served as practice trials.

Quantification and Microscopy

Thoracic spinal cord dorsal and ventral column sections, as well as CC were imaged using an Olympus BX61 confocal microscope (Olympus America Inc., Center Valley, PA) using a 10x and 40x objective. Z-stack projections were compiled using SlideBook 6 software (Intelligent Imaging Innovations, Inc., Denver, CO). Immunostaining was quantified using unbiased stereology[292]. All images (RGB) were converted to grayscale, split, and separated by color channel using imageJ version 2.2.0-rc-46/1.50g (NIH). To avoid experimenter bias, auto-adjustment of brightness and contrast, as well as threshold of staining signal, was carried out by ImageJ. MBP^+, $GFAP^+$, $CD45^+$, and $CXCL1^+$, staining intensity was measured as percent area of positive immunoreactivity within the region of interest and intensity of signal determined by ImageJ. $CC1^+$ cell numbers were automatically counted within a region of interest using ImageJ.

Electrophysiology

To assess functional conductivity across the corpus callosum (CC), electrophysiological recordings of compound action potentials (CAPs) were measured as previously described[292,295]. Coronal brain slices were prepared from adult (3 to 4 month) old C57BL/6 female mice. Briefly, mice were deeply anesthetized under isoflurane and decapitated. The brain was removed and submerged in partially frozen "slushy" solution of slicing buffer containing (in mM): 87 NaCl, 75 sucrose 2.5 KCl, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 1.3 ascorbic acid, 0.1 kynurenic acid, 2.0 pyruvate, and 3.5 MOPS, bubbled with 5% CO_2 + 95% O_2[296]. Coronal slices (350 µm) were prepared using a Leica VT 1000S Vibratome (Bannockburn, IL) and subsequently incubated for 45 minutes at 35 °C in slicing buffer. Following incubation, slices were allowed to cool to room temperature for 15 minutes then transferred to artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 NaH_2PO_4, 26.0 $NaHCO_3$, and 15 glucose,

oxygenated with 5% CO_2 + 95% O_2. Slices were equilibrated in the standard ACSF for a minimum of 15-20 minutes prior to electrophysiological recordings. During electrophysiological recordings, slices were continuously perfused with oxygenated ACSF maintained at a flow rate of 1 mL/min. For recording CAPs, an Axon Digidata 1550 was used with a Multiclamp 700B Amplifier and PClamp 10.4 Software (Molecular Devices, Sunnyvale, CA). Continuous recordings for CC conduction experiments were low-pass filtered at 10 kHz and digitized at 200 kHz. All experiments were conducted at room temperature (24-26 °C). To stimulate the CC fiber tract, a concentric bipolar stimulating electrode (FHC Neural microTargeting Worldwide, Bowdoin, ME, USA) was placed approximately 1 mm away across from a recoding electrode (glass micropipette filled with ACSF) with a resistance of 1-3 MΩ. To elicit CAPs, an episodic stimulation protocol was created consisting of 8 consecutive sweeps, each 12 ms long, with a 5-sec delay between sweeps and an immediate stimulus (0.01 ms duration) after the start of each sweep[271]. Stimulus intensity was adjusted manually using an ISO-Flex stimulator (A.M.P.I). Standardized input-output plots were generated in current clamp mode for each slice by averaging at least 4 consecutive sweeps together to reduce the signal-to-noise ratio. Brain slices that exhibited near zero voltage even when stimulated with the maximal current were not included in the analysis.

Statistical Analysis

All statistics were performed using Prism 6 software (GraphPad Software, La Jolla, CA). Differences in EAE clinical scores were determined by two-way unbalanced ANOVA with Dunnett's multiple comparisons test[287]. Luminex data and immunohistochemistry data were analyzed by ordinary one-way ANOVA with Dunnett's multiple comparisons test either if data satisfied assumptions of normal distribution (D'Agostino & Pearson omnibus normality test) and equal variances among all groups or Kruskal Wallis with Dunn's multiple comparisons test. CAP

recording analysis was carried out per previously published work[277,295] using Clampfit 10.4 software (Molecular Devices, Sunnyvale, CA), OriginPro 2016 64Bit (OriginLab Corporation) and GraphPad Prism 6 (GraphPad Software). The averaged mean amplitude was compared using one-way ANOVA with post hoc tests using Tukey's multiple comparison test. All data are presented as mean ± SEM for two independent experiments. Differences were considered significant at * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$, **** $p \leq 0.0001$.

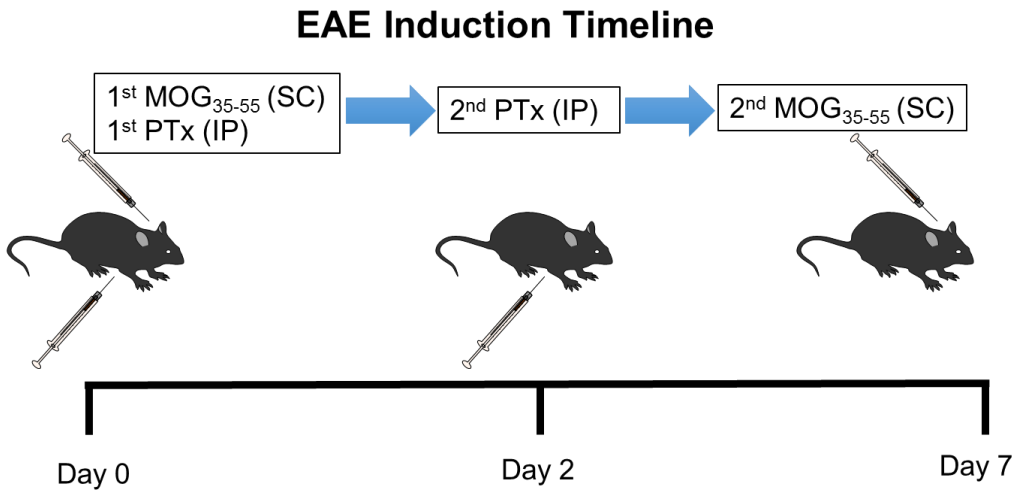

Figure 6. Experimental autoimmune encephalomyelitis (EAE) induction timeline. C57BL/6 mice are immunized with $MOG_{35–55}$ (200 µg/mouse) and *M. tuberculosis* (TB; 200 µg/mouse) in Complete Freund's Adjuvant (CFA), as well as Pertussis toxin (PTx). On post-induction Day 0, mice receive two $MOG_{35–55}$ emulsion injections [subcutaneous (s.c.); 0.05 mL/injection], one near the left axillary lymph nodes and one near the left inguinal lymph nodes. PTx (500 ng/mouse) is also administered [intraperitoneal (i.p.); 0.30 mL]. On Day 2, mice receive another PTx injection. Finally, on Day 7 each mouse receives two $MOG_{35–55}$ emulsion injections (s.c.; 0.05 mL each), this time near the right axillary and inguinal lymph nodes.

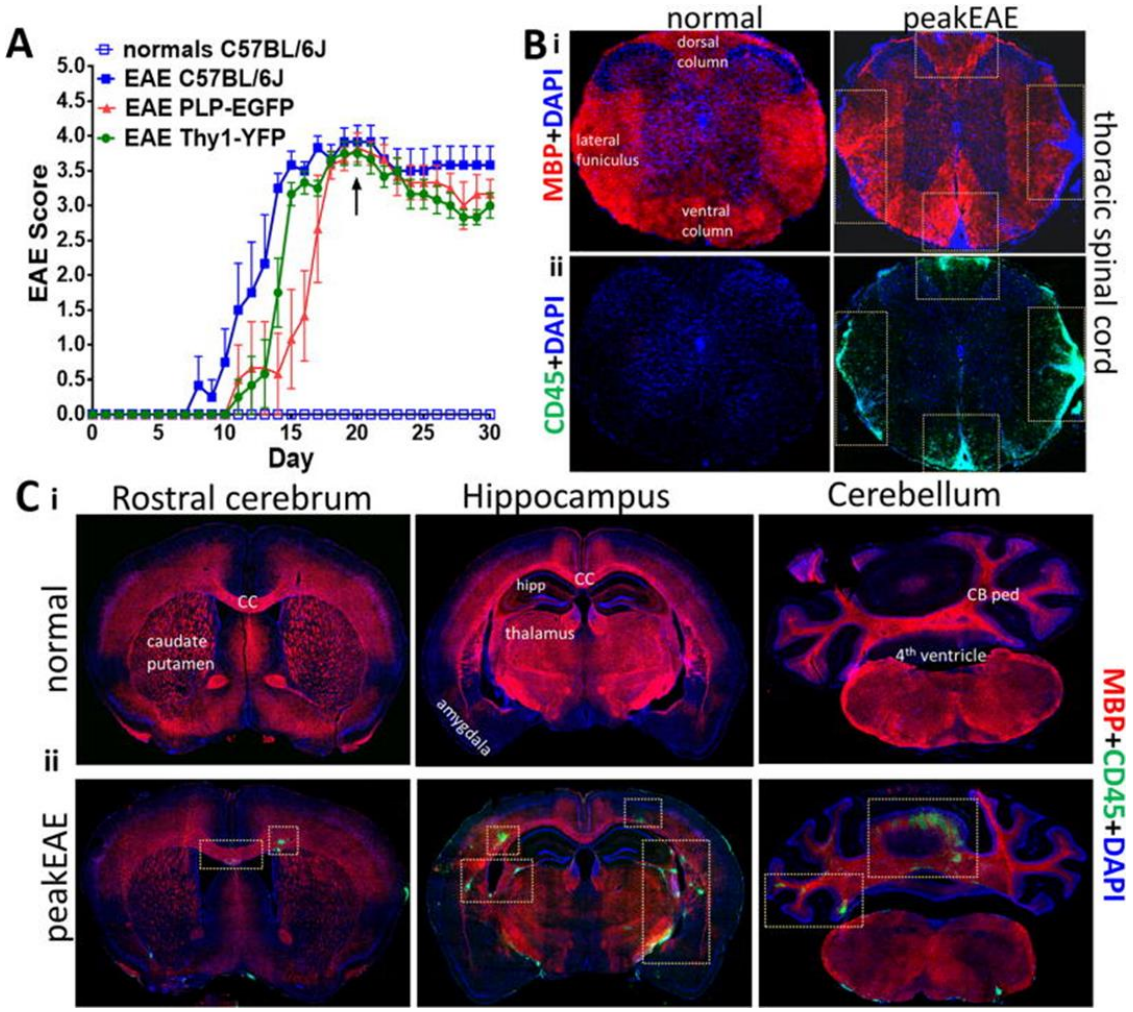

Figure 7. EAE pathology. (A) C57BL/6J (closed blue square), PLP-EGFP (red), and Thy1-YFP (green) mice underwent EAE induction, and C57BL/6J (open blue square) were used as normal controls (n = 5–6 mice/group). Clinical scores were assessed daily and represented as mean±SEM. C57BL/6J, PLP-EGFP, and Thy1-YFP mice responded similarly to EAE induction, with clinical disease onset at Days 8–15 and peak disease at Days 15–20 (black arrow). (B) Thoracic spinal cord coronal sections and (C) Coronal brain sections from normal and peak clinical disease EAE (Day 21) mice were stained for myelin basic protein (MBP; red) to stain myelin and leukocyte antigen marker CD45+ to stain microglia/leukocytes (ii; green), as well as DAPI (blue). EAE mice exhibit decreased MBP+ and increased CD45+ cell reactivity from normal and peak EAE mice. Increased inflammatory lesions (CD45+; green) are observed in all brain sections depicted here at peak EAE similar to as seen in[183]. 10X magnification images.

Table 1. Clinical EAE Scores		
Score	**Scoring Method**	**Observation**
0	Hold mouse by the base of the tail	Unaffected; mouse can “helicopter” tail
0.5	Hold mouse by the base of the tail	Some loss of tail tone
1.0	Hold mouse by the base of the tail	Complete tail limpness, with no evidence of limb weakness
1.5	Hold mouse at base of the tail between index finger and thumb, resting the heel of your palm flat on a surface. Attempt to roll the mouse once by rolling its tail between your fingers.	Can roll mouse, but with some struggling
2.0	Hold mouse at base of the tail between index finger and thumb, resting the heel of your palm flat on a surface. Attempt to roll the mouse once by rolling its tail between your fingers.	No hind limb paralysis upon ambulation, but mouse fails to remain upright when the examiner attempts to roll the mouse
2.5	Hold mouse at base of tail and place its forepaws on edge of the cage. Be prepared to catch the mouse should it fall.	Climbs into cage with difficulty; normal ambulation
3.0	Hold mouse at base of tail and place forepaws on edge of cage. Be prepared to catch the mouse should it fall.	Inability to climb over cage edge; partial paralysis of hind limbs; waddles upon ambulation (but does not drag limbs)
3.5	Observe ambulation	Partial paralysis of hind limbs as evidenced by dragging one limb upon ambulation
4.0	Observe ambulation	Complete paralysis of both hind limbs; dragging body by forearms; still capable of moving around the cage
4.5	Observe mouse behavior	Responsive but not moving/stationary, listless, rapid breathing (consult institutional veterinarian; consider euthanasia)
5.0	Observe mouse behavior	Immobile and unresponsive; Moribund (immediate euthanasia recommended)

Table 2. List of Antibodies used for Immunohistochemistry and Flow Cytometry			
Antibody	**Manufacturer**	**Cat. No.**	**Dilution**
Immunohistochemistry			
MBP	EMD Millipore	AB9345	1:500
NF200	Sigma-Aldrich	N4142	1:500
GFAP	EMD Millipore	AB5541	1:500
Iba1	EMD Millipore	MABN92	1:500
CD45	BD Biosciences	55039	1:500
CC-1	Genetex/EMD Millipore	GTX16794/OP80	1:50
CXCL1	R&D Systems	MAB453	1:250
CXCR2	Abcam	AB14935	1:250
Flow Cytometry			
CD11b	eBioscience	12-0112-81	1:300
CD45	eBioscience	56-0451-82	1:300
CD4	eBioscience	17-0042-82	1:300
IFNγ	Biolegend	505821	1:300
IL-17A	eBioscience	53-71722-80	1:300
CD16/CD32 (Fc Block)	BD Biosciences	553141	1:200

Chapter 3: An increase in chemokine CXCL1 by ERβ ligand treatment could be a key mediator in promoting axon myelination in a mouse model of multiple sclerosis

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Abstract

Estrogen receptor β (ERβ) ligands promote remyelination in mouse models of multiple sclerosis. Recent work using experimental autoimmune encephalomyelitis (EAE) has shown that ERβ ligands induce axon remyelination, but impact peripheral inflammation to varying degrees. To identify if ERβ ligands initiate a common immune mechanism in remyelination, central and peripheral immunity and pathology in mice given ERβ ligands at peak EAE were assessed. All ERβ ligands induced differential expression of cytokines and chemokines, but increased levels of CXCL1 in the periphery and in astrocytes. Oligodendrocyte CXCR2 binds CXCL1 and has been implicated in normal myelination. In addition, despite extensive immune cell accumulation in the CNS, all ERβ ligands promoted extensive remyelination in mice at peak EAE. This finding highlights a component of the mechanism by which ERβ ligands mediate remyelination. Hence, interplay between the immune system and central nervous system may be responsible for the remyelinating effects of ERβ ligands. Our findings of potential neuroprotective benefits arising from the presence of CXCL1 could have implications for improved therapies for multiple sclerosis.

Introduction

Multiple sclerosis (MS) is an autoimmune central nervous system (CNS) demyelinating disease[297]. Experimental autoimmune encephalomyelitis (EAE) is a widely utilized, T cell-mediated mouse model that shares key pathological features of MS, including development of inflammatory lesions, demyelination, axon pathology, and gliosis[298]. In both MS and EAE, pro-inflammatory cytokines produced by autoreactive T helper (Th) cells induce oligodendrocyte (OL) apoptosis, resulting in demyelination and neurodegeneration[299–301].

Current approved therapies for relapsing-remitting MS prevent relapses and slow disability progression primarily by reducing inflammation. However, they fail to reverse axonal pathology or restore myelination[302]. No directly neuroprotective or remyelinating agents are currently available[303].

Recently, estrogens have emerged as attractive candidates for MS therapy that fulfill this need. Estrogenic effects are primarily mediated by ligand-activated nuclear estrogen receptor (ER)α and -β isoforms. Nonselective endogenous estrogens produced during pregnancy reduce MS and EAE relapses and severity through primarily ERα-mediated suppression of inflammation[304–306]. Furthermore, treatment of nonpregnant female MS patients with pregnancy levels of the placenta-derived estrogen hormone estriol reduces circulating CD4+ T cell numbers and pro-inflammatory TNF-α and IFN-γ, while increasing antiinflammatory IL-5 and IL-10[307]. In EAE mice, selective ERα ligands are broadly antiinflammatory and decrease leukocyte infiltration into the CNS[263]. Unfortunately, estrogenic signaling through ERα is also feminizing in males and associated with proliferative effects that increase cancer risk, limiting the clinical usefulness of endogenous

estrogens[308]. In contrast, selective ERβ ligands offer many of estrogen's benefits without these deleterious side effects (see Figure 8 for structure and selectivity of ERβ vs. ERα ligands).

Treatment with highly selective ERβ ligands modulates autoimmunity, improves neurological deficits, and increases myelination in mice with EAE, despite ongoing infiltration of autoreactive leukocytes [263,272,277,309]. For example, diarylpropionitrile (DPN), which displays >70-fold ERβ selectivity over ERα, ameliorates late-stage EAE clinical disease[263,264,272,292], but does not affect production of IFNγ, TNFα, or IL-6 by peripheral mononuclear cells or reduce CNS infiltration of $CD45^+$ leukocytes[263,264]. Notably, while overall immunity was not significantly altered, the frequency of antigen-presenting dendritic cells within the CNS was reduced by DPN, which also impeded their production of TNFα[264].

In addition to their immunological effects, ERβ agonists also mediate therapeutic effects through action on CNS populations. In both EAE and cuprizone models of MS, DPN or chloroindazole (IndCl) treatment increased callosal OLs and myelination, while simultaneously improving functional measures[263,277,292]. In contrast to DPN, either prophylactic or therapeutic treatment with IndCl reduced leukocyte infiltration into the CNS of EAE mice[277], decreased peripheral Th1 and Th17 cytokine production (15), and suppressed expression of IL-1β, IL-6, and IL-23 by microglia[276]. DPN and IndCl treatment during EAE increased brain-derived neurotrophic factor levels and activated the PI3K/Akt/mammalian target of rapamycin pathway, which is involved in OL progenitor (OPC) proliferation and differentiation[277]. The nonsteroidal agonist WAY-202041 (WAY) exhibits >200-fold selectivity for ERβ over ERα, and also decreases inflammation and shows promise for the treatment of MS[273]. Our recent results show that WAY has similar neuroprotective effects to DPN and IndCl in chronic EAE.

Previously, ERβ ligand-mediated immune effects have been assessed after peak disease when demyelination and CNS inflammation has plateaued [263,277]. To understand how ERβ ligands modify EAE pathogenesis and to expose a potential common immunomodulatory role of ERβ ligands, leukocyte populations and secreted cytokines/chemokines were analyzed from mice treated with one of several ERβ ligands from EAE onset to peak disease. In the present study, we show that therapeutic ERβ ligands enhance peripheral and CNS concentrations of the C-X-C motif chemokine, CXCL1, with similar remyelinating and neuroprotective properties.

Results

Effect of Therapeutic ERβ Ligand Treatment on EAE Clinical Score and Uterine Weight.

A persistent disease course starting at day 15–16 with chronic motor deficits was observed in vehicle-treated EAE mice. Therapeutic IndCl and WAY treatment administered at peak disease decreased clinical disease severity over time[277] (Figure 9A, Table 3). Not previously reported, therapeutic WAY treatment induced an increase in myelin density, increase in the OL population, and decrease in "g-ratio" of white-matter tracts (Figure 10), similar to EAE groups treated with DPN[292] or IndCl[277].

To address therapeutic response of different ERβ ligand treatment on peak EAE pathology and immune response, treatment was started at day 8 and suspended on day 21 (Figure 9B, Table 4). Estradiol pretreatment (pre-E2) completely abrogated clinical disease[277]. All other treatment groups displayed onset of clinical disease beginning 7–10 d postinduction, with peak disease (defined as 2 to 3 sequential days of maximal clinical score) occurring between days 18 and 21 (Figure 9 A and B). All therapeutic ERβ ligands administered at onset of disease did not attenuate clinical symptoms by day 21 (Figure 9B). Pre-E2 treatment and not ERβ ligands increased uterine weight by nearly fourfold (Figure 9C), as previously observed[277].

Improved Axon Myelination and No Effect on Leukocyte Infiltration and Astrogliosis.

Reduced axon numbers and myelination at peak EAE has been reported[183,292]. Similarly, vehicle-treated mice at peak disease (Figure 9B) displayed extensive demyelination, indicated by reduced myelin basic protein (MBP+) staining intensity, proximal to CD45+ (a pan-leukocyte marker) perivascular cuffs and leukocyte infiltrates that were not observed in normal animals (Figure 11A). Significant loss of MBP+ and NF200+ axons was observed in the vehicle-treated mice, while ERβ ligand treatment rescued both myelin and axons (Figure 11 B and C).

There was an increase in CD45+ leukocyte infiltration in the white matter of vehicle-treated and ERβ ligand-treated mice, whereas pre-E2 treatment had minimal CD45+ immunoreactivity (Figure 12 A and C). Similarly, immunoreactivity for the myeloid cell marker ionized calcium-binding adaptor molecule 1 Iba1+ and the astrocyte marker glial fibrillary acidic protein (GFAP) was significantly elevated in vehicle- and ERβ ligand-treated mice (Figure 12). Pre-E2 abrogated this increase (Figure 12 D and E).

No Change in CNS-Infiltrating Leukocyte Populations.

To profile CNS infiltrating leukocytes after ERβ ligand treatment, mononuclear cells were isolated at peak EAE disease and analyzed by flow cytometry. Vehicle-treated mice showed elevated CD11b+CD45hi activated microglia/macrophage frequency (gate R2) (Fig. 13 A and C). Pre-E2 significantly reduced CD11b+CD45hi frequency (Figure 13C), while ERβ ligand and vehicle treated groups displayed similar frequency of activated microglia/macrophages (Figure 13C). Similarly, increased CD4+IFN-γ+ Th1 and CD4+IL-17+ Th17 frequencies were observed in vehicle-treated mice but not pre-E2 (Figure 13 D and E). ERβ ligands did not affect Th1 or Th17 frequency (Figure 13 D and E).

Modification of Cytokine and Chemokine Production by Peripheral Leukocytes.

To characterize the effects of ERβ ligands on the peripheral immune system, splenocytes were harvested from mice 21 d after EAE induction and assessed by flow cytometry. Splenic CD11b+CD45+ macrophage/monocyte composition was attenuated by pre-E2, but not ERβ ligand treatment (Figure 14 A and B). Th1 frequency was significantly reduced in pre-E2 and ERβ ligand treated groups vs. vehicle (Figure 14C), while Th17 frequency was not affected (Figure 14).

Cytokine and chemokine expression was analyzed to assess the role of leukocyte effector function in ERβ ligand derived benefits during peak disease[310].

Pro-inflammatory cytokines. The Th1 cytokine IFNγ was significantly up-regulated in vehicle-treated mice vs. normal. In contrast, pre-E2, post-IndCl, WAY, and DPN decreased IFNγ concentration relative to vehicle (Figure 15 A, i). IL-2 was significantly increased in vehicle-treated mice vs. normal and was attenuated only by pre-E2 treatment (Figure 15 A, ii). Vehicle-treated mice produced higher levels of TNFα and IL-17 vs. normal (Figure 15 A, iii and iv). Pre-E2, IndCl, WAY, and DPN produced similar levels of TNFα and IL-17 as vehicle treatment (Figure 15 A, iii and iv). Increased IL-1β was observed in vehicle-treated and pre-E2 groups vs. normal. Interestingly, all ERβ ligand groups exhibited greater IL-1β production vs. vehicle (Figure 15 A, v).

Anti-inflammatory cytokines. Skewing immune response toward production of Th2 cytokines, such as IL-4, IL-5, and IL-10, with parallel suppression of Th1 cytokines ameliorates EAE[311]. IL-4 was up-regulated only in vehicle-treated mice (Figure 15 B, i). Similarly, neither IL-5 nor IL-10 was changed by E2 or ERβ ligand treatment (Figure 15 B, ii and iii).

Chemokines. CXCL1 was significantly up-regulated by ERβ ligands relative to vehicle (Figure 15 C, i). No change was observed in pre-E2 groups. CXCL9 and CXCL10, closely related IFNγ–inducible T cell and monocyte chemoattractants, were increased in vehicle-treated mice vs. normal. CXCL9 levels were similar in all treatment groups compared with vehicle; however, pre-E2 and IndCl treatment significantly reduced CXCL10 levels, with no change by WAY or DPN treatment (Figure 15 C, ii and iii).

ERβ Ligands Up-Regulate CXCL1. Due to its role in OPC recruitment and up-regulation by splenic leukocytes in response to ERβ ligand treatment, CXCL1 expression was examined in white matter from mice at peak EAE by immunohistochemistry (IHC). In the CNS, astrocytes are a primary source of CXCL1 during white-matter development[312] and neuroinflammation[313]. Compared with vehicle, IndCl- and DPN-treated mice exhibited increased CXCL1+ immunoreactivity that appeared to overlap with GFAP (Figure 16 A and C)[314]. In addition, CXCL1 immunoreactivity colocalized with IL-1β and GFAP in astrocytes during ERβ ligand treatment (Figure 17). In addition, supernatant from primary astrocyte cultures stimulated with IL-1β contained increased levels of CXCL1. The same supernatant when added to OL cultures induced an increase in MBP expression (Figure 18).

CXCR2 receptor is critical for developmental OPC positioning and differentiation in the CNS[120]. OL primary cultures in the presence of CXCR2 antagonist, SB 225002 and IL-1β astrocyte-conditioned media showed decreased differentiation and enhanced OL apoptosis (Figure 18). To determine the distribution of CXCR2 during peak EAE disease in response to ERβ ligand treatment, CXCR2 expression by OPCs/OLs was assessed by colabeling with the OL lineage-specific transcription factor, Olig2 in spinal cords. Olig2+ nuclei were comparable between all groups evaluated and CXCR2 was detected in a subset of Olig2+ nuclei (Figure 16 B and D). The fraction of CXCR2+Olig2+ cells increased in all treatment groups (Figure 16E), consistent with previous reports that OPC/OL CXCR2 is up-regulated in MS[126].

Discussion

In this study, we characterized the therapeutic impact of three ERβ ligands in a clinically relevant mouse model of MS. Our results suggest that the protective effects of ERβ ligands on remyelination and neuroprotection may be mediated by changes in cytokine and chemokine production. Comparisons among the ERβ ligands tested show improved myelination. Consistent with previous studies[263,277], this occurred with little impact on the composition or polarization of CNS or splenic leukocytes, but involved reduction of peripheral IFNγ production and increased CXCL1 expression in the CNS and spleen. Individual ERβ ligands displayed differential effects on the production of the IFNγ–inducible CXCL10, with IndCl being the only compound tested to suppress production aside from pretreatment with E2. These data indicate that individual ERβ ligands may have various effects on leukocyte activity in EAE, while also pointing to a potential immunological mechanism common to ERβ-mediated remyelination. Specifically, three findings that were observed with all ERβ ligands tested stand out: (i) increased myelination, (ii) reduced IFN-γ, and (iii) increased CXCL1. These data suggest that ERβ ligands may facilitate an environment within the inflamed CNS where OPCs are recruited to the site of ongoing demyelination by astrocytic CXCL1 and allowed to differentiate into myelinating OLs through attenuation of cytotoxic IFNγ production.

Although the ligands used in this study were all selective for ERβ, their activity in the peripheral and CNS may differ[263,276,277,315]. For example, IndCl, but not DPN or WAY, was effective at repressing inflammatory response gene products in microglia and astrocytes[276]. The differential immune effects observed by treatment with the ERβ ligands tested in the present study may be due to how these compounds recruit cofactors. As an example, IndCl promotes entry of ERβ into the C-terminal binding protein-dependent transrepression pathway while DPN does not[276]. However, while transcriptional cofactor recruitment differs, these ERβ ligands converge in their ability to

induce OPC/OL survival and myelination during demyelinating disease[277,316]. Importantly, this was shown to be mediated by direct action on OLs as conditional knockout of ERβ from these cells abrogated the clinical benefits of DPN[316]. Quiescent microglia are distributed throughout the homeostatic CNS and are characterized by coexpression of the myeloid cell integrin/complement receptor CD11b and low levels of CD45. In the present study, unlike pre-E2, no ERβ ligand tested abrogated the rise in activated microglia/macrophages recovered from the CNS or spleen of vehicle-treated mice.

Unexpectedly, ERβ ligands had divergent effects on peripheral and CNS T lymphocyte populations. Pre-E2 reduced the frequency of Th1 cells in splenic and CNS mononuclear cells. In contrast, pre-E2 reduced CNS-infiltrating Th17 cells, but had no effect on splenic lymphocytes. Similarly, ERβ ligand treatment decreased Th1 cell frequency, as well as pro-inflammatory cytokine IFNγ in the periphery, with no change in Th1 populations in the CNS. This finding may reflect CNS infiltration at disease onset or reduced Th1 differentiation resulting from decreased IFN-γ concentrations. Another ERβ ligand, 4-(2-phenyl-5,7-bis[trifluoromethyl]pyrazolo[1,5-a]pyrimidin-3-yl)phenol (PHTPP), was shown to suppress Th17 response by acting directly on CD4+ T cells[309] while IndCl altered CD4+ T cell ERβ expression in vitro[309]. We did not observe a suppression of Th17 frequency or peripheral IL-17 secretion with ERβ ligands. Additional study is required to assay the effect of ERβ ligands on CD8+ populations; however, E2 has been shown to increase their activity in a viral of lung infection[317].

Pro-inflammatory cytokines, such as IFN-γ, drive EAE by potentiating antigen presentation and secretion of additional cytokines and chemokines[310]. Of potential importance to the mechanism whereby ERβ ligands promote remyelination, splenocytes from ERβ ligand treatment produced

less IFNγ in response to MOG35–55 peptide. IFNγ elicits OL apoptosis in vitro[318] and ectopic CNS expression results in OL death and impaired myelination[319]. These data indicate that IFNγ–induced death of OLs/OPCs may represent a potential etiology for remyelination failure in EAE and MS. However, IFNγ may possess pleiotropic effects on OLs during demyelinating disease[320]. Thus, ERβ ligand-mediated peripheral IFNγ reduction may play a key role in supporting remyelination by slowing Th1 differentiation, while lowered levels of CNS IFNγ may improve OL survival.

Interestingly, IL-1β, which is associated with various inflammatory and demyelinating disorders[321] and responsive to E2 signaling[322], was significantly increased by ERβ ligand treatments. While IL-1β is cytotoxic to mature OLs in vitro, neither demyelination nor OL loss during cuprizone demyelination are attenuated in mice lacking IL-1β[321]. However, remyelination fails due to lack of IL-1β–mediated expression of insulin growth factor-1[86], pointing toward a complex role for this cytokine. Our results suggest that IL-1β in the presence of the ERβ ligands may be associated with myelin repair by promoting mature OL differentiation and remyelination during EAE.

Chemokines and their cognate receptors play a critical role in the recruitment and trafficking of leukocytes in the context of disease conditions. ERβ ligands increased CXCL1 and decreased CXCL10. Abundant CXCL10 has been detected at early time points in the development of EAE and MS lesions[323] and promotes neuronal and OL apoptosis[324]. Here, only IndCl decreased CXCL10 levels.

CXCL1, a major neutrophil chemoattractant expressed by astrocytes and microglia, affects chemoattraction through its receptor CXCR2, which is present on diverse cellular populations, including leukocytes, keratinocytes, dermal fibroblasts, neutrophils, and OLs[125,325]. E2 has been shown to be a negative regulator of CXCL1/CXCR2 signaling pathways[326–329] through

predominantly ERα-dependent pathways[326]. However, CXCL1-mediated functions extend beyond chemoattraction. In MS, both CXCL1+astrocytes and CXCR2+ OLs have been detected in lesions[78], indicating that it may play a role in recruitment of OPCs to demyelinating sites. CXCL1 overexpression by astrocytes attenuates EAE disease severity[122], while CXCR2 deficient mice exhibit reduced white-matter volume and myelin proteins in the spinal cord[312]. Furthermore, CXCR2 signaling promotes OPC survival by increasing levels of the antiapoptotic protein, Bcl-2[324]. Together, these results indicate a potential role for astrocyte derived CXCL1 in promoting OL survival in ERβ ligand treated EAE mice.

Our finding that ERβ ligand therapy promotes increased astrocytic CXCL1 production may represent an additional facet of how these compounds induce remyelination. OPC recruitment and differentiation by CXCL1, in combination with attenuated IFNγ production reducing OL apoptosis, may account for at least one avenue whereby ERβ ligands exert their clinical benefits. In summary, our data show that ERβ ligand neuroprotection/remyelination may be partly mediated by skewing the pro-inflammatory phenotype to a protective phenotype. Our results demonstrate that the interplay between CNS- and immune-derived signals is central to the induction and regulation of neuroinflammatory diseases such as MS. The possibility that ERβ ligands modulate the cytokine and chemokine milieu to potentially promote repair/remyelination opens up exciting therapeutic options.

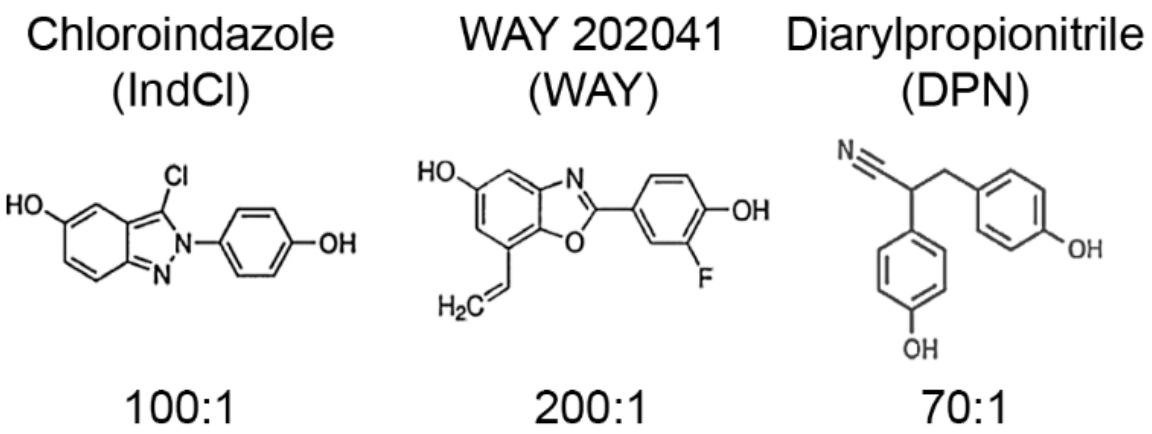

Figure 8. **Structure and selectivity of ERβ ligands** Chloroindazole (IndCl), WAY 202041 (WAY) and Diarylpropionitrile (DPN): ERβ vs. ERα.

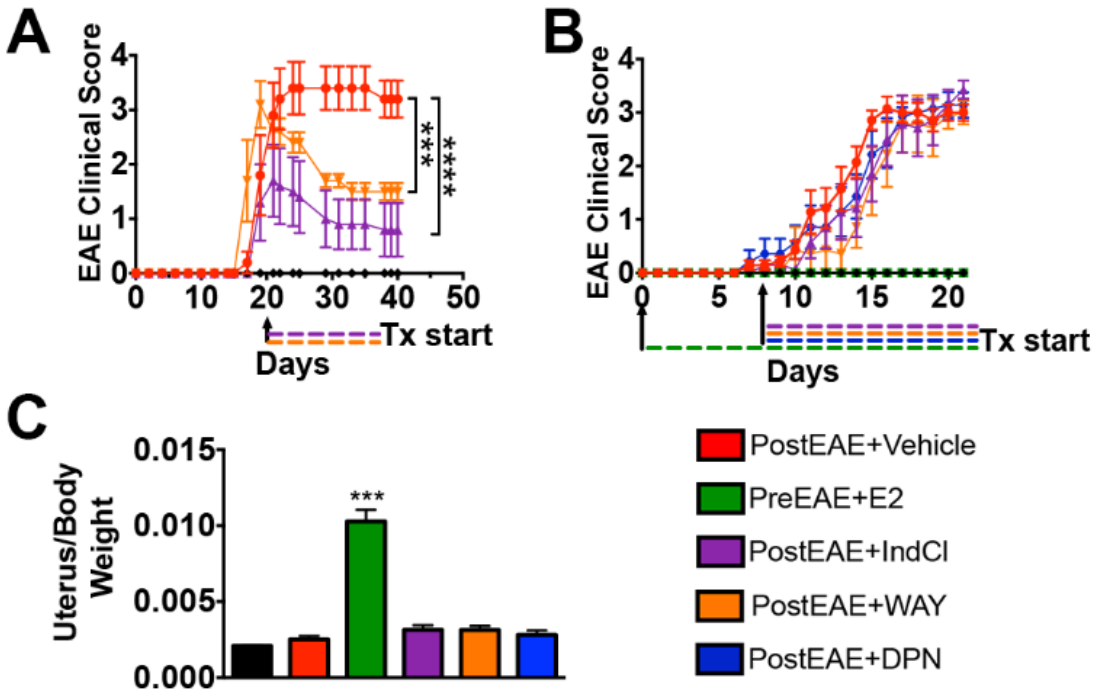

Figure 9. Therapeutic ERβ ligand treatment decreases EAE severity with no effect on uterine weight. (A) Treatment with ERβ ligands, IndCl (post-EAE+IndCl; purple), and WAY (post-EAE+WAY; orange) began on day 21 until day 40. Onset of clinical disease occurred between days 17 and 19 in vehicle-treated EAE mice (post-EAE+vehicle; red), with peak severity at day 21. Treatment with IndCl and WAY significantly decreased disease severity vs. vehicle beginning by day 24 through 40 (IndCl-purple stars; WAY-orange stars). Differences in EAE clinical scores were determined by two-way unbalanced ANOVA with Dunnett's multiple comparisons test (48). n = 8–10 mice per group; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (B) Therapeutic treatment with ERβ ligands, IndCl, WAYand DPN (post-EAE+DPN; blue) began on day 8 until day 21. Prophylactic E2 (pre-EAE+E2; green) prevented onset of clinical disease. Vehicle treated EAE mice displayed onset of clinical disease symptoms between days 7 and 10 with disease severity peaking around day 17. During peak disease, IndCl, WAY, and DPN treatment did not affect EAE clinical scores. (C) Assessment of postperfusion uterus to body weight. Only pre-E2–treated female mice exhibited a 4× increase in uterus:body weight ratio. n = 8–10 mice per group; ***P < 0.001.

Table 3. Values for Disease Onset and Cumulative Disease Index for EAE Day 40 (chronic disease)

Treatment	Disease Incidence	Disease Onset	Cumulative Disease Index
Normal	-	-	-
Vehicle	100%	19	38
IndCl	80%	19	14
WAY	100%	17	26
Vehicle vs IndCl	-	p=0.9923	p=0.0050
Vehicle vs WAY	-	p=0.0685	p=0.1582

Table 4. Values for Disease Onset and Cumulative Disease Index for EAE Day 21 (peak disease)

Treatment	Disease Incidence	Disease Onset	Cumulative Disease Index
Normal	-	-	-
Vehicle	100%	10.4	30.7
PreE2	-	-	-
IndCl	100%	12.3	26.9
WAY	100%	13.2	23.6
DPN	100%	10.4	28.6
Vehicle vs IndCl	-	p=0.6928	p=0.7929
Vehicle vs WAY	-	p=0.443	p=0.341
Vehicle vs DPN	-	p>0.9999	p=0.9701

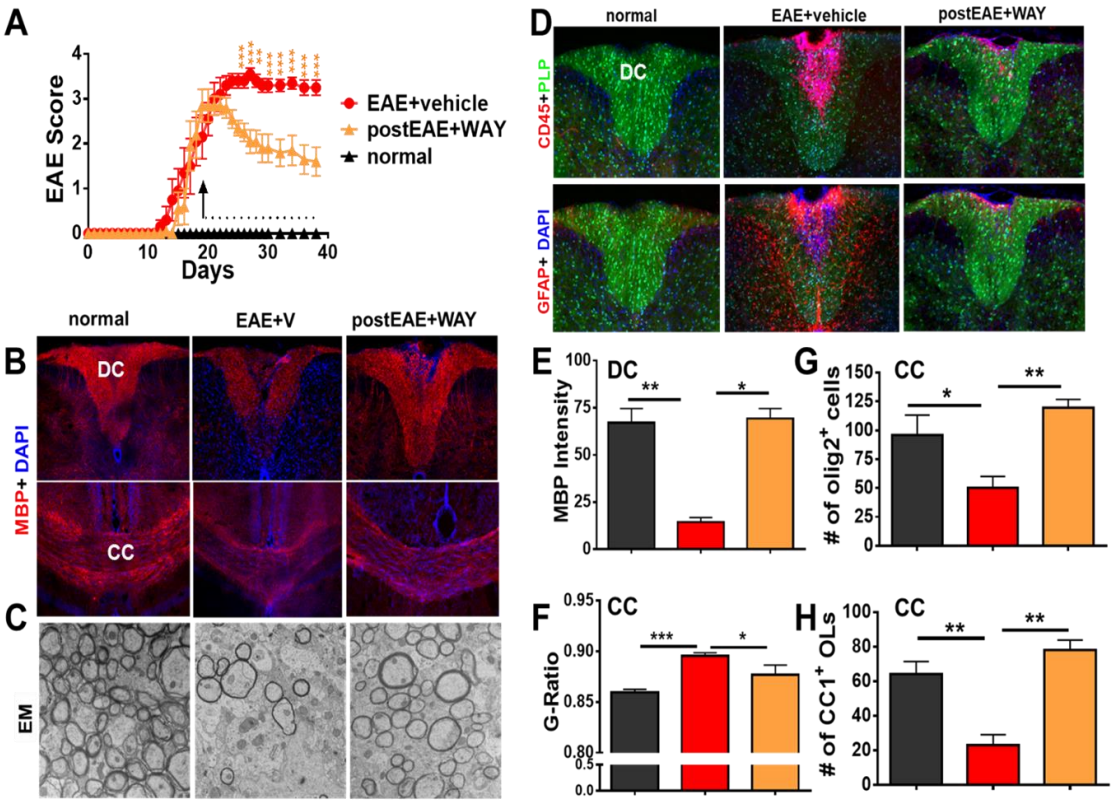

Figure 10. Therapeutic treatment with WAY decreases EAE clinical disease. (A) Therapeutic treatment with WAY (10 mg/kg/d; orange) began on day 18 until day 38. Treatment with WAY significantly decreased disease progression over time. One of four representative EAE experiments is shown. n = 8–10 mice/group. ***P < 0.001, 2way ANOVA with Dunnett's multiple comparisons test. (B) Representative 10x magnification images of the dorsal column (DC) of thoracic spinal cord sections, and corpus callosum (CC) containing brain sections collected on day 38. Sections were immunostained with myelin basic protein (MBP; red) and nuclear stain (DAPI; blue). (C) Representative electron micrographs of CC axons imaged at 14,000× magnification reveal fewer myelinated axons in vehicle-treated EAE versus normal CC. Comparatively, there were more thinly myelinated axons in the WAY-treated groups. (D) Representative 10x magnification images of the dorsal column (DC) reveals increased intensity of glial fibrillary acidic protein (GFAP;red), and cluster of differentiation 45 (CD45; red) in vehicle treated EAE mice compared to normal control mice. Treatment with WAY exhibited similar GFAP and CD45 fluorescence intensity as vehicle treated mice. (E, G, and H) Quantification of the relative fluorescence intensity of MBP, Olig2 (all OL lineage cells) and CC1 (mature OLs) from normal, vehicle, and therapeutic WAY-treated EAE mice. MBP intensity, number of Olig2+ and CC1+ OLs was significantly decreased in vehicle treated EAE mice compared to normal controls but was maintained to near normal levels with therapeutic WAY treatment. n = 6 to 8 mice/group; *P < 0.05, **P < 0.01, Kruskal Wallis Analysis. (F) G-ratio was calculated for a minimum of 500 callosal axons/mouse. Significantly higher g-ratio was observed in EAE+vehicle mice, indicative of more unmyelinated axons compared to normal group. WAY-treated callosal axons had a significantly lower g-ratios representative of increased axon myelination. n = 4 mice/group; *P < 0.05, ***P < 0.001, ANOVA.

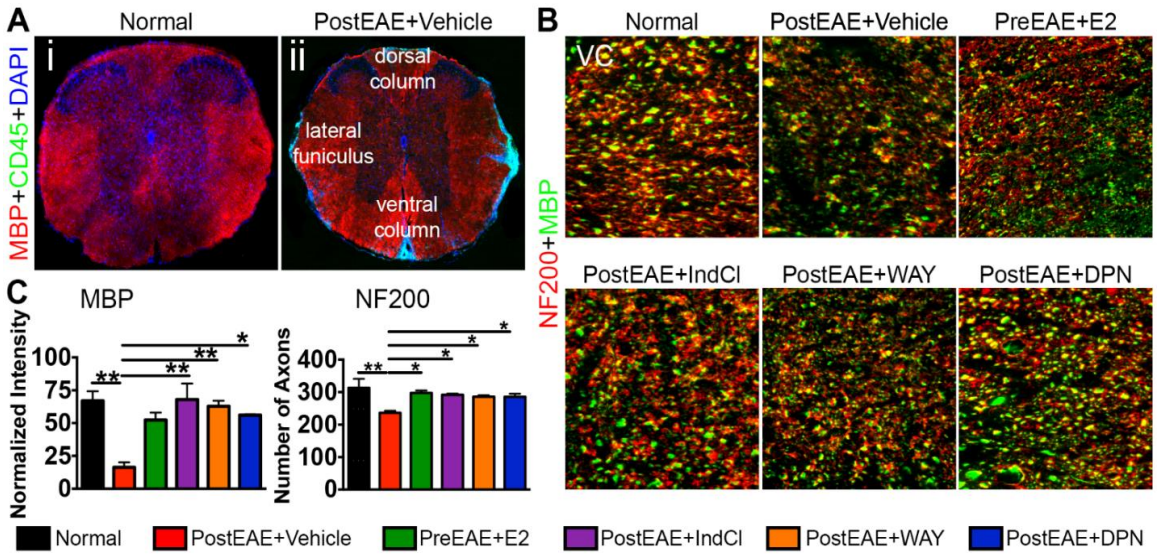

Figure 11. Therapeutic ERβ ligand treatment improves myelination during peak EAE disease. (A) Representative 10× magnification images of thoracic spinal cords collected at peak disease (day 21). Experimental groups shown in Fig. 2B, were immunostained for MBP (red), CD45 (green), and DAPI (blue), with 4× normal (i) and vehicle (ii) images to show pathology. (B) Magnified 40× images showing ventral column immunostained with MBP (red) and NF200 (green). (C) Quantification of MBP intensity and NF200+ axon numbers revealed significant decreases in only vehicle treated groups. n = 5 mice per group; *P < 0.05, **P < 0.01.

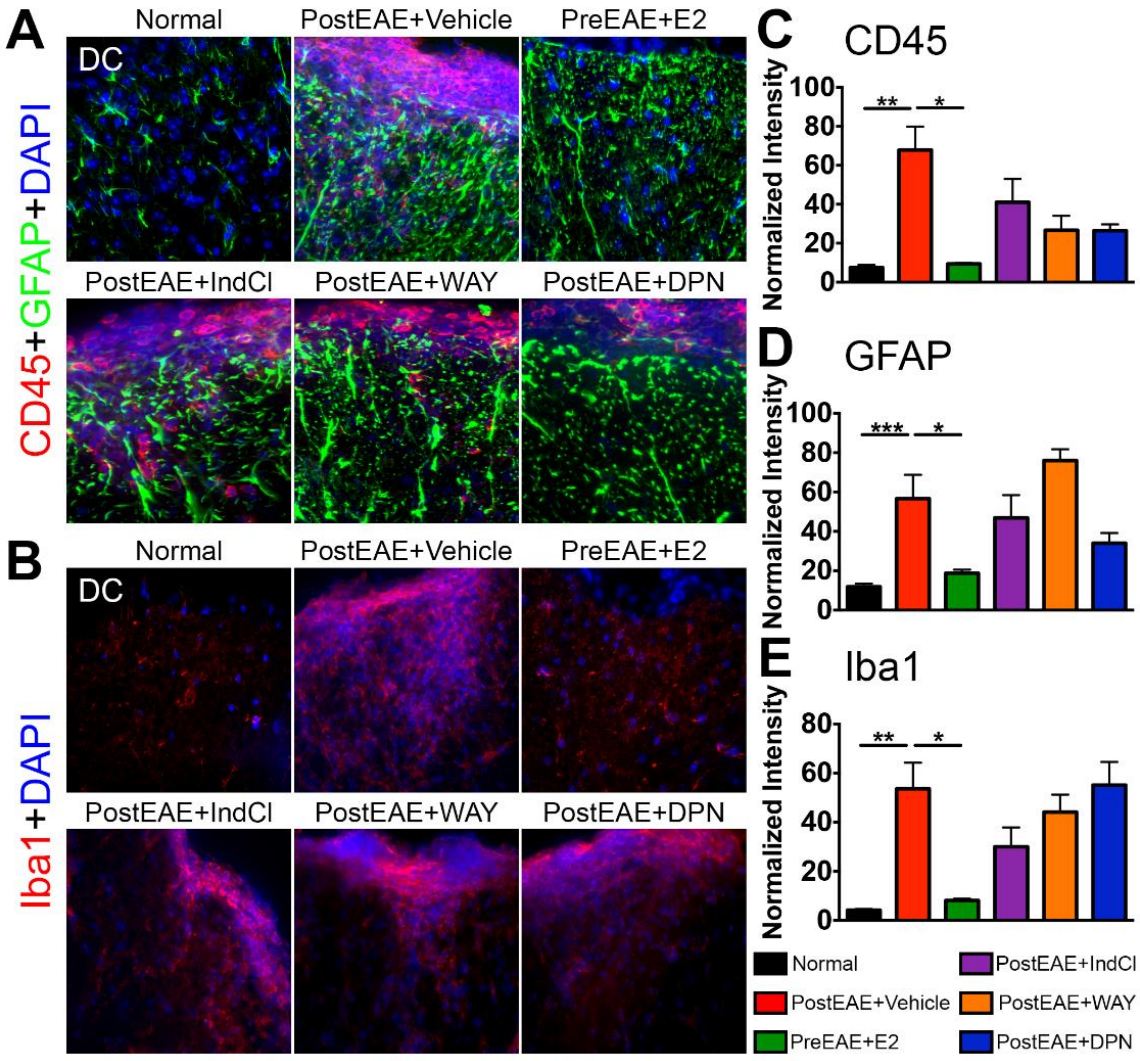

Figure 12. Treatment with ERβ ligands do not exhibit significant changes in CNS immune cell populations during peak EAE disease. (A and B) Representative 40× magnification images of thoracic spinal cords collected at peak disease (day 21) from experimental groups shown in Figure 9B were immunostained for astrocytes (GFAP, green), leukocytes (CD45, red), microglia/macrophages (Iba1, red), and DAPI (blue). (C–E) Quantification of normalized intensities of CD45, GFAP, and Iba1. Vehicle-treated mice exhibited increased CD45, Iba1, and GFAP intensity that was decreased only with pre-E2 treatment. n =5 mice per group; *P < 0.05, **P < 0.01, ***P < 0.001.

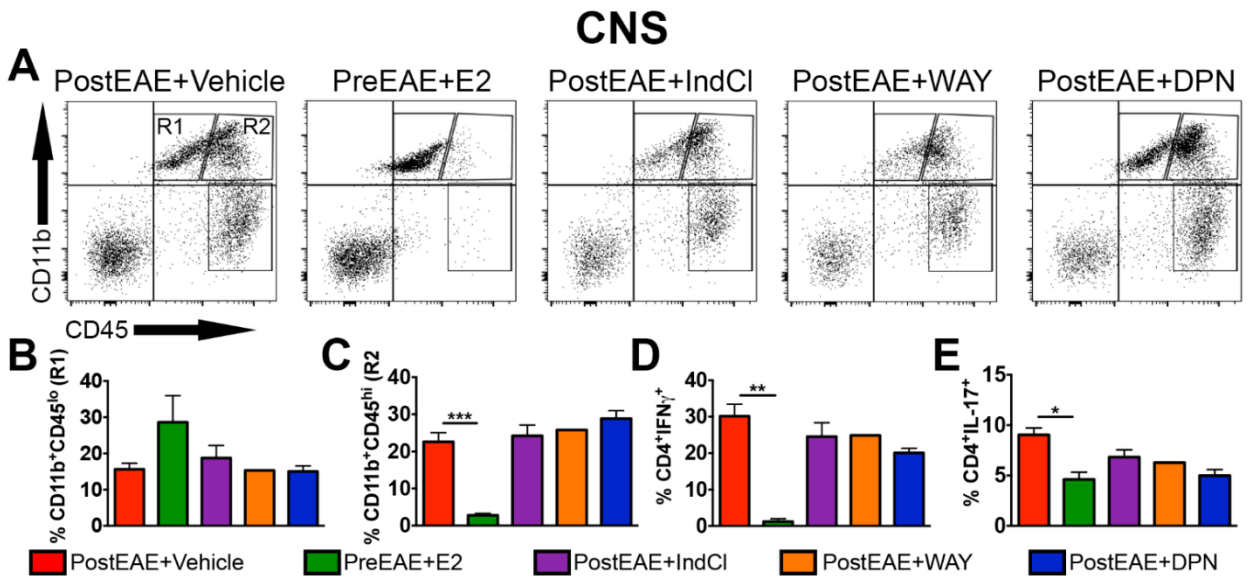

Figure 13. Therapeutic ERβ ligand treatment effect on CNS inflammatory immune cell numbers during peak EAE disease. (A) Representative plots of CD11b (y axis) and CD45 (x axis) distribution in the CNS from vehicle, pre-E2, and therapeutic ERβ ligand treated mice collected from peak EAE mice. In the CNS, resting microglia (R1) are represented by CD11b+CD45lo populations, while activated microglia/macrophages (R2) are represented by CD11b+CD45hi. (B and C) Quantification of resting (CD11b+CD45lo; R1) and activated microglia/macrophages (CD11b+CD45hi; R2) from prophylactic E2 and therapeutic ERβ ligands treated EAE mice. Pre-E2 decreased activated macrophages compared with vehicle. (D and E) Th1 (CD4+IFN-γ+) and Th17 (CD4+IL-17+) frequency in the CNS was decreased by pre-E2 treatment. ERβ ligands did not affect Th1 or Th17 frequency. n = 8–10 mice per group; *P <0.05, **P < 0.01, ***P < 0.001.

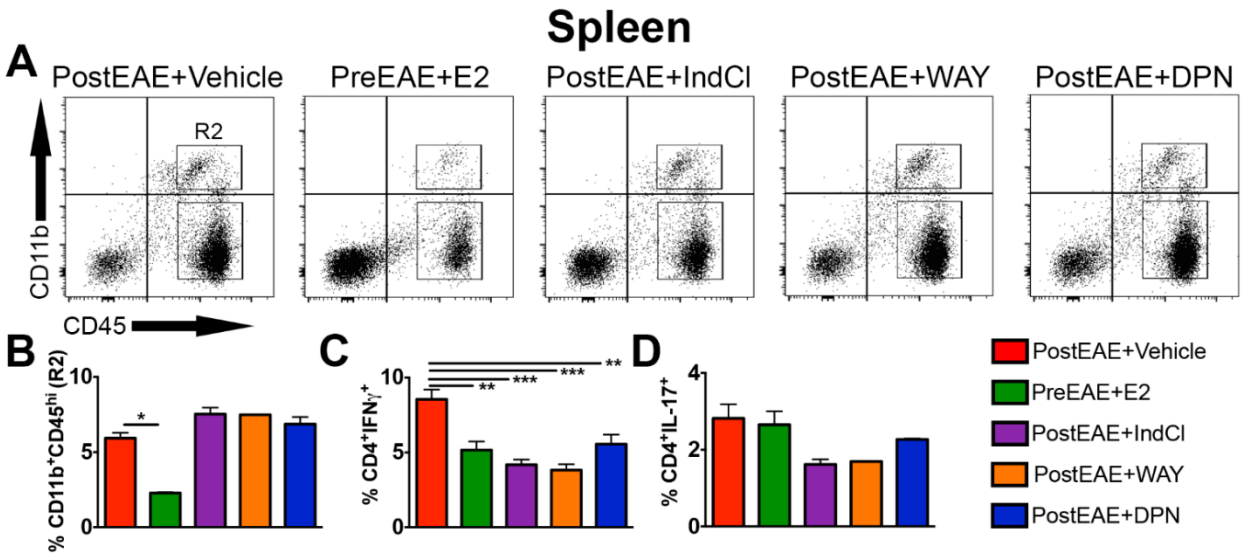

Figure 14. Effect of therapeutic ERβ ligand treatment on splenic immune cell numbers during peak EAE. (A) Representative plots of splenic CD11b (y axis) and CD45 (x axis) distribution in vehicle, prophylactic E2, and therapeutic ERβ ligand treated mice collected at peak disease. Activated monocytes/macrophages (R2) are represented by CD11b+CD45hi. (B) Quantification of activated microglia/macrophage frequency was decreased only in pre-E2–treated mice. (C and D) ERβ ligands decreased Th1 frequency. No change was observed in Th17 frequency in any group vs. vehicle. n = 8–10 mice per group; *P < 0.05, **P < 0.01, ***P < 0.001.

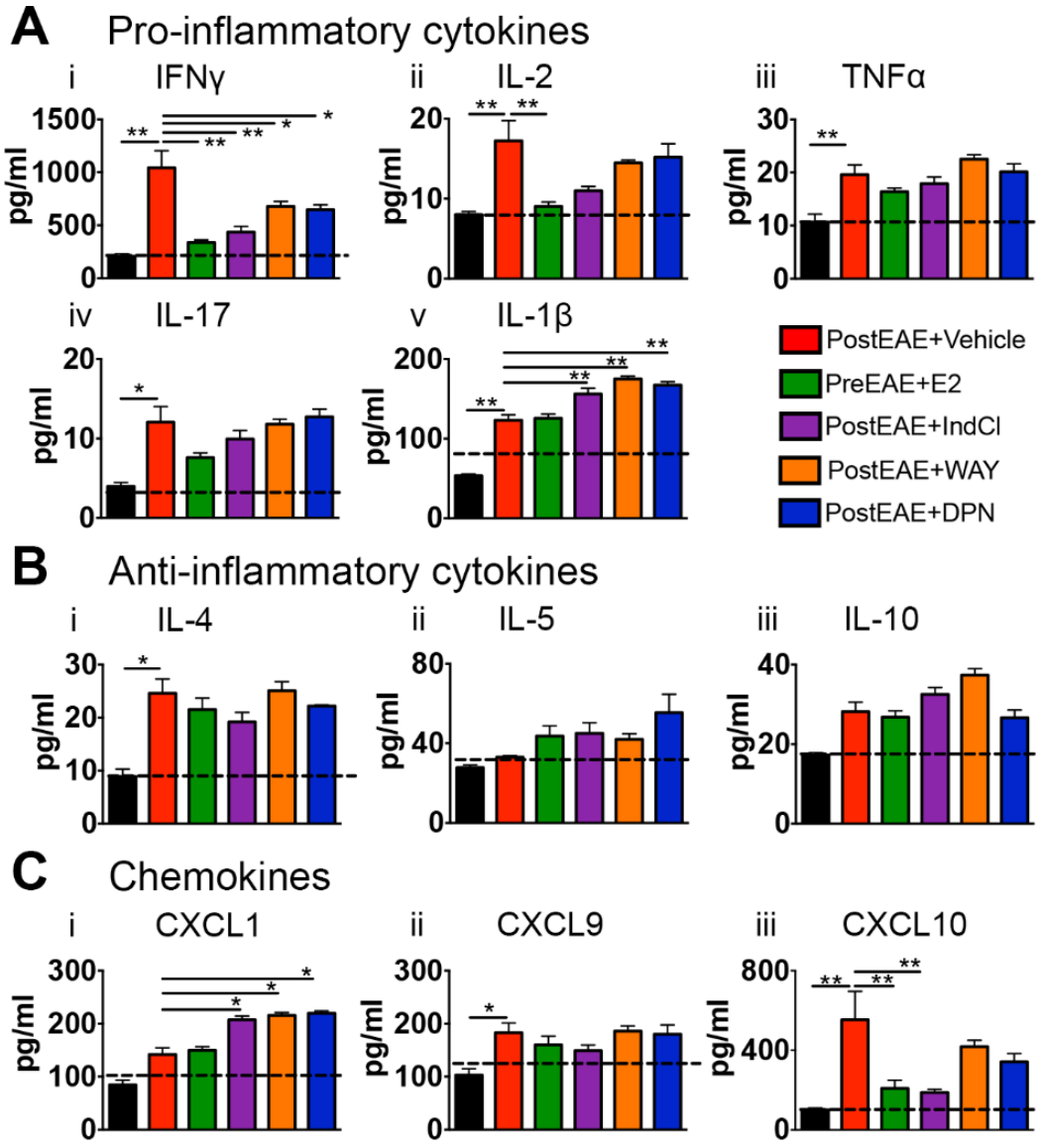

Figure 15. Chemokine production by peripheral immune cells during peak EAE disease. (A) Splenocytes collected from vehicle-treated mice at peak disease stimulated with MOG35–55 for 48 h showed increased IFN-γ, IL-2, TNF-α, IL-17, and IL-1β vs. normal. (i) Pre-E2 and therapeutic ERβ ligand treatment decreased IFN-γ. (ii) Only pre-E2 groups decreased IL-2 vs. vehicle. (iii and iv) No change was observed in TNF-α and IL-17 in any treatment group. (v) ERβ ligands increased IL-1β levels vs. vehicle. (B, i–iii) Vehicle treated mice exhibited increased levels of anti-inflammatory cytokine IL-4 vs. normal. No change was observed in IL-4, IL-5, or IL-10 production in any treatment groups. (C) Vehicle treated mice exhibited increased levels of CXCL9 and CXCL10 with no increase in CXCL1 levels. (i) ERβ ligands, but not preE2, increased CXCL1. (ii) Similar levels of CXCL9 was observed in all treatment groups. (iii) Only pre- E2 and therapeutic IndCl groups reduced CXCL10 vs. vehicle. n = 5–8 mice per group; *P < 0.05, **P < 0.01.

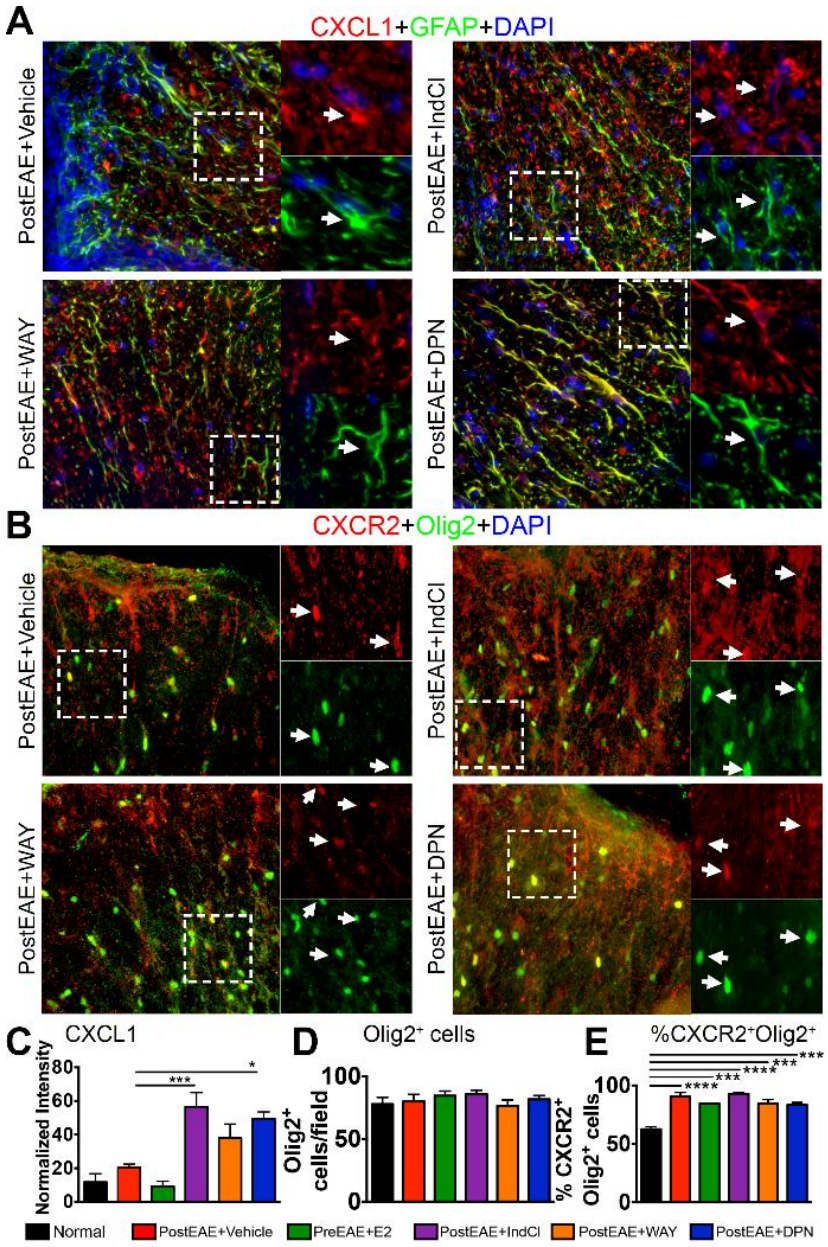

Figure 16. Modulation of CXCL1 and its receptor CXCR2 in the CNS from ERβ ligand-treated EAE mice. (A) Representative 40× images of thoracic spinal cord ventral column at peak disease (Figure 9B) immunostained for CXCL1 (red), GFAP (green), and DAPI (blue). (A and B) White dashed boxes from merged images were split and enlarged 3×. Arrows indicate costaining between CXCL1 and GFAP. (B) Magnified 40× images of CXCR2 (red) and OPCs/OLs (Olig2, green), arrows indicate CXCR2+Olig2+ cells. (C) ERβ ligands increased CXCL1 staining intensity vs. vehicle. (D and E) Olig2+ cell numbers were comparable among groups, while the fraction of Olig2+CXCR2+ OPCs/OLs were increased in groups with EAE. n = 5–8 mice per group; *P < 0.05, ***P < 0.001, ****P < 0.0001.

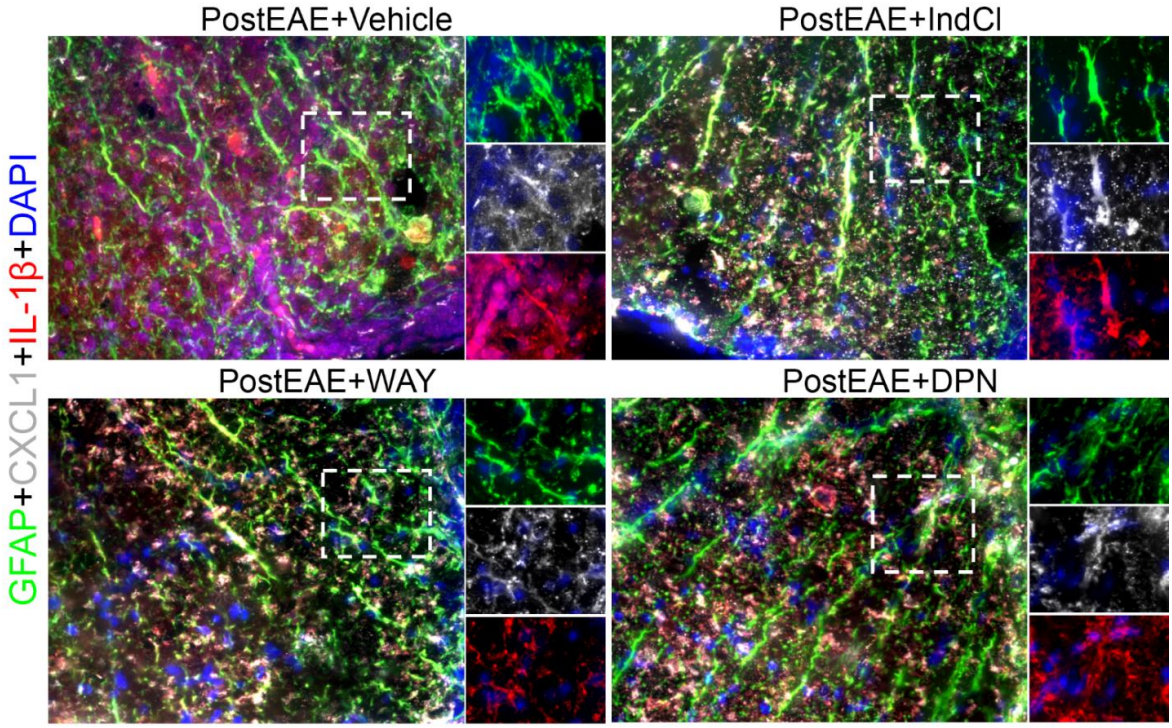

Figure 17. The effect of IL-1β on CXCL1 production by astrocytes with ERβ ligand treatment. Representative 40x images of the ventral column of the thoracic spinal cord at peak EAE disease (Figure 9B) immunostained with GFAP (green), CXCL1 (white), IL-1β (red) and DAPI (blue). White boxes within the 40x images indicate areas of colocalization of GFAP, CXCL1 and IL-1β.

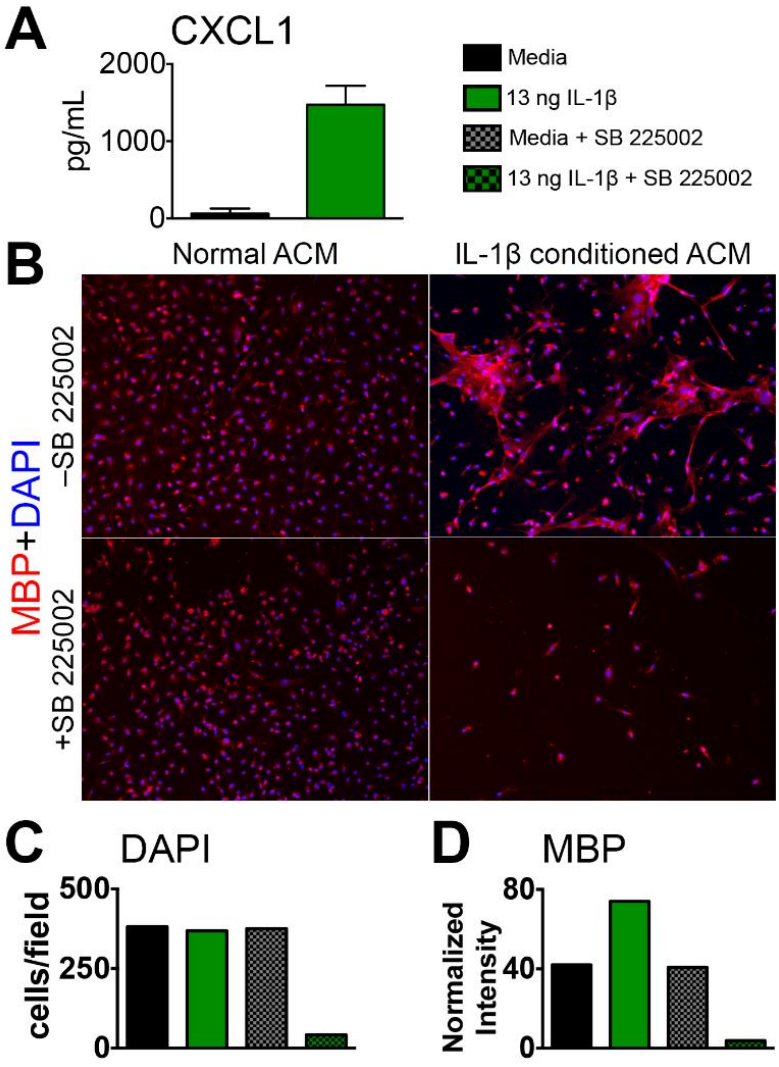

Figure 18. The effect of CXCL1 on primary OL survival and differentiation in vitro. (A) The amount of secreted CXCL1 (pg/ml) from astrocyte conditioned media (media, black) or from IL-1β treatment (green) was measured by ELISA. (B) Representative 10x images of *in vitro* OLs immunostained with MBP (red) and DAPI (blue) with astrocyte conditioned media (ACM) and IL-1β treated ACM, with and without CXCR2 antagonist, SB 225002. (C) Quantification of DAPI+ cells from (B) reveals no differences between ACM, IL-1β treated ACM with or without SB 225002. (D) Quantification of MBP intensity from (B) reveals an increase in MBP density with IL-1β ACM treatment compared to media alone. When media or IL-1β ACM is added to the culture containing SB-225002, there is no change exhibited in media alone, but a decrease in OL survival and differentiation with IL-1β treatment.

Chapter 4: Analogues of ERβ ligand chloroindazole exert immunomodulatory and remyelinating effects in a mouse model of multiple sclerosis

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Abstract

Pharmaceutical agents currently approved for the treatment of multiple sclerosis reduce relapse rates, but do not reverse or prevent neurodegeneration nor initiate myelin repair. The highly selective estrogen receptor (ER) β ligand chloroindazole (IndCl) shows particular promise promoting both remyelination while reducing inflammatory cytokines in the central nervous system of mice with experimental autoimmune encephalomyelitis. To optimize these benefits, we developed and screened seven novel IndCl analogues for their efficacy in promoting primary oligodendrocyte (OL) progenitor cell survival, proliferation, and differentiation *in vitro* by immunohistochemistry. Two analogues, IndCl-*o*-chloro and IndCl-*o*-methyl, induced proliferation and differentiation equivalent to IndCl and were selected for subsequent *in vivo* evaluation for their impact on clinical disease course, white matter pathology, and inflammation. Both compounds ameliorated disease severity, increased mature OLs, and improved overall myelination in the corpus callosum and white matter tracts of the spinal cord. These effects were accompanied by reduced production of the OL toxic molecules interferon-γ and chemokine (C-X-C motif) ligand, CXCL10 by splenocytes with no discernable effect on central nervous system-infiltrating leukocyte numbers, while IndCl-*o*-methyl also reduced peripheral interleukin (IL)-17. In addition, expression of the chemokine CXCL1, which is associated with developmental oligodendrogenesis, was upregulated by IndCl and both analogues. Furthermore, callosal compound action potential recordings from analogue-treated mice demonstrated a larger N1 component amplitude compared to vehicle, suggesting more functionally myelinated fibers. Thus, the *o*-Methyl and *o*-Chloro IndCl analogues represent a class of ERβ ligands that offer significant remyelination and neuroprotection as well as modulation of the immune system; hence, they appear appropriate to consider further for therapeutic development in multiple sclerosis and other demyelinating diseases.

Introduction

Multiple sclerosis (MS) is an autoimmune, demyelinating, and neurodegenerative disease of the central nervous system (CNS) with no known cause or cure. Experimental autoimmune encephalomyelitis (EAE) recapitulates the inflammation, demyelination, and neurodegeneration observed in MS and is among the most common inducible animal models of MS1. The EAE model has been used to develop many of the currently approved MS treatments, including interferon (IFN)-β, glatiramer acetate, fingolimod, and the anti- cluster of differentiation (CD) 20 monoclonal antibody, ocrelizumab[330,331]. However, although these therapeutics attenuate inflammation, they neither prevent neurodegeneration nor initiate remyelination.

Accumulating evidence indicates that estrogens are both neuroprotective and immunomodulatory, making them attractive candidates for the treatment of MS. Estrogens skew the inflammatory T helper (Th) 1 response prevalent in MS towards an anti-inflammatory Th2 profile[332,333]. Furthermore, in preclinical studies, treatment with pregnancy levels of the placenta-derived estrogenic hormone estriol attenuated EAE disease severity[250,334]. However, although they display immense potential for treating MS, endogenous estrogen therapy possesses several undesirable or deleterious side effects[308]. In addition to feminizing male recipients, treatment with endogenous estrogens increase the risk of developing breast and endometrial cancers in females[308]. Importantly, the carcinogenic effects of estrogens are mediated through estrogen receptor (ER)α and not ERβ, suggesting that therapies targeting specific ER subtypes may impart the benefit of estrogen treatment, while circumventing these side effects[335].

In support of this notion, chloroindazole (IndCl), a halogen-substituted phenyl-2H-indazole core with up to 100-fold relative binding affinity for ERβ over ERα[275], has demonstrated promise as an

immunomodulatory, pro-myelinating, and neuroprotective agent in mouse models of MS[277,289]. In C57BL/6 mice with EAE, IndCl attenuated disability scores and improved rotarod performance[276,277].

This was accompanied by reduced frequency of CNS-infiltrating CD45+ leukocytes and decreased production of inflammatory cytokines by antigen reactivated splenocytes[277,289]. Similarly, IndCl suppressed lipopolysaccharide or interleukin (IL)-1β-induced upregulation of inducible nitric oxide synthase, IL-1β, IL-6, and IL-23, in cultured human and murine microglia and astrocytes[276,315].

In addition to reducing inflammation, IndCl and other ERβ ligands act on oligodendrocytes (OLs) directly to support their proliferation, differentiation, and overall myelination activity[276,316]. Mice with EAE that received IndCl treatment showed increased myelin basic protein (MBP) and mature OL numbers in the spinal cord and corpus callosum (CC)[277,289]. Concomitantly, IndCl increased the number of actively dividing OL progenitor cells (OPCs) in the subventricular zone neurogenic niche and adjacent white matter lesions[277]. Critically, electrophysiological evaluation showed that the pro-myelinating effects of IndCl correlated with functional recovery, as compound action potential recordings from treated mice exhibited improved callosal axon conduction[277]. Additionally, unlike estrogens or ERα ligands, IndCl may directly oppose oncogenesis and is anti-proliferative in several disease models[336]. For instance, in a recent study, IndCl reduced inflammation and inhibited the establishment of endometrial lesions in a mouse model endometriosis[337].

In this report, we have investigated the therapeutic efficacy of IndCl analogues using cell culture, mouse behavior, functional electrophysiology, and CNS histology. In doing so, we have included for comparison some IndCl analogues in the hopes of discovering a remyelinating ERβ ligand that

would be suitable for pre-clinical development and transition from bench to bedside. Our interest in including IndCl analogues is supported by the rather different activity found between similar selective ERβ ligands such as diarylpropionitrile and IndCl[277]. To this end, we prepared and evaluated seven IndCl analogues that were modified to contain an additional substituent on the 2′ position of the 4′-hydroxyphenyl ring, and one having an additional chlorine substituent on the 4 position (Figure 19A). These seven analogues, all of which exhibited ERβ-preferential binding affinities, were initially screened in primary OPC cultures for survival, proliferation and differentiation. From this initial set, only two, IndCl-o-chloro (IndCl-o-Cl) and IndCl-o-methyl (IndCl-o-Me), showed activity comparable or superior to IndCl and were thus selected for in vivo testing in mice with EAE. Herein, we delineate their immunomodulatory and neuroprotective effects.

Results

Treatment with IndCl analogues stimulates differentiation of OPCs in vitro

Primary OPC cultures were used as a cell-based assay to characterize the differentiating effects of the new IndCl analogues and to select those most suitable for more extensive studies to be performed in comparison with the parent ligand, IndCl. Primary OPCs prepared from mouse neonatal cortex were treated with one of seven IndCl analogues or control at a concentration of 10 nM[293,338]. After 72 hours, cells were fixed and labeled with an antibody against MBP and the number of MBP+ labeled OLs were counted. (Figure 19 B and C)[275,276]. Treatment with IndCl, IndCl-o-Cl, IndCl-o-Me, and IndCl-o-I increased the number of MBP+ cells and branching processes, indicative of efficient OL differentiation as compared to vehicle-treated cultures (Figure 19 B and C). By contrast, the other 4 analogues did not affect cell differentiation as compared to vehicle-treated groups (Figure 19 B and C). The total number of cells in culture was not altered by any treatment (Figure 19 B and D).

IndCl analogues ameliorate EAE severity more effectively than IndCl and improve rotarod performance without affecting uterine weight

IndCl has been shown to reduce motor disability in EAE mice when administered prophylactically or therapeutically[277]. Having established that IndCl-o-Cl and IndCl-o-Me exhibited comparable effects to IndCl in vitro, their impact was next evaluated in vivo using eight-week-old female C57BL/6 mice in which EAE had been induced following an established protocol[287]. As a positive control for non-ER isoform specific estrogenic signaling, mice were given prophylactic E2 subcutaneously at the time of initial immunization with MOG35–55 peptide, which continued throughout the course of experiments (PreEAE + E2 group). All other groups received therapeutic daily subcutaneous doses of vehicle (PostEAE + vehicle), E2 (PostEAE + E2), IndCl

(PostEAE + IndCl), IndCl-o-Cl (PostEAE + IndCl-o-Cl), or IndCl-o-Me (PostEAE + IndCl-o-Me) that began at the onset of clinical symptoms (day 8; Figure 20A) or at peak disease (day 17; Figure 20B) and continued throughout the course of experiments. The timing of the different dosage regimens is illustrated schematically in Figure 20 A and B.

Disease course was greatly attenuated in mice that received prophylactic but not therapeutic E2 treatment, compared to those that received vehicle only, in which accumulating motor deficits appeared between post-immunization days 8–12 and persisted for the duration of experiments (Figure 20A). Both therapeutic IndCl-o-Cl and IndCl-o-Me, when administered at onset of clinical symptoms significantly reduced EAE clinical scores beginning at post-immunization day 23, roughly two weeks after treatment (Figure 20A). This is consistent with previously published reports using IndCl and other ERβ ligands, which demonstrated significant protective effects at later stages of disease[263,264,272,277]. IndCl and analogue treatment administered at peak disease (day 17), also reduced EAE clinical severity significantly compared to vehicle treatment (Figure 20B).

As a complementary assay of motor function, mice (from Figure 20A set) were tested on a rotarod device following a previously described protocol[277]. Normal mice and those that received prophylactic E2 did not fall off the rotarod within the time allotted, whereas vehicle and therapeutic E2 and IndCl treated mice had a tendency to fall from the cylinder abruptly. Both IndCl-o-Cl and IndCl-o-Me treatment improved rotarod performance compared to vehicle and IndCl by day 20 post-immunization with IndCl-o-Me treatment group exhibiting the greatest improvement in motor function (Figure 20C).

Estrogens increase uterine weight by acting primarily through ERα[339]. In order to determine whether analogues tested possessed ERα signaling properties that could contribute to the improved motor performance observed, uterine weight was assessed. As expected, prophylactic and therapeutic E2 treatment significantly increased uterus to bodyweight ratios (Figure 20D). In contrast, neither IndCl nor its analogues significantly increased this ratio (Figure 20D).

IndCl analogues increase myelination in spinal cord white matter during EAE

Treatment with IndCl or other ERβ ligands enhances axon myelination within CNS white tracts of mice with EAE[272,277,316]. To establish the pro-myelinating effects of the IndCl analogues tested, thoracic ventral column white matter (Figure 21B) was assessed for MBP immunoreactivity from mice treated during onset of clinical symptoms (day 8) or peak disease (day 17). Mice that received vehicle treatment showed significantly reduced MBP staining intensity as well as loss of NF200 + axons compared to normal, consistent with previous studies[183,277,287] (Figure 21A–D). Therapeutic IndCl, IndCl-o-Cl, and IndCl-o-Me treatment, either on the onset of disease or during peak disease, and prophylactic E2 treatment, increased MBP staining intensity and NF200+ axon numbers relative to vehicle. All IndCl ligands tested had comparable remyelinating effects when treatment was started early disease or peak disease (Figure 21A–D).

IndCl analogues modify peripheral cytokine and chemokine responses in EAE

During MS and EAE, peripherally activated leukocytes secrete inflammatory cytokines and chemokines as they migrate into the CNS, where they contribute to demyelination and axon damage[299]. To characterize the effects of IndCl analogues on the peripheral immune response, splenocytes were isolated from mice 21 days post-immunization (from Figure 20A set) and stimulated ex vivo with MOG35–55 peptide for cytokine and chemokine analysis using a magnetic

bead-based 20-plex cytokine/chemokine detection assay. Effects on cytokines related to inflammation, CD4+ T cell polarization, immune regulation, and chemokines associated with OL apoptosis and myelination that were measured in collected supernatants are presented below.

Pro-inflammatory Cytokines. As expected, splenocytes from vehicle-treated mice exhibited greater production of IFNγ, IL-2, TNFα, IL-6, IL-17 and IL-1β relative to normal. Prophylactic E2 reduced IL-2, IL-6, IL-17, and IFNγ concentrations, but had no effect on IL-1β or TNFα, whereas therapeutic E2 reduced IL-6, IL-17, and IFNγ only. IndCl and both the o-Me and o-Cl analogues decreased IFNγ concentrations in supernatants relative to vehicle, while decreased IL-6 production was observed in splenocytes of all treatment groups except for IndCl. IndCl-o-Me stood out among ERβ ligands tested as also reducing IL-17 production. None of the ERβ ligands included in this study affected IL-2 or TNFα. Interestingly, IndCl treatment alone led to increased IL-1β production compared to vehicle (Figure 22A).

Anti-inflammatory Cytokines. Skewing the adaptive immune response toward a Th2 profile, which is characterized by production of cytokines such as IL-4, IL-5, and IL-13, ameliorates EAE disability[311]. Therefore, concentrations of these cytokines, along with the key anti-inflammatory and immunoregulatory cytokine IL-10[340], in supernatants were assessed. Splenocytes from vehicle-treated mice exhibited increased IL-4 production compared to normal, but IL-10, IL-13, and IL-5 levels remained unchanged. Neither prophylactic nor therapeutic E2 significantly altered Th2 cytokine or IL-10 production relative to vehicle. Similarly, IndCl had no effect on cytokine concentrations. In contrast to IndCl, splenocytes from IndCl-o-Cl and IndCl-o-Me-treated mice exhibited attenuated IL-13 production compared to vehicle (Figure 22B).

Chemokines. CXCL1 and CXCL10 are leukocyte chemoattractants with critical, but largely divergent effects on OPC survival. CXCL1 signaling through its receptor, CXCR2, is essential for homeostatic white matter development, OPC proliferation, and survival[324]. In contrast, CXCL10 induces OPC cell death in vitro, which is augmented by the addition of IFNγ[324]. Splenocytes from vehicle-treated mice displayed no change in CXCL1, but significantly upregulated CXCL10 production compared to normal. Splenocytes from prophylactic and therapeutic E2 treated mice produced increased concentrations of CXCL1 and decreased CXCL10 relative to vehicle. Similarly, splenocytes from both IndCl and analogue-treated mice exhibited increased CXCL1 and decreased CXCL10 levels compared to vehicle-treated animals (Figure 22C).

IndCl analogue treatment does not affect leukocyte infiltration or astrogliosis in thoracic spinal cord white matter

IndCl has been shown to reduce several indicators of inflammation during EAE, including staining intensity of the pan-leukocyte marker CD45 and the degree of glial fibrillary acidic protein (GFAP)+ astrogliosis present in the dorsal column white matter[277]. To assess whether IndCl analogues exert similar anti-inflammatory effects, CD45+ leukocyte, and GFAP+ astrocytes were assessed in thoracic spinal cord dorsal column sections from normal and EAE mice sacrificed at day 21 (when treatment was started early EAE) and day 35 (when treatment was started peak EAE) postEAE.

Leukocytes. In mice given vehicle only, dorsal column white matter displayed extensive CD45+ infiltration into spinal cord parenchyma, with staining intensity significantly elevated relative to normal controls. Prophylactic and therapeutic treatment with E2, as well as therapeutic treatment

with IndCl and the analogues, either on the onset of disease or during peak disease, significantly decreased CD45+ staining intensity compared to vehicle-treated mice (Figure 23A and B).

Astrogliosis. GFAP+ staining intensity was significantly increased in dorsal column white matter from vehicle-treated mice, indicating widespread astrogliosis. In line with its effects on other measures of inflammation, prophylactic E2 significantly reduced GFAP+ staining intensity. By contrast, therapeutic E2, IndCl, or IndCl analogue treatment, either on the onset of disease or during peak disease, did not modify GFAP+ staining intensity at either of the postEAE time points (Figure 23C and D).

Therapeutic IndCl analogues enhance astrocytic CXCL1 expression during EAE

Under inflammatory conditions, such as those generated by MS, astrocytes undergo NF-κB-dependent upregulation of CXCL1, which is thought to recruit OPCs to the site of demyelinating injury[78,341]. Thus, having observed that IndCl raised its production by peripheral leukocytes, we next evaluated CXCL1 expression in thoracic ventral column white matter. Vehicle-treated mice exhibited CXCL1+ staining intensity was comparable to normal (Figure 23 C and E). In contrast with its effect on CXCL1 production by peripheral leukocytes, prophylactic E2 treatment caused no change in CXCL1 staining intensity relative to vehicle (Figure 23 C and E). However, there was a small but significant increase in CXCL1 intensity with therapeutic E2 treatment. Notably, IndCl, IndCl-o-Cl, and IndCl-o-Me treatment, either on the onset of disease or during peak disease, significantly increased ventral column CXCL1 staining intensity compared to vehicle, with staining intensity appearing to co-localize more extensively with GFAP+ astrocytes (Figure 23 C and E).

IndCl analogues upregulate astrocytic CXCL1 and stimulate OPC survival and differentiation in vitro

To determine the direct effect of IndCl analogues on CXCL1 production by astrocytes and its impact on OPC survival and differentiation, we utilized primary astrocyte and OPC/OL cultures. Primary astrocytes from postnatal day 0–4 pups were isolated and treated with 13 ng/mL IL-1β, which induces astrocytic CXCL1 production[78,289], or 13 ng/mL IL-1β concurrently with 10 nM IndCl-o-Cl or IndCl-o-Me for 48 hours. After stimulation, CXCL1 concentration was quantified in supernatant (astrocyte conditioned media; ACM) by ELISA (Figure 24A). Astrocytes treated with IL-1β, IL-1β + IndCl-o-Cl and IL-1β + IndCl-o-Me significantly increased CXCL1 production compared to untreated astrocytes (Figure 24A). 10 ng/mL exogenous CXCL1, which has been shown to increase OPC differentiation and survival in vitro[314], was added to primary OPC cultures as a positive control. Exogenous CXCL1 treated OPCs/OLs and those treated with ACM from IL-1β, IL-1β + IndCl-o-Cl and IL-1β + IndCl-o-Me groups significantly increased MBP expression compared to ACM alone. To test whether CXCL1 mediated this phenomenon, CXCR2, the high affinity receptor for CXCL1 which is expressed by OL lineage cells, was blocked by the selective CXCR2 antagonist, SB22500238. CXCR2 antagonism significantly decreased MBP staining intensity in all groups. Total number of DAPI+ nuclei were unaffected by the addition of SB225002 in all groups aside from those that received ACM from IL-1β only-treated astrocytes (Figure 24 B–D).

IndCl analogues increase mature OL numbers and restore myelination in callosal white matter tracts in EAE mice

IndCl and other ERβ ligands have been shown to increase white matter and subventricular zone OPC/OL populations and enhance callosal myelination in translational models of MS[260,277,292]. To

test whether IndCl analogues promote similar gains in mature OL numbers and myelination, callosal white matter tracts were assessed for adenomatous polyposis coli (CC-1) and MBP immunoreactivity, respectively. Additionally, ultrastructural analysis of the CC was performed by EM imaging to confirm the integrity of axon myelination.

Mature OLs. Vehicle-treated mice exhibited significant loss of CC1+ mature OLs relative to normal mice. Prophylactic E2 treatment did not show a decrease in CC1 cells; however, E2 treatment after disease induction was unable to rescue the decrease in CC1 cells. By contrast, therapeutic treatment with IndCl and IndCl analogues, either on the onset of disease or during peak disease, rescued the loss of CC1+ cells observed in vehicle-treated mice (Figure 25A and C).

MBP+ myelination. Corresponding with loss of CC1+ mature OLs, MBP+ staining was decreased in vehicle-treated mice relative to control. The presence of prophylactic E2 prevented the EAE-induced decrease in MBP+ staining, while therapeutic E2 was unable to rescue the decrease in MBP staining intensity as seen in vehicle-treated EAE CC (from Figure 20A set). Also, consistent with CC1 data, IndCl, IndCl-o-Cl, and IndCl-o-Me treatment all resulted in increased MBP+ staining with respect to vehicle (Figure 25 D and E).

EM analysis. Within a given field imaged, g-ratios were calculated by comparing mean ratio of inner axonal diameter to total outer diameter for all myelinated and non-myelinated fibers in the CC of groups of mice from Fig. 2A only. Roughly 50% of callosal fibers were non-myelinated or thinly myelinated in vehicle-treated mice compared to 10% in normal, resulting in a g-ratio that was significantly increased in vehicle-treated mice[183,292]. Prophylactic E2 reduced g-ratio relative to the vehicle-treatment level; however, therapeutic E2 did not decrease g-ratio significantly as

compared to vehicle-treated group. Treatment with IndCl or either analogue, by contrast, decreased both non-myelinated axons numbers and g-ratio relative to vehicle (Figure 26 A and B).

IndCl analogues improve fast and slow components of commissural axon conduction during EAE

Large white matter tracts, such as the CC, are especially vulnerable to demyelination and axonal damage in MS and EAE[183,342]. Compound action potential (CAP) recordings are a valuable technique for assessing demyelination and damage in these areas through their impact on functional conductivity[271,295,343]. Thus, callosal CAPs were recorded from Figure 20A set-normal, IndCl, pre-E2, IndCl-o-Cl and IndCl-o-Me-treated mice brain slices corresponding approximately to plates 29–48 in the atlas of Franklin and Paxinos (2004)[344] (Figure 27). N1 and N2 peak amplitudes (representing fast myelinated and slower un/partially myelinated fibers, respectively) were reduced in slices from vehicle-treated mice compared to normal but were not affected by prophylactic or therapeutic E2 treatment (Figure 27B–D). Slices from IndCl and analogue-treated mice showed significant improvement in N1 amplitude, but only IndCl-o-Cl treatment also increased N2 amplitude (Figure 27B–D).

Discussion

In search of therapeutic agents capable of reversing the progression of MS, we discovered that IndCl, a novel highly selective ERβ ligand, reduces CNS inflammation, promotes remyelination, and ameliorates disease in the EAE and cuprizone models of MS[277]. These findings prompted the current study, in which we sought to identify IndCl analogues optimized for these functions and to investigate the mechanism of their activity in greater detail. To do so, we examined the therapeutic efficacy of seven analogues of IndCl with single substitutions to the phenol ring (and one that was di-substituted), each of which retained selectivity for ERβ binding over ERα. After an initial OPC differentiation screening assay, we found that three analogues, IndCl-o-Cl, IndCl-o-Me, and IndCl-o-I had activity equivalent to that of IndCl itself, and from these, we selected the best two, IndCl-o-Cl and IndCl-o-Me, for subsequent in-depth evaluation of their effects in mice with EAE. These studies found that the IndCl analogues tested shared many therapeutic qualities in common with their parent compound, but also displayed several unique benefits not observed with IndCl treatment that speak to their promise for ultimate clinical utility (a summary of which can be found in Table 1). In these studies, none of the chloroindazole compounds demonstrated any apparent cellular or in vivo toxicities, while structurally they all conform to a pharmacophore model typical for ERβ-selective ligands[315,336]. IndCl itself has been extensively studied in cellular and in vivo models of endometriosis and was found to have good, ERβ-dependent efficacy with no apparent toxicities[337].

Common effects of IndCl and analogues

Consistent with past reports, treatment with IndCl or either of the two analogues tested improved parameters related to myelination and inflammation with respect to vehicle alone[276,277]. Examination of thoracic dorsal horn and CC white matter showed increased myelination and greater

numbers of mature callosal OLs in these groups, suggesting myelination induced by IndCl or its analogues was a product of preserving or replenishing OL populations. While the precise mechanism whereby IndCl analogues promote these changes remains uncertain, previous studies have found that IndCl and another ERβ ligand, diarylpropionitrile, produced similar effects on myelination through induction of PI3K/Akt/mTOR signaling[272,277,316].

Importantly, these findings corresponded with improvement in functional measures of myelin recovery. Ultrastructural analysis of callosal white matter revealed greater numbers of myelinated fibers and thicker myelin sheaths overall, indicating that IndCl and analogue-induced myelin production was correctly targeted to axons. Similarly, CAP recordings from these groups demonstrated improvement in N1 peak amplitude, suggesting more myelinated axons or a larger response from those present in the evoked fiber volley[271,345]. Consistent with previous reports examining ERβ ligands in EAE, these benefits were observed in the presence of ongoing cellular inflammation, denoted by the lack of effect on astrogliosis or leukocyte infiltration.

Examination of cytokines secreted by splenic leukocytes from these animals suggested this maybe partially due to suppression of IFNγ and CXCL10 production, both of which are potent mediators of OL death[318,324,346]. IFNγ is a major pro-inflammatory cytokine and is found in MS lesions as well as in activated blood mononuclear cells in progressive MS patients[347,348]. However, IFNγ may have a protective role in late EAE by regulating myelin debris removal by CNS antigen presenting cells[349]. Furthermore, low levels of IFNγ protected cultured OLs against oxidative stress, thus preventing their death[350]. IFNγ was significantly decreased by all ERβ ligands compared to vehicle, suggesting a role for these ligands in protecting OLs. CXCL10, also known as Interferon gamma-induced protein 10 (IP-10), an IFNγ dependent chemoattractant for T lymphocytes, is upregulated

in the cerebrospinal fluid and CNS lesions of MS patients[351]. Similar to what is observed with E2 and IndCl treatment during EAE, antibody-mediated systemic blockade of CXCL10 signaling has been reported to prevent recruitment of activated CD4+ T cells and diminished EAE severity[352]. IndCl and analogues significantly decreased CXCL10 levels in the periphery. Additionally, the induction of CXCL1 both in vivo within the periphery and CNS of mice treated with IndCl or its analogues as well as in vitro may play a role in promoting the pro-myelinating effects observed. Interestingly, CXCL1 upregulation in the spinal cord was noted in mice that received IndCl or analogue treatment, but not E2, suggesting that this effect may be antagonized by ERα signaling[289].

Although best known as a neutrophil chemoattractant[353], astrocyte-derived CXCL1 signaling through its receptor, CXCR2, on OPCs is essential for normal developmental myelination[120,354]. Several lines of evidence suggest CXCL1 may be harnessed for its therapeutic potential in the adult CNS. CXCL1+ astrocytes and CXCR2+ OPCs have been noted at the borders of active, but not silent, MS lesions where spontaneous myelination has been documented[78]. Additionally, CXCL1 contributes to OPC proliferation and migration[314], and CXCR2 signaling protects OPCs from IFNγ and CXCL10-induced apoptosis by increasing levels of the anti-apoptotic protein, Bcl-2 in vitro[324]. CXCL1 overexpression by GFAP+ astrocytes ameliorate EAE disease severity during late disease (day 30 onwards), similar to what is observed with ERβ ligand treatment[122]. IL-1β is associated with the pathophysiology of various inflammatory and demyelinating disorders[355,356]. Although IL-1β has been shown to be cytotoxic to mature OLs in vitro, it is crucial in CNS repair, as IL-1β−/− mice fail to remyelinate properly, possibly through the induction of astrocyte and microglia–macrophage-derived insulin growth factor-1[321]. We have demonstrated that ACM from IL-1β treated cultures induced CXCL1 expression which promoted OPC differentiation to MBP+ OLs. When CXCR2 is blocked with SB225002, we observed significant OL death, suggesting the

importance of CXCR2 in promoting OL survival and differentiation, as previously demonstrated[324]. However, when IndCl analogues were added in combination with IL-1β treatment, there was no significant difference in the number of OLs in the presence nor absence of SB225002, although MBP intensity is significantly reduced with SB225002 treatment. These results suggest that besides increasing CXCL1 production, which enhances OPC recruitment and differentiation, ERβ ligands may also skew the pro-inflammatory environment to one associated with myelin repair by promoting OL survival and myelination.

Together, these findings suggest that IndCl-based compounds stimulate functional remyelination by altering inflammatory responses associated with OL apoptosis, while upregulating cytokine programs involved in developmental myelination. Future studies will address whether the results described above represent lynchpins of the pro-myelinating functions of IndCl-family molecules and whether additional factors play a role in their therapeutic effects.

Differential effects between IndCl, IndCl-o-Cl, and IndCl-o-Me

While both IndCl and its two analogues improved myelination and modulated cytokine production associated with both demyelination and remyelination, key differences emerged in their impact on neurological disability, cytokine milieu, and electrophysiological measures. In contrast with previous reports, only IndCl-o-Cl and IndCl-o-Me reduced clinical disease severity, while IndCl did not significantly alter clinical disability (Figure 20A)[277,289]. Although it is unclear why IndCl performed differently, one source of variation comes from the earlier time point at which treatments were initiated in the present study. This may arise from IndCl's weak effect on leukocyte infiltration and CNS cytokine production. Earlier studies, initiated treatment during peak or chronic EAE, which features ongoing inflammatory leukocyte infiltration, but reduced cytokine production[357]. Initiation of IndCl therapy during the acute phase of EAE may have been less effective at

attenuating the prolific production of inflammatory cytokines characteristic of this time point[357]. However, IndCl, similar to IndCl-o-Cl and IndCl-o-Me, reduced clinical disease severity when they were administered during peak disease (Figure 20B), Additional study is required to determine how ERβ ligand signaling alters disease kinetics at earlier versus later stages of EAE.

Related to their effect on clinical disease, IndCl-o-Cl and IndCl-o-Me reduced production of cytokines related to Th17 differentiation. Th17 cells represent a CD4+ T cell population that are induced and activated by exposure to IL-1β, IL-6, IL-23, and transforming growth factor β[358]. In EAE and MS, Th17 cells exacerbate blood-brain barrier permeability, demyelination, and axon damage through release of factors that potentiate the cytotoxic properties of ongoing inflammatory processes[359]. Both IndCl analogues reduced peripheral IL-6 production, potentially contributing to the decreased IL-17 also observed with analogue treatment. No such decrease was seen in either parameter with IndCl. Given the much greater selectivity for ERβ over ERα exhibited by IndCl, it is possible this reflects weak partial ERα agonism by the analogues tested, due to their somewhat reduced ERβ binding selectivity (Figure 19A). Interestingly, IndCl treatment has shown to increase peripheral IL-1β production which we have previously shown is important for CXCL1 production and has a positive effect on myelination and immunomodulation[289].

In addition to reducing peripheral Th1 and Th17 cytokines, IndCl-o-Cl and IndCl-o-Me had the unexpected effect of also suppressing peripheral production of the Th2 cytokine, IL-13. Driving Th2 polarization is protective in EAE and MS, and elevated cerebrospinal fluid concentrations of IL-13 correlate with improved measures of neuronal integrity and cortical inhibition in MS patients in patients with MS. However, IL-13 also upregulates major histocompatibility complex II on monocytes, and global IL-13 knockout lowers susceptibility to EAE in female mice[360,361]. Thus, the consequences of its reduction in the current study warrant further investigation.

Among the IndCl-related compounds studied, IndCl-o-Cl displayed a potential benefit not observed with other treatments. Callosal CAP recordings revealed that in addition to improving the fast, myelinated component, IndCl-o-Cl also rescued slower conduction by small, unmyelinated, or partially myelinated fibers. As lower motor neuron loss and reduction of remaining neurites is a feature of similar EAE paradigms, this result suggests IndCl-o-Cl may exert neuroprotective effects that outstrip the other IndCl-based molecules included in this study.

Through our examination of the functional, histopathological, and immunological basis of the pro-myelinating effects of IndCl-based ERβ ligands, we have shown that two of the IndCl analogues tested exhibit therapeutic benefits exceeding their parent compound. While treatment with IndCl, IndCl-o-Cl and IndCl-o-Me resulted in enhanced myelination, IndCl-o-Cl and IndCl-o-Me improved neurological outcomes and suppressed inflammatory cytokine production better than their parent compound. Further support that modification of the base IndCl molecule differentially effects its impact on demyelinating disease is evidenced by IndCl-o-Cl uniquely demonstrating support of unmyelinated axon health in the form of improved N2 amplitude. Thus, IndCl itself, but even more so the two analogues IndCl-o-Me and IndCl-o-Cl, represent a class of ERβ ligands that offer potent remyelination and neuroprotection as well as modulation of the immune system that may be fine-tuned by additional refinement and substitution. The lack of any discernable side effects for the compounds we have thus far studied, and in other work for IndCl itself, is also of note[337]. For these reasons, this family of molecules appear appropriate to consider for further therapeutic development in the treatment of MS and other diseases affecting myelination and neurodegeneration.

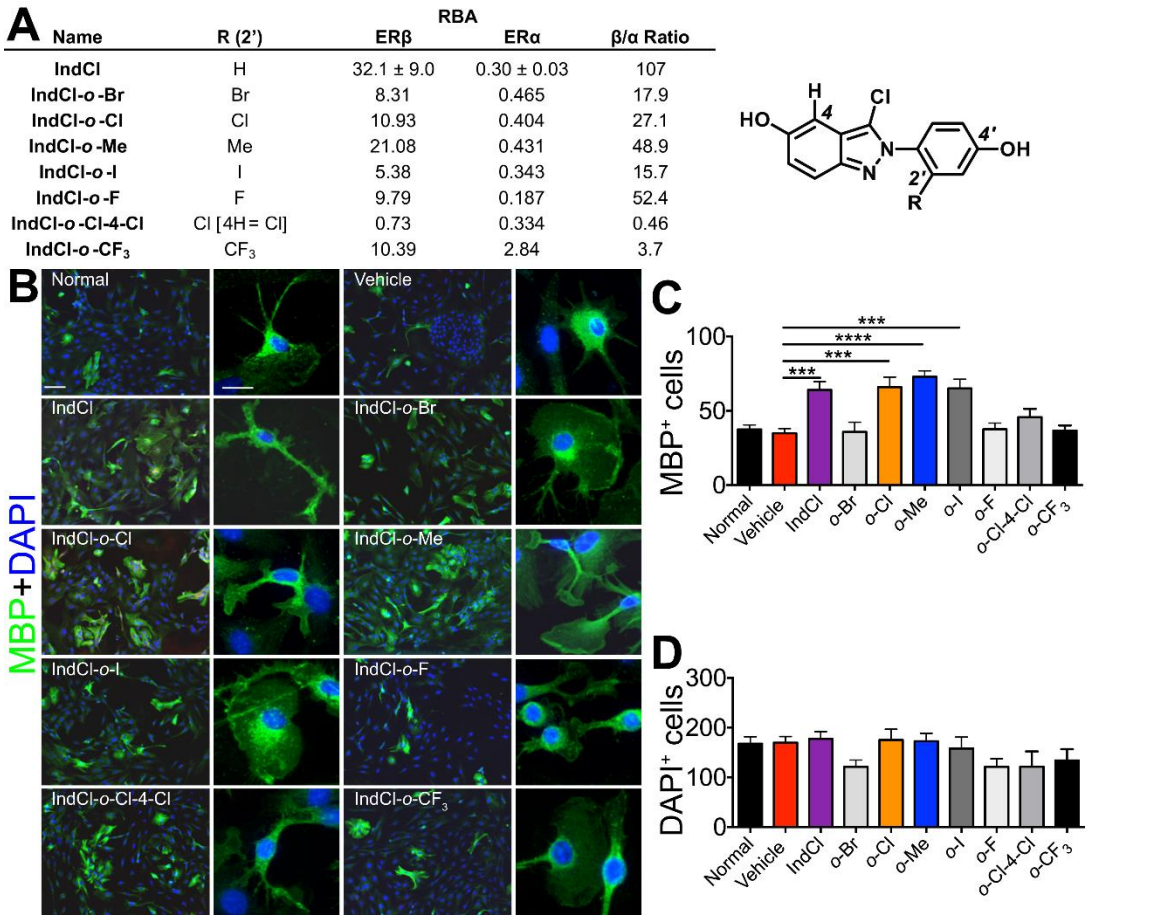

Figure 19. Estrogen receptor β (ERβ) ligand IndCl and analogue structure and effect on cell survival. (A) Estrogen receptor binding affinity for IndCl and seven analogues, IndCl-*o*-Br, IndCl-*o*-Cl, IndCl-*o*-Cl, IndCl-*o*-IMe IndCl-*o*-I, IndCl-*o*-F, IndCl-*o*-Cl-4-Cl and IndCl-*o*-CF3. (B) Some estrogen receptor β (ERβ) ligands increase primary mouse oligodendrocyte differentiation: Representative images of primary OPCs/OLs from wells containing differentiating media alone (normal media), vehicle, positive control IndCl, or the 7 different IndCl analogues. OLs were immunostained with myelin basic protein (MBP; green) and co-stained with nuclear stain DAPI (blue). (C-D) Effects of treatment on the number of MBP+ OLs and the number of total cells were quantified. Analogue IndCl-*o*-Cl, IndCl-*o*-Me and IndCl-*o*-I showed a significant increase in the number of MBP+ OLs with an increase in the percentage of branched OLs, compared to vehicle treated cells. No significant differences in total number of cells were observed between groups. There were 3 wells/treatment group. n = 3 independent experiments. One-Way ANOVA with Dunnett's multiple comparisons test. ***P < 0.001.

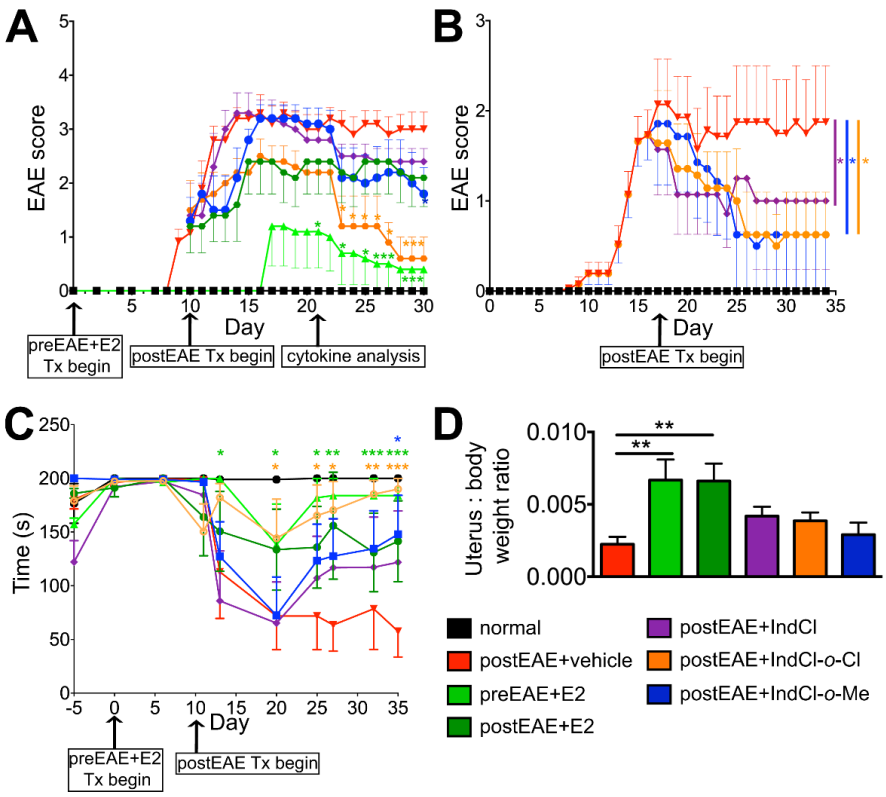

Figure 20. Therapeutic treatment with IndCl analogues ameliorates EAE disease, improves rotarod performance, and does not increase uterine weight. (A, B) Mice were immunized with MOG35–55. Normal mice did not receive MOG35–55 or treatment. (A) Therapeutic treatment with ERβ ligands, IndCl (5 mg/kg/d; purple), IndCl-*o*-Cl (5 mg/kg/d; orange) and IndCl-*o*-Me (5 mg/kg/d; blue) and 17β-estradiol (E2; 0.05 mg/kg/d; dark green) began at the onset of clinical disease (day 8) until day 30. Prophylactic E2 (0.05 mg/kg/d; light green) delayed onset of clinical disease. Vehicle-treated EAE mice (red) displayed onset of clinical disease symptoms between days 7–10, with disease severity peaking around day 15. During peak disease, IndCl (purple), IndCl-*o*-Cl (orange) and IndCl-*o*-Me (blue) treatment did not significantly affect EAE clinical symptoms, but decreased disease progression over time. (B) Therapeutic treatment with ERβ ligands, began at peak disease (day 17) and was continued daily till day 35. Vehicle-treated EAE mice displayed onset of clinical disease symptoms around day 9–10 with peak disease occurring on day 17. All ERβ ligands significantly attenuated clinical disease severity compared to vehicle treatment. One of two representative EAE experiments is shown. n = 8–10 mice/group, Two-Way ANOVA with Dunnett's multiple comparisons test. (C) To assess motor function, mice were subjected to the rotarod motor performance test. Vehicle-treated EAE mice displayed an abrupt and consistent decrease in time (seconds) remaining on the rotarod. While EAE mice treated with IndCl-*o*-Cl remained on the rotarod significantly longer indicative of improved motor function. Data are representative of experiments repeated three times. n = 8–10 mice/group, Ordinary One-Way ANOVA with Dunnett's multiple comparisons test. (D) Assessment of post-perfusion uterus to body weight ratios from normal and EAE mice treated with prophylactic E2 (dark green), or therapeutic E2 (light green), IndCl (purple), IndCl-*o*-Cl (orange) and IndCl-*o*-Me (blue). Both prophylactic and therapeutic E2 treated female mice showed a fourfold increase of uterus to body weight ratio with no differences between all other treatment groups. n = 8–10 mice/group, One-Way ANOVA with Dunnett's multiple comparisons test analysis, *P < 0.05, **P < 0.01.

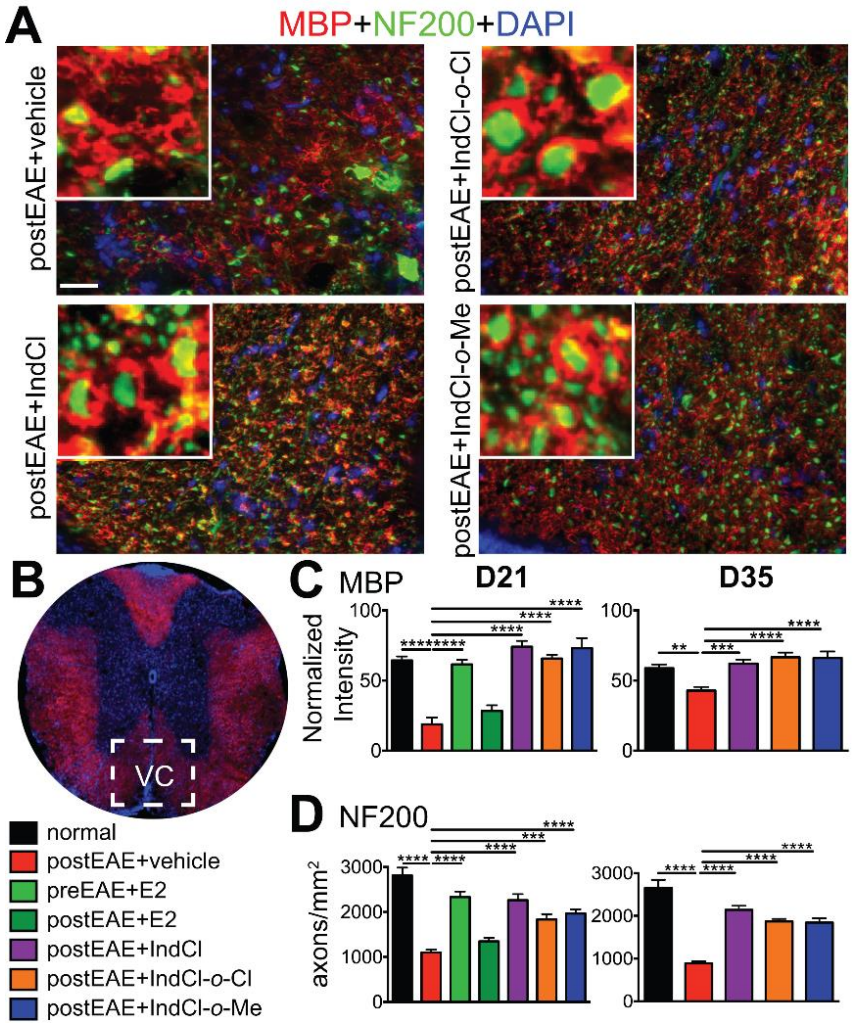

Figure 21. Therapeutic treatment with IndCl analogues improves myelination in the spinal cord of peak disease EAE animals. (A) Representative 40x magnification coronal images of the ventral column of thoracic spinal cord (area delineated by white square in 21B), showing axons stained with MBP (red), neurofilament 200(NF200; green) and nuclear DAPI stain (blue). Inset (white box) depicts zoomed in magnification images to show myelin wrapped axons. Scale bar represents 10 μM. (C,D) Quantification of MBP intensity and NF200 axons was performed at day 21 (Fig. 2A) and day 35 (Fig. 2B) postEAE. (C) Vehicle-treated EAE mice exhibited significantly decreased MBP intensity which was maintained at near normal levels with prophylactic E2 at day 21 and therapeutic IndCl, IndCl-*o*-Cl and IndCl-*o*-Me treatment at both time points. (D) Quantification of NF200 numbers reveals a significant decrease in the number of axons in the vehicle-treated mice. Prophylactic E2 treatment showed significant recovery of axons at day 21 with therapeutic IndCl, IndCl-*o*-Cl and IndCl-*o*-Me treatment exhibiting significantly increased NF200+ axon staining at both time points. n = 5–7 mice/ group, One-Way ANOVA with Dunnett's Multiple Comparisons Analysis, **P < 0.01, ***P < 0.001, ****P < 0.0001.

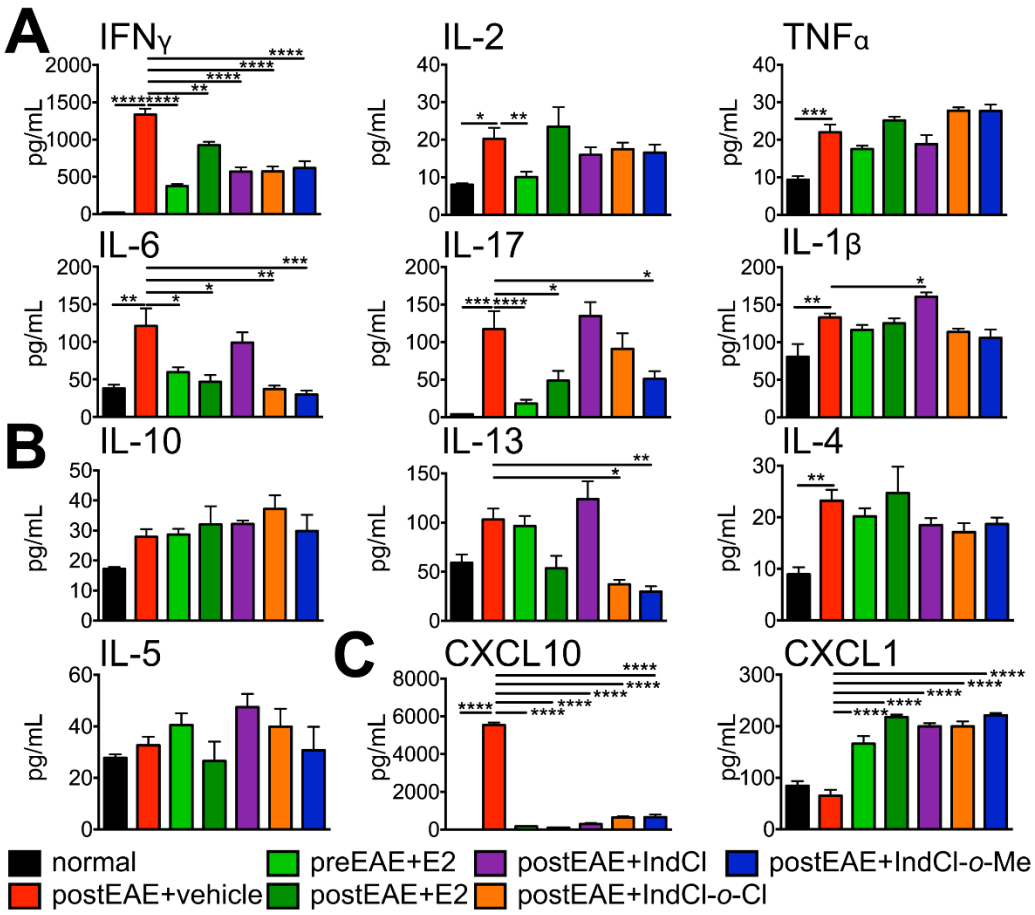

Figure 22. Therapeutic treatment with IndCl analogues decreases pro-inflammatory cytokine IFNγ, pro-inflammatory chemokine CXCL10 and increases chemokine CXCL1 production by peripheral immune cells during peak EAE disease. Cytokine production by MOG35–55-stimulated splenocytes was assessed from EAE mice culled on post induction day 21 (from Figure 20A set). (A) Vehicle-treated mice exhibited significantly increased levels of pro-inflammatory cytokines: IFNγ, IL-2, TNFα, IL-6, IL-17, and IL-1β compared to normal controls. Prophylactic E2 significantly decreased IFNγ, IL-2, IL-6 and IL-17 levels compared to vehicle, with therapeutic E2 decreasing IFNγ, IL-6, IL-17 levels. Therapeutic IndCl treatment significantly decreased IFNγ levels compared to vehicle. Treatment with IndCl-*o*-Cl significantly decreased IFNγ and IL-6, with IndCl-*o*-Me significantly decreasing IFNγ, IL-17, IL-6 levels compared to vehicle. (B) Anti-inflammatory cytokine production of IL-10, IL-13, IL-4, and IL-5 revealed no significant differences in any of the treatment groups compared to vehicle, except for IL-13 which significantly decreased in IndCl-*o*-Cl and IndCl-*o*-Me treated mice. (C) Vehicle-treated mice exhibited significantly elevated levels of CXCL10 compared to normal controls. Prophylactic E2 and therapeutic treatment with E2 and ERβ ligands significantly reduced CXCL10 levels compared to vehicle. Prophylactic and therapeutic treatment with E2 and ERβ ligands showed a significant increase in chemokine CXCL1 levels compared to vehicle. Data are representative of experiments repeated twice. n = 4–6 mice/group, Kruskal Wallis Analysis with Dunn's Multiple Comparisons Analysis and One-Way ANOVA with Dunnett's Multiple Comparisons Analysis, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

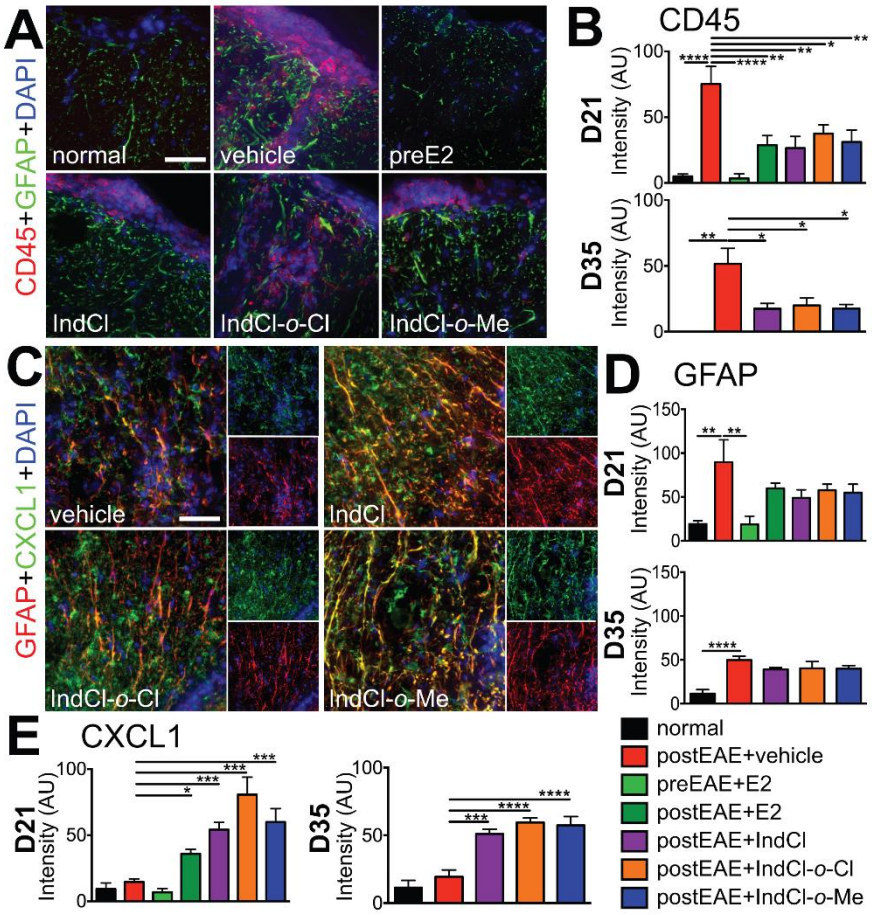

Figure 23. Therapeutic treatment with IndCl analogues does not decrease CNS inflammation, but increases CXCL1 production by astrocytes in the CNS. (A) Representative 40x magnification images of the spinal cord dorsal column reveals increased intensity of cluster of differentiation (CD)45 and glial fibrillary acidic protein (GFAP), in vehicle-treated EAE mice compared to normal control mice. (B) Prophylactic E2 (green) and therapeutic E2 (dark green) treatment decreased CD45 intensity at day 21 postEAE. IndCl (purple), IndCl-*o*-Cl (orange) and IndCl-*o*-Me (blue) significantly decreased CD45 intensity at both time points compared to vehicle-treated mice. (C) Representative 40x magnification coronal images of the ventral column of thoracic spinal cord collected at peak disease (day 21). Sections collected from vehicle, IndCl, IndCl-*o*-Cl and IndCl-*o*-Me were immunostained with chemokine (C-X-C motif) ligand 1 (CXCL1; green), glial fibrillary acidic protein (GFAP; red), and nuclear stain (DAPI; blue). Scale bar represents 10 μM for A&C. (D) Quantification of the relative fluorescence intensity of GFAP from normal, vehicle, prophylactic E2, therapeutic IndCl, IndCl-*o*-Cl and IndCl-*o*-Me treated EAE mice. Vehicle-treated mice exhibited increased GFAP fluorescence intensity at both time points that was significantly decreased only with prophylactic E2 treatment at day 21 ERβ ligand treatment with IndCl, IndCl-*o*-Cl and IndCl-*o*-Me exhibited similar degrees of intensity of GFAP as vehicle treated EAE mice. (E) Quantification of the relative fluorescence intensity of CXCL1 revealed a significant increase in CXCL1 intensity in therapeutic E2 at day 21 and therapeutic IndCl, IndCl-*o*-Cl and IndCl-*o*-Me at both time points as compared to vehicle-treated EAE mice. n = 5–8 mice/group, One-Way ANOVA with Dunnett's Multiple Comparisons Analysis, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

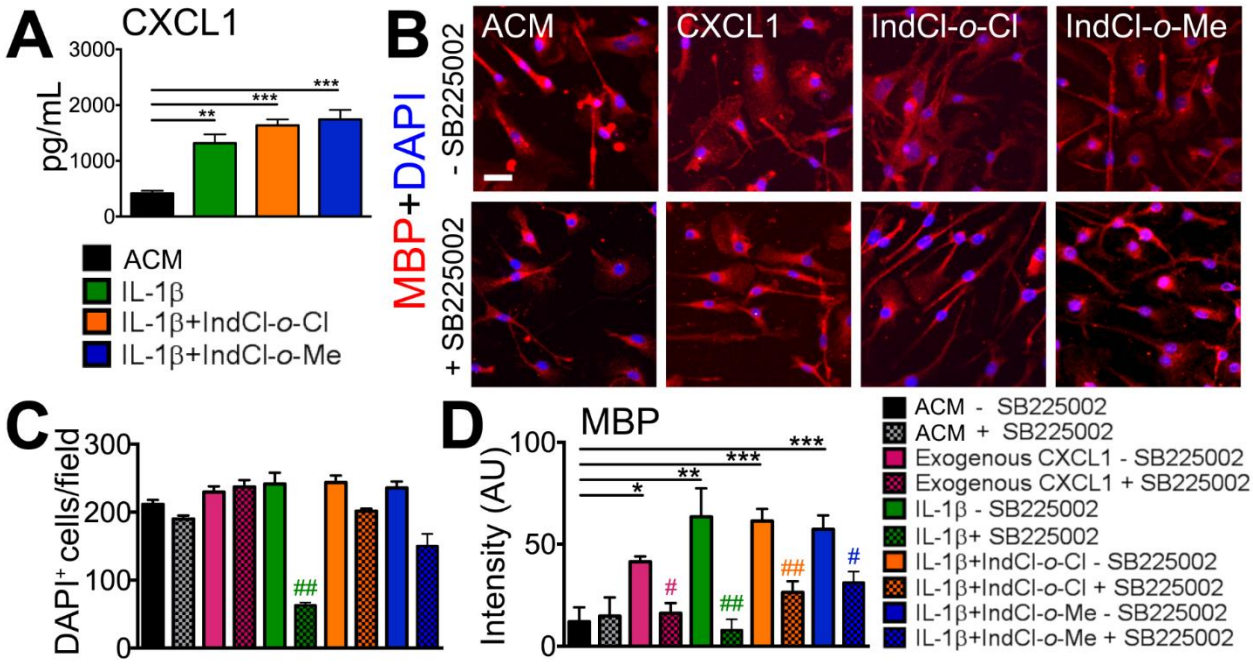

Figure 24. ERβ-induced astrocytic CXCL1 upregulation increases OL survival and differentiation. (A) Primary astrocytes were cultured with media alone, 13 ng/mL IL-1β, 10 nM IL-1β + IndCl-o-Cl, or 10 nM IL-1β + IndCl-o-Me for 48 hours, after which supernatant (astrocyte conditioned media; ACM) was collected and CXCL1 concentration (pg/ml) was measured by ELISA. Astrocytes treated with IL-1β, IL-1β + IndCl-o-Cl, or IL-1β + IndCl-o-Me significantly increased CXCL1 levels compared to untreated astrocytes. (B) Representative 20x images of primary OPCs cultured in the presence of ACM from (A) for 48 hours immunostained for MBP (red) and DAPI (blue) with and without 100 nM CXCR2 antagonist, SB225002. Scale bar represents 10 μM. (C) Quantification of DAPI + cells from (B) revealed a significant decrease in total cells with IL-1β treatment compared to ACM alone in the presence of SB225002 (# indicates significance between cultures treated with SB225002 vs those without). There were no significant differences between all other treatment groups. (D) Quantification of MBP intensity from (B) showed increased staining intensity in OPCs cultured with ACM from IL-1β, exogenous CXCL1, IL-1β + IndCl-*o*-Cl and IL-1β + IndCl-*o*-Me treated astrocytes compared to ACM alone. When ACM from IL-1β, exogenous CXCL1, IL-1β + IndCl-*o*-Cl and IL-1β + IndCl-*o*-Me treated groups was added to the cultures containing SB225002, there was no change exhibited in ACM alone, but a decrease in OL differentiation was observed in all treatment groups. n = 3 wells/group, One-Way ANOVA with Dunnett's Multiple Comparisons Analysis and unpaired t-test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

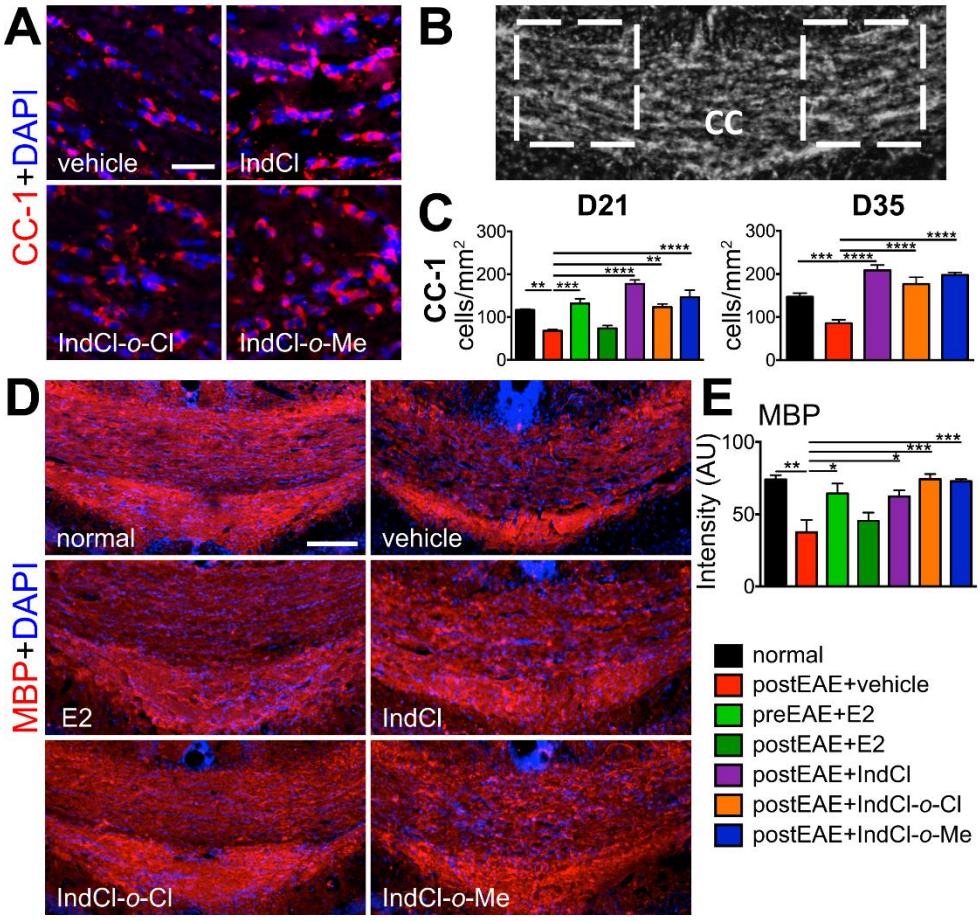

Figure 25. Therapeutic treatment with IndCl analogues improves the number of mature oligodendrocytes and increases myelin intensity. Mature OLs numbers (A–C) and myelination levels (D-E) were assessed by staining for adenomatous polyposis coli (CC-1; red) and myelin basic protein (myelin; red) from day 21 and day 35 postEAE groups. White-dashed boxes within the normal CC (B), which depict areas examined at 40X magnification in (A), reveal a significant increase in numbers of mature OLs (red) in therapeutic IndCl, IndCl-*o*-Cl and IndCl-*o*-Me treated EAE mice at both time points (C). (D-E) To assess myelination levels within the CC, representative 10X magnification images of midline-crossing CC from coronal brain sections (from Figure 20A set) stained for MBP; red is shown (D). All treatments, except therapeutic E2, improved MBP+ intensity (E). n = 8 mice/group, One-Way ANOVA with Dunnett's Multiple Comparisons Analysis, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Scale bar represents 10 μM for A and 100 μM for D.

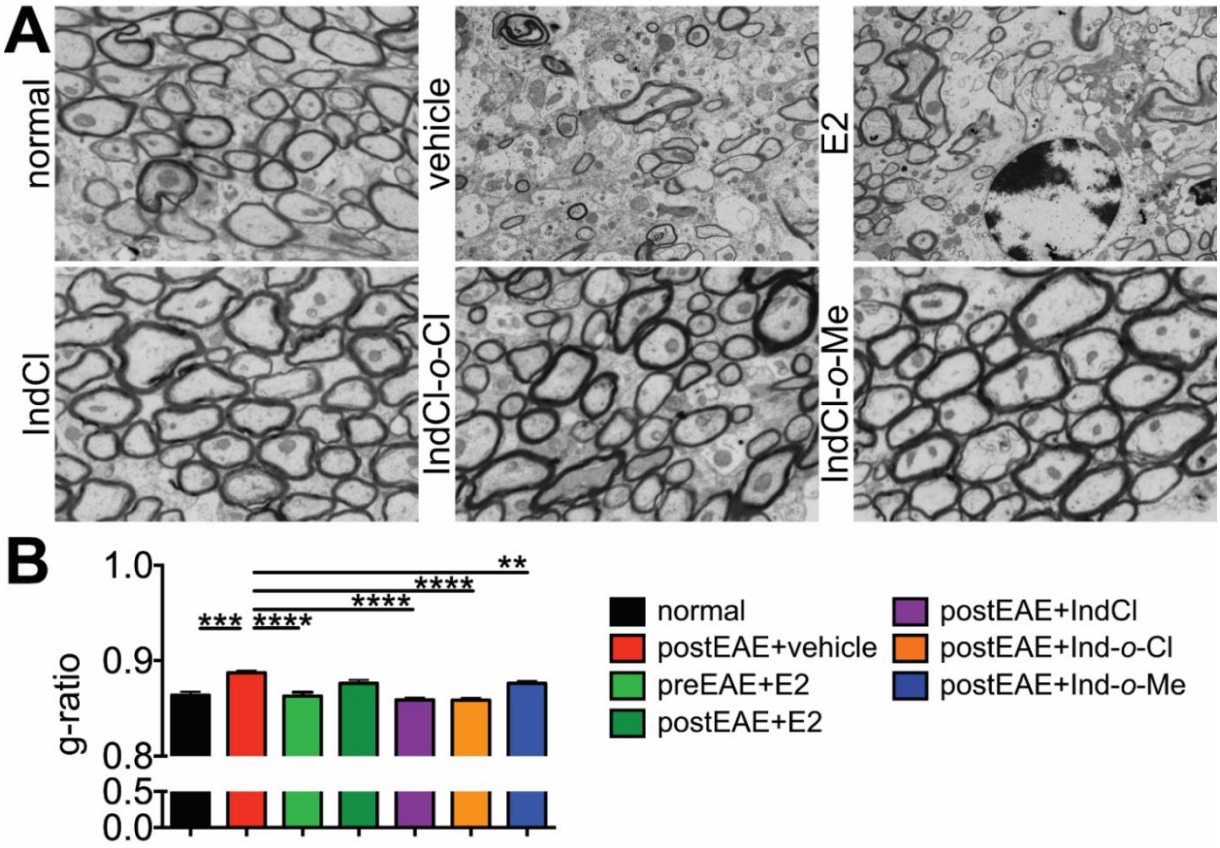

Figure 26. Improvement of axon myelination in IndCl analogues-treated EAE corpus callosum. (A) Representative electron micrographs of CC axons imaged at 14,000× magnification (brains from groups of mice from Figure 20A set), (B) Prophylactic E2 reduced g-ratio relative to the vehicle-treatment level; however, therapeutic E2 did not decrease g-ratio significantly as compared to vehicle-treated group. Treatment with IndCl or either analogue, by contrast, decreased both non-myelinated axons numbers and g-ratio relative to vehicle. A minimum of 500 axons were measured per mouse. Scale bar represents 1 μM. n = 4–8 mice/group, One-Way ANOVA with Dunnett's Multiple Comparisons Analysis, **P < 0.01, ***P < 0.001, ****P < 0.0001.

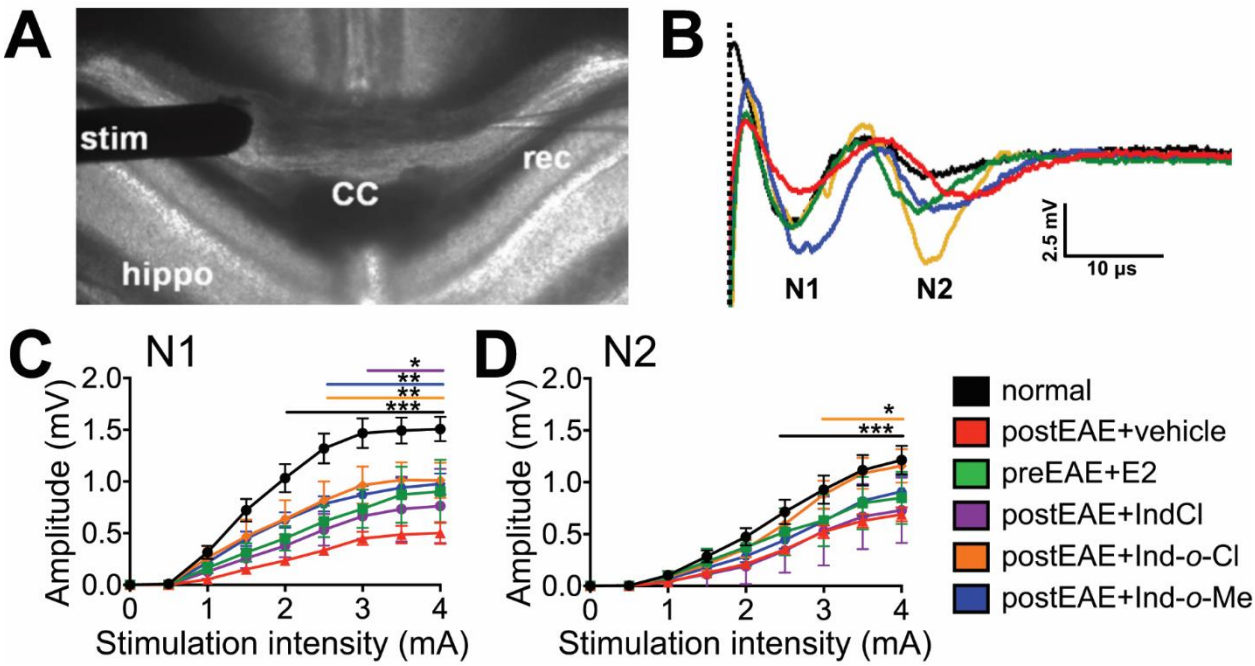

Figure 27. Treatment with novel estrogen receptor beta ligands increases EAE-induced callosal conduction. (A) Compound action potentials (CAPs) were recorded across the corpus callosum (CC) in caudal brain slices (350-µm thick) plate 48–55 (Paxinos and Franklin atlas, 200441) containing the hippocampus (hippo). A recording electrode (rec) was placed 1 mm away from a bipolar stimulating electrode (stim), and voltage traces were recorded with increasing current stimulus of 0–4 mA in steps of 0.5 mA. (B) Voltage traces acquired with 4 mA stimulation intensity from normal (black), 30 days postEAE + vehicle (red), preEAE + E2 (green), postEAE + IndCl-*o*-Cl (orange), postEAE + IndCl-*o*-Me (blue), and postEAE + IndCl, (purple) brain slices (from Fig. 2A set). Dashed line indicates the end of the stimulus artifact and the beginning of the CAPs. The faster myelinated axon peak is indicated by "N1", and the CAP component "N2" denotes the slower partially myelinated or unmyelinated axons peak. N1 (C) and N2 (D) CAP amplitudes of callosal axons recorded from vehicle-treated EAE slices (red) were significantly smaller than in normal controls (black). Similar to IndCl (purple), IndCl-*o*-Cl (orange) and IndCl-*o*-Me (blue) treatment resulted in improved N1. N2 amplitudes of IndCl-*o*-Cl had a small but significant increase as compared to postEAE + vehicle group. n = 6–12 animals per group, Two-Way ANOVA with post hoc tests using Tukey's multiple comparison test, *P < 0.05, **P < 0.01, ***P < 0.001.

Chapter 5: Discussion and Future Directions

The work presented in this thesis was begun to examine the role of ERβ ligands in promoting remyelination by modulating the immune system, in particular altering levels of pro-inflammatory cytokines and activating CXCL1/CXCR2 signaling. The most widely used mouse model was utilized; EAE consists of an immune mediated event that is followed by demyelination and eventual neurodegeneration. In EAE disease, ERβ ligand treatment stimulated an environment where OPCs were able to survive, and differentiate into mature remyelinating OLs, thus making them promising candidates for MS and other autoimmune demyelinating diseases.

The MS treatment pipeline has made significant progress within the past 26 years with more efficacious treatment options available. Current therapeutic treatments for MS have been focused mainly on modulating or suppressing the immune response which may slightly reduce the relapse rate and delay disease progression. However, the rate of progressive disability and early mortality remains alarming[362]. An unmet need is the development therapies promoting myelin repair, thus, several remyelination drugs are currently under clinical development[363]. The development of selective estrogen receptor modulators (SERMs) may be especially beneficial while minimizing adverse effects to achieve the dual immunomodulation and neuroprotective benefits of estrogens. An ideal SERM would be an ERβ ligand. Treatment with ERβ ligands have demonstrated neuroprotection in chronic EAE disease, promoting OL survival, sparring axons and myelin, with minimal effects on immune response[263,364,365]. Additionally, through high throughput drug screening assays, SERMs have been identified as compounds that promote OL maturation and myelination [366].

Given the robust effects of ERβ ligand treatment on EAE, an effort was made to determine what cell type(s) mediate ERβ ligand neuroprotective effects. ERβ is expressed on a variety of cells including neurons, oligodendrocytes, dendritic cells, microglia, astrocytes, and T cells and epithelial cells[264,272,309,316,367–372]. In many animal models of MS, ERβ ligand treatment is neuroprotective, but the mechanisms behind this neuroprotection remains unclear. Conditional knockout of ERβ in OL lineage cells prevented ERβ ligand induced attenuation of clinical disease and myelination as well as prevented activation of the PI3K/Akt/mTOR pathway[316], suggesting that ERβ-ligand induced neuroprotection is mediated through direct action of ERβ on OL lineage cells. ERβ deletion from astrocytes or neurons, however, did not mediate ERβ ligand improvement on EAE disease or myelination[372]. Furthermore, when ERβ was removed from CD11c+ dendritic cells, the protective effect of ERβ ligand treatment during EAE was lost[368]. CXCL1 plays an important role in myelination and is predominately expressed in activated astrocytes,[78,354,373,374] (Figure 28). Thus, a conditional knockout, in which ERβ was removed in GFAP+ astrocytes, was created to determine if ERβ is required on astrocytes to mediate neuroprotection via the CXCL1/CXCR2 pathway during EAE.

*Selective deletion of ERβ from astrocyte*s

To determine whether upregulation of CXCL1 by astrocytes is due to ERβ direct action on these cells, ERβ was selectively ablated from astrocytes using mice that express Cre recombinase under the control of the mouse GFAP promoter that express Cre recombinase under the control of the mouse GFAP promoter (mGFAP-Cre line 77.6[375] mated to mice with loxp sites flanking the Esr2 (ERβ) gene, thereby generating GFAP+ astrocyte-specific ERβ-/- (GFAP:ERβ-cKO) Tg mice (Figure 29A). Tissue samples acquired from these mice revealed homozygous $ERβ^{fl/fl}$ (loxP/loxP; floxed), and heterozygous $ERβ^{fl/+}$ subjects at 230-, and both 230- and 180-bp fragment,

respectively. Presence of GFAP-specific Cre recombinase gene was detected by a ~400 –bp fragment while a ~ 200 bp fragment represents an internal positive control. To verify gene deletion in GFAP+ astrocytes, immunohistochemistry was performed. A representative section of spinal cord white matter revealed lack of ERβ expression on GFAP+ astrocytes in GFAPCre+,ERβ$^{fl/fl}$ mice compared with robust ERβ expression in GFAPCre-,ERβ$^{fl/fl}$ (Figure 29B).

Deletion of ERβ in astrocytes does not affect IndCl-induced improvement in EAE clinical disease

Active EAE was induced in GFAP:ERβ-cKO and littermate control mice using a previously described protocol[287]. Within each group, mice were treated with IndCl, or vehicle beginning at 16 days post EAE induction. Mice were scored daily according to the standard EAE grading scale[287]. Normal mice did not display EAE clinical symptoms, while in both Tg and littermate control groups, vehicle treated mice exhibited clinical symptoms beginning on day 11-12, with peak disease at around day 16. Treatment with IndCl began on day 16 until day 35 and significantly attenuated disease severity over time in both cKO and littermate control groups, suggesting that ERβ on astrocytes is not required for EAE clinical protection, as previously described[372] (Figure 30).

Deletion of ERβ in astrocytes does not alter IndCl-induced upregulation of CXCL1 on astrocytes in the CNS

CXCL1 expression was examined in thoracic ventral column white matter from mice at day 35 post EAE induction by immunohistochemistry. Compared with vehicle, IndCl treated GFAP:ERβ-cKO mice and control mice exhibited increased CXCL1+ immunoreactivity that appeared to overlap with GFAP. To quantify reactive astrogliosis and microgliosis, GFAP and Iba1, respectively, was assessed. Quantification of GFAP+ and Iba1+ staining intensity revealed no significant differences

between vehicle and IndCl treated GFAP:ERβ-cKO and control EAE mice (Figure 31). Together, these findings suggests that IndCl acts on another cell other than astrocytes to regulate chemokine CXCL1 production.

De*letion of ERβ in astrocytes but has no effect on mature OL populations*

Myelination and mature OL numbers were examined in the corpus callosum of all EAE mice by immunohistochemistry. IndCl treatment of GFAP:ERβ-cKO and littermate control EAE mice rescued the decrease in CC-1+ cells observed in vehicle treated mice. Myelination, as indicated by MBP+ immunoreactivity was also rescued with IndCl treatment in both genotypes (Figure 32). Interestingly, IndCl treated GFAP:ERβ-cKO mice displayed reduced MBP+ immunoreactivity compared to IndCl treated littermate controls, suggesting that IndCl may partially act on astrocytes to enhance myelination.

Conclusions

The complex immune mediated attack on OLs during demyelination is often accompanied by spontaneous remyelination by OPCs[376]. Estrogens and SERMs, exert neuroprotective effects in EAE when signaling through ERβ. ERβ ligands decrease clinical disease, demyelination and axonal loss in EAE[263,264,292]. The mechanism by which these ligands induce neuroprotection are still under investigation. However, recent evidence from our group suggests that ERβ ligand neuroprotection may involve the pro-myelinating chemokine CXCL1. (Karim et al 2018; Karim et al 2019). Uncovering how ERβ ligands modulate CXCL1 production is of vital importance. In the present study, we examined the impact of ERβ deletion in astrocytes, specifically in modulating CXCL1 production in ERβ ligand treated EAE mice. We found no differences in EAE severity, CXCL1 upregulation, or CC-1 mature OL number between IndCl treated GFAP:ERβ-cKO and littermate

controls. However, in contrast to IndCl treated littermate controls, IndCl treated GFAP:ERβ-cKO mice displayed a slight but significant reduction in MBP+ immunoreactivity in the corpus callosum. Together, these finding suggest that ERβ acting on other cell types including neurons, oligodendrocyte or immune cells may be responsible for ERβ ligands neuroprotective and remyelinating effects. This is particularly important as CXCL1 expression by astrocytes requires IL-1β and TNFα induced activation of NF-κB/STAT1 signaling pathway[126,377,378]. IL-1β and TNFα are primarily expressed by microglia and macrophages and are important mediators in regulating early inflammation and repair after injury[378–382]. Thus, these findings do not exclude additional effects of ERβ ligands mediated by ERβ in other cell types including microglia and macrophages.

Importantly, in various studies, ERβ ligands demonstrated neuroprotection even in the presence of ongoing inflammation, indicating that ERβ may act on multiple cell types to exert its beneficial effects. This may be partially due to suppression of Th1, Th17 and microglial and astrocyte cytokine and chemokine production including IL-6, TNFα, and IL-17[276,277]. Specifically, our studies demonstrate IndCl immunomodulatory effects by suppressing IFNγ and CXCL10 expression, both of which are potent inducers of OL apoptosis[318,324]. Together, these results suggest a role for ERβ-selective agonists like IndCl in modulating inflammation and exerting neuroprotective effects.

Experimental demyelination and remyelination in other murine mouse models of MS

EAE is the most extensively studied mouse model of MS. It is a useful model of CNS inflammation and demyelination. EAE can also be induced by the use of adjuvant and synthetic myelin peptides to mimic certain subtypes of MS disease, making murine EAE the most relevant model of MS. The chronic $MOG_{35–55}$-EAE model is capable of recapitulating aspects of all three subtypes of MS. RR-

MS is the most common form of the disease, accounting for 85% of MS patients, and is marked by acute episodes of disability followed by recovery[383]. The onset stage of $MOG_{35–55}$-EAE can serve as a model of these early relapses and offers the opportunity to monitor possible key effectors in MS progression and test interventions prior to permanent CNS damage. Typically, RRMS patients progress to a chronic disease known as SPMS, during which they develop permanent motor and cognitive impairments[384]. A third subtype, PPMS, affects 15% of patients[384] and presents with a chronic disease course at onset, devoid of remittances. As such, the chronic nature of $MOG_{35–55}$-EAE recapitulates the permanent damage observed in both SPMS and PPMS. Thus, the development of $MOG_{35–55}$-EAE has provided an invaluable model for studying both the progression and treatment of multiple clinical forms of MS. Examples of how EAE has played a critical role in elucidating MS pathology include the identification of the aryl hydrocarbon receptor (AHR) as a ligand-dependent transcription factor needed for the development of Th17 and T regulatory (Treg) responses, and the discovery of retinoid related orphan receptor gamma (RORγ) as a critical transcription factor for Th17 cell development[385,386]. Additionally, multiple therapeutics, including glatiramer acetate, an amino acid copolymer[188], and natalizumab, an antibody against the adhesion molecule α4β1-integrin (very late antigen 4 (VLA-4), CD49d/CD29)[190,213], demonstrated efficacy in EAE models prior to proceeding to clinical trials. It has also been reported that all currently-approved MS treatments reduce EAE symptoms to a certain extent[387].

Inflammation and demyelination are pathological hallmarks of MS. To identify new therapeutic strategies, understanding the mechanisms involved in regeneration must be thoroughly investigated[388]. Although EAE is an excellent model for recapitulating MS, it is often difficult to separate the complex inflammatory aspect from repair and remyelination responses[330]. Thus, toxin induced MS models may be used to provide this information. The most frequently used model of

toxic demyelination is induced by exposure to cuprizone (CPZ) as it produces a global insult, unlike lysolecithin and ethidium bromide which result in focal demyelination[389]. Administration of 0.2-0.5% (w/w) of CPZ in standard rodent chow induces oxidative stress in OLs resulting in their death and consequent demyelination[390–392], with removal of CPZ diet resulting in spontaneous remyelination[393,394]. During CPZ-induced demyelination, microglia and astrocytes are activated and OLs undergo apoptosis as little as 3-7 days post CPZ exposure[85,395], with prominent demyelination of the corpus callosum, occurring 3-5 weeks post CPZ exposure[396,397]. Concurrently with demyelination, rapid spontaneous remyelination proceeds, regardless whether CPZ is present or not [201,397,398]. In this model, demyelination and robust remyelination is more predictable, consistent and reproducible[389,399]. However, CPZ induces demyelination by directly killing mature oligodendrocytes in the rodent brain, with no effect in the spinal cord and without involvement of the adaptive immune system [392,400]. As inflammation if necessary for successful remyelination[401,402], the CPZ model is unlikely to mimic the complex pathology of MS in humans. Nonetheless, several studies have used CPZ induced demyelination to investigate the therapeutic neuroprotective and remyelinating properties of estrogens without the interference of the multifaceted immune response in EAE. Estradiol treatment of five and eight-week CPZ fed male mice prevented OL loss and demyelination in the corpus callosum[403,404]. Additionally, IndCl treatment of nine week CPZ fed male mice improved remyelination and increased OL numbers[277], suggesting that IndCl is capable of enhancing myelination in both innate and adaptive inflammatory demyelinating models such as EAE and primary innate demyelination models such as CPZ, rendering IndCl a promising treatment for MS and other autoimmune demyelinating disorders.

Signaling components of CXCR2

CXCL1/CXCR2 mediates its signaling via ERK, JAK/STAT and PI3K pathways. Addition of exogenous CXCL1 to in vitro human fetal OPCs stimulated OPC proliferation and activation of the ERK1/2 pathway, primarily in astrocytes[314]. CXCL1/CXCR2 signaling has been widely described for its roles in immunity, cancer and CNS development. The interaction of CXCL1, secreted by astrocytes with CXCR2 on OL lineage cells has been shown to induce proliferation, migration and differentiation of OPCs and to prevent their apoptosis. Additionally, CXCR2 neonate deficient animals showed a reduced number of differentiated OLs, as well as ectopic positioning of OPCs[354]. Adult mice deficient in CXCR2 exhibited a decrease in spinal cord white matter, reduced thickness of myelin, with decreased levels of MBP, and PLP[312]. Furthermore, CXCR2 signaling protects against OPC apoptosis by impairing pro-apoptotic proteins and elevating expression of anti-apoptotic protein Bcl-2[324], also seen in the OPC/OL rich corpus callosum of EAE mice treated with IndCl[289,290]. Signaling through CXCR2 also protects OPCs from both IFNγ and CXCL10 mediated apoptosis[324], suggesting that CXCL1/CXCR2 signaling is important for normal OL distribution and differentiation in the neonate and adult mouse. Similar decreases in IFNγ and CXCL10 in IndCl and analogue treated EAE mice is observed. Although CXCL1/CXCR2 is vital for OL development and repair, several studies have demonstrated its role in the onset of MS and EAE disease[405,406]. Overexpression of CXCL1 in mature OLs induced neutrophil infiltration and astrogliosis[407]. Similarly, overexpression in astrocytes induced neutrophil activation associated with increased clinical severity and histologic disease in acute EAE[408]. In contrast, inducible astrocytic CXCL1 overexpression attenuated clinical severity as well as increased OLs associated with enhanced remyelination during chronic EAE disease[122]. The difference in results in both studies is most likely due to differences in methodology, including the duration of EAE disease (acute vs chronic), and *cxcl1* transgene employed (sustained vs transient). CXCR2+ neutrophils are often the first set of

cells to migrate in the region of injury in response to local expression of CXCL1. These cells potentiate the inflammatory response and secrete mediators that activate leukocytes and exacerbate disease. Localized inhibition of CXCR2 signaling reduced the number of infiltrating leukocytes into lesion areas, thus reducing lesion size and enhancing myelination[409]. These studies suggest that CXCR2 signaling is a major contributor to immune mediated demyelination. A possible explanation for the incongruity of these results may be due to chemokine concentration and receptor desensitization[410,411]. For example, elevated levels of CXCL1 may lead increased leukocyte infiltration into the CNS with a transient increase in OPC proliferation, while sustained CXCL1 expression may lead to desensitization of CXCR2, negating OPCs responses but also reducing leukocyte infiltrating, promoting functional recovery in these models. While the literature is conflicted regarding the role of this chemokine in demyelinating disease, ERβ ligands may reprogram the response of various CNS resident and leukocytic populations to emphasize the pro-myelinating qualities of this pleiotropic molecule. However, as ERβ ligands modulate production of various inflammatory molecules, including IFNγ and CXCL10[289,290], further studies are necessary to fully elucidate the role of the CXCL1/CXCR2 signaling pathway in ERβ ligand-induced remyelination (Figure 33).

Future Studies

Future studies are needed to assess if CXCL1 induction in astrocytes is a key component of the mechanism by which ERβ ligands promote remyelination. In this work, ERβ ligands upregulated CXCL1 production by astrocytes which indirectly led to increased OL survival and OPC differentiation. To test the extent that CXCL1 contributes to ERβ ligand-induced myelination, CXCL1 will be selectively ablated from $GFAP^+$ astrocytes to generate GFAP:CXCL1-cko mice. After successful recombination, GFAP:CXCL1-cko mice will be treated with various ERβ ligands

to examine the direct or indirect role of CXCL1 and ERβ ligands on repair and remyelination. As astrocytic CXCL1 deletion may interfere with normal OPC/OL recruitment, positioning and differentiation, compact myelin surrounding axons will be severely decreased in GFAP:CXCL1-cko mice that receive vehicle, with ERβ ligands slightly improving axon myelination but with variable myelin thickness and potential abnormal myelination. In vitro primary OPC and OL cultures will be used as confirmation of in vivo results. Furthermore, CXCR2 is the high affinity receptor for CXCL1 and is essential for normal myelination during development, as well as promotes OPC survival in response to inflammatory cytotoxic insult. Thus, future studies will investigate the role of CXCR2 signaling on ERβ ligand induced myelination during autoimmune demyelination. As such, CXCR2 will be conditionally knocked out from PDGFRα+ OPCs to generate PDGFRα:CXCR2-cKO mice and from proteolipid protein containing OLs (PLP^+) to generate PLP:CXCR2-cKO mice. EAE will then be induced in both mouse lines and treated with ERβ ligands. As CXCR2 facilitates OPC recruitment and OPC/OL survival; OPC numbers are likely to be comparable to WT. However, as the magnitude CXCR2-mediated protection is unclear in mature OLs, we expect that myelination may not be as robust as WT.

ERβ ligands including IndCl and analogues, IndCl-o-Cl and IndCl-o-Me, increase OL numbers, axon remyelination, along with an increase in CXCL1, a decrease in IFNγ and CXCL10 levels during EAE [289,290] (Figure 34). Uncovering how ERβ ligands modulate CXCL1 production to initiate OPC proliferation, differentiation, and myelin repair would expand the milieu of potential targets for MS and other diseases with myelin pathology, such as leukodystrophies and schizophrenia, thereby revolutionizing drug discovery in this field. Thus, the development of selective ERβ ligands, such as IndCl and analogues thereof, with similar immunomodulatory,

remyelinating, and neuroprotective qualities will offer targeted therapies to patients with MS and other demyelinating diseases.

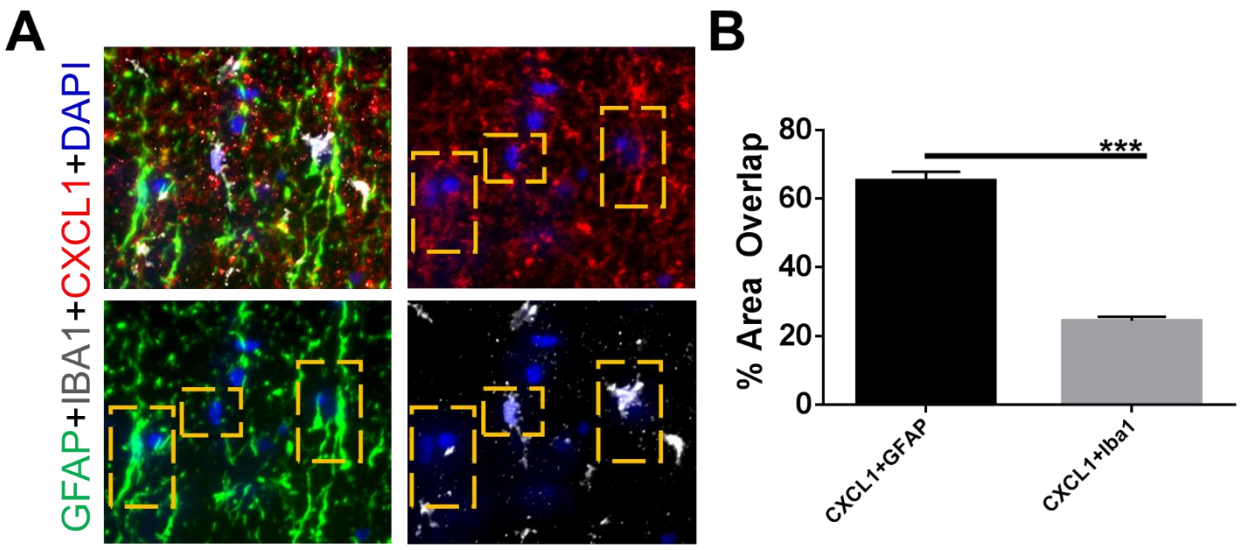

Figure 28. Therapeutic ERβ ligand treatment significantly increases CXCL1 on astrocytes, but not microglia during peak EAE disease. (A) 40x magnification images of the ventral spinal cord column treated with therapeutic ERβ treated EAE mice stained with CXCL1 (red), and GFAP (green) and Iba1 (green) and DAPI (blue). (B) Quantification of the percent area overlap of CXCL1+GFAP and CXCL1+Iba1 from peak EAE mice reveals a significant % overlap between CXCL1 and GFAP+ astrocytes, with very little expression on Iba1+ microglia. n=3 mice/group, unpaired t test, ***P < 0.001.

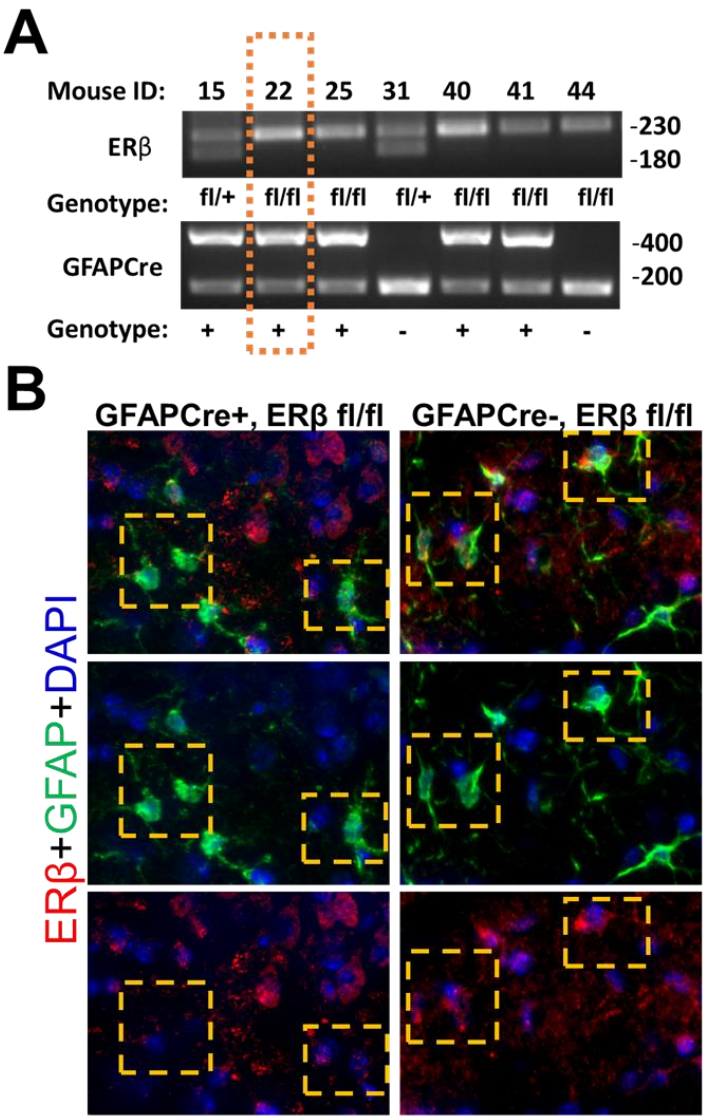

Figure 29. Generation of mice with conditional knockout of ERβ in astrocytes. (A) ERβ is selectively ablated from astrocytes using mice that express Cre recombinase under the control of the mouse GFAP promoter (mGFAPCre line 77.6) mated to mice with loxp sites flanking the Esr2 (ERβ) gene, thereby generating GFAP+ astrocyte-specific ERβ-/- (GFAP:ERβ-cKO) Tg mice. Genotyping results are shown with GFAP:ERβ-cKO+ lanes 22, 25, 40, and 41. Gel area within orange dashes represents a sample from a GFAP:ERβ-cKO mouse. (B) Spinal cord sections from mice (from A group) were immunostained for ERβ (red) and GFAP (green) and counterstained with nuclear stain- DAPI (blue). $GFAPCre^{+}ER\beta^{fl/fl}$ spinal cord show no ERβ staining in GFAP+ cells as compared to $GFAPCre^{-}ER\beta^{fl/fl}$.

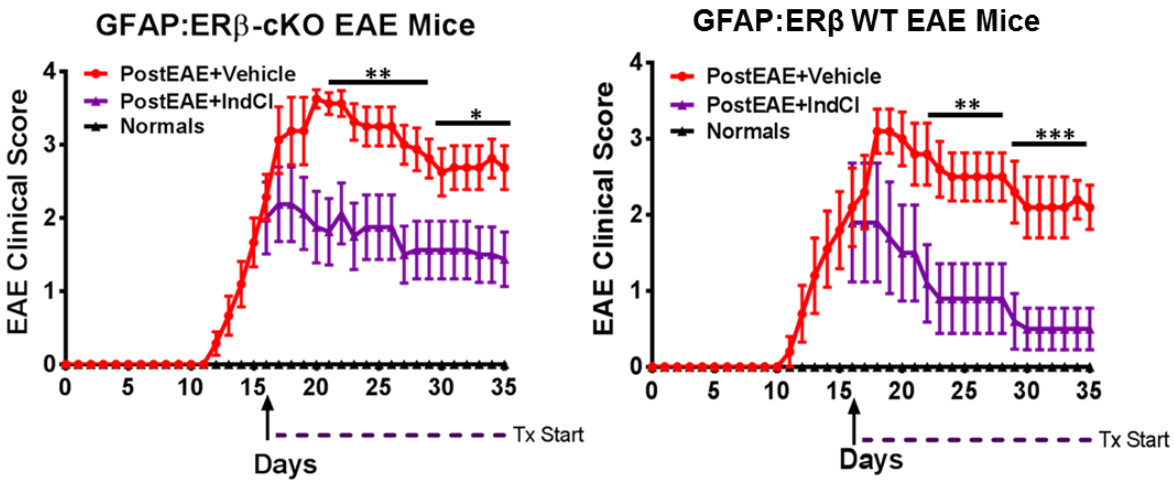

Figure 30. ERβ ligand does not act directly on astrocytes for clinical disease protection. EAE was induced in GFAP:ERβ-cKO and GFAP:ERβ WT control age and sex matched mice as previously described[287]. Therapeutic treatment with IndCl (5 mg/kg/d; purple), began on day 16 and continued to day 35. A decrease in clinical disease score was exhibited by IndCl treatment compared to vehicle treated EAE mice in both cKO and WT mice, *P < 0.05, **P < 0.01, ***P < 0.001.

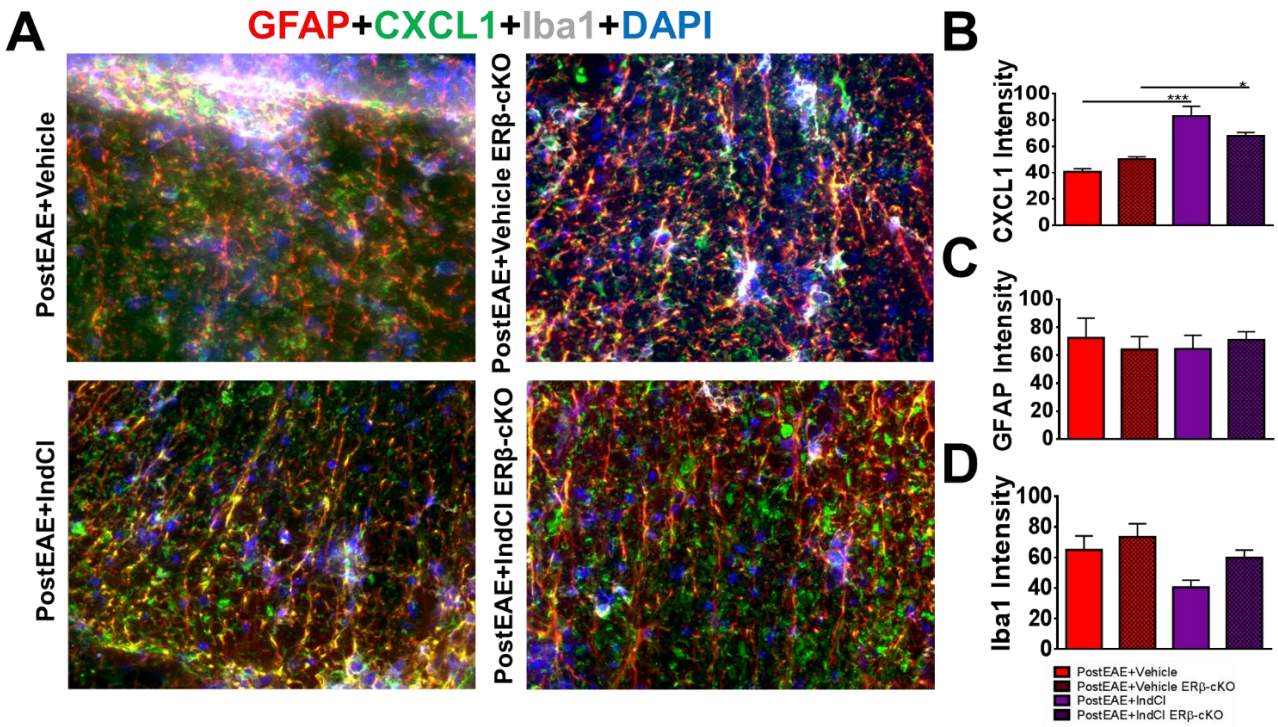

Figure 31. IndCl-treated GFAP:ERβ CKO and WT EAE mice exhibit comparable levels of CXCL1 expression and inflammation. (A) Representative 40x magnification coronal images of the ventral column of thoracic spinal cord collected at chronic disease (day 25). Sections collected from vehicle, and IndCl of GFAP:ERβ-cKO and littermate controls were immunostained with chemokine (C-X-C motif) ligand 1 (CXCL1; green), glial fibrillary acidic protein (GFAP; red), and nuclear stain (DAPI; blue). (B) Quantification of the relative fluorescence intensity of CXCL1 revealed a significant increase in CXCL1 intensity with therapeutic IndCl, for both genotypes as compared to vehicle-treated EAE mice. (C) Quantification of the relative fluorescence intensity of GFAP from vehicle and therapeutic IndCl treated EAE mice. ERβ ligand treatment with IndCl exhibited similar degrees of intensity of GFAP as vehicle treated EAE mice in both GFAP:ERβ-cKO and littermate controls . (D) Quantification of the relative fluorescence intensity of Iba1 revealed similar intensity with therapeutic IndCl, for both genotypes as compared to vehicle-treated EAE mice. n = 5–8 mice/group, One-Way ANOVA with Dunnett's Multiple Comparisons Analysis, *P < 0.05, ***P < 0.001.

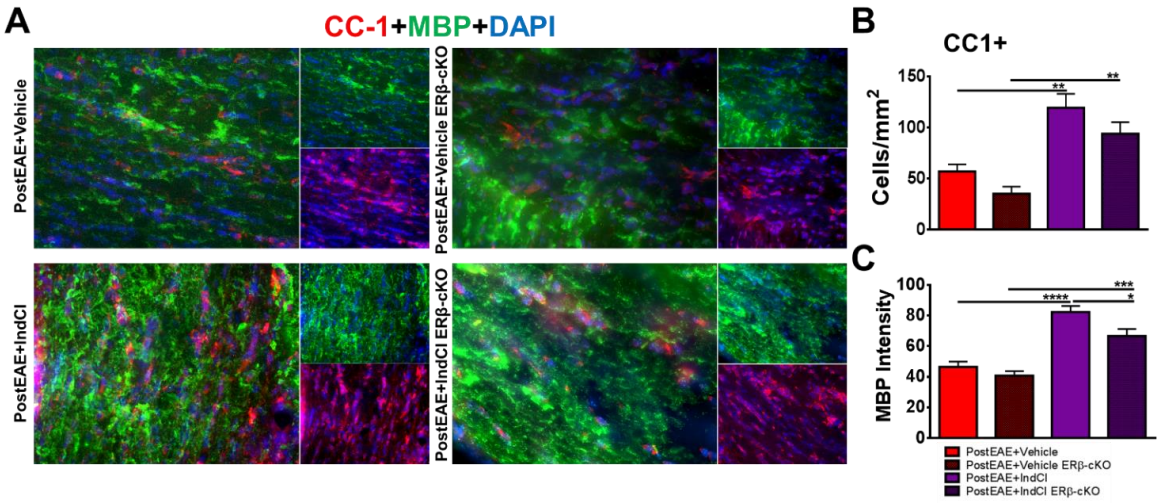

Figure 32. Selective deletion of ERβ in astrocytes does not alter IndCl-induced improvement in mature OL number and myelin density. (A) Representative 40x magnification images of the corpus callosum (CC) of vehicle, and IndCl treated GFAP:ERβ CKO and littermate control WT EAE at day 36. Mature OLs were stained with adenomatous polyposis coli (CC1; red), and myelin with myelin basic protein (myelin; green). (B) Quantification of CC-1 from (A) reveals significant increase in the number of mature OLs (red) with therapeutic IndCl treatment in both GFAP:ERβ CKO and littermate control WT EAE mice. (C) IndCl treatment significantly enhanced MBP intensity in both genotypes compared to vehicle treatment. Interestingly, there was a significant difference in MBP intensity between IndCl treated GFAP:ERβ CKO EAE mice and IndCl treated littermate control WT EAE mice. n = 5–8 mice/group, One-Way ANOVA with Dunnett's Multiple Comparisons Analysis, *P < 0.05, **P < 0.01, ***P < 0.001.

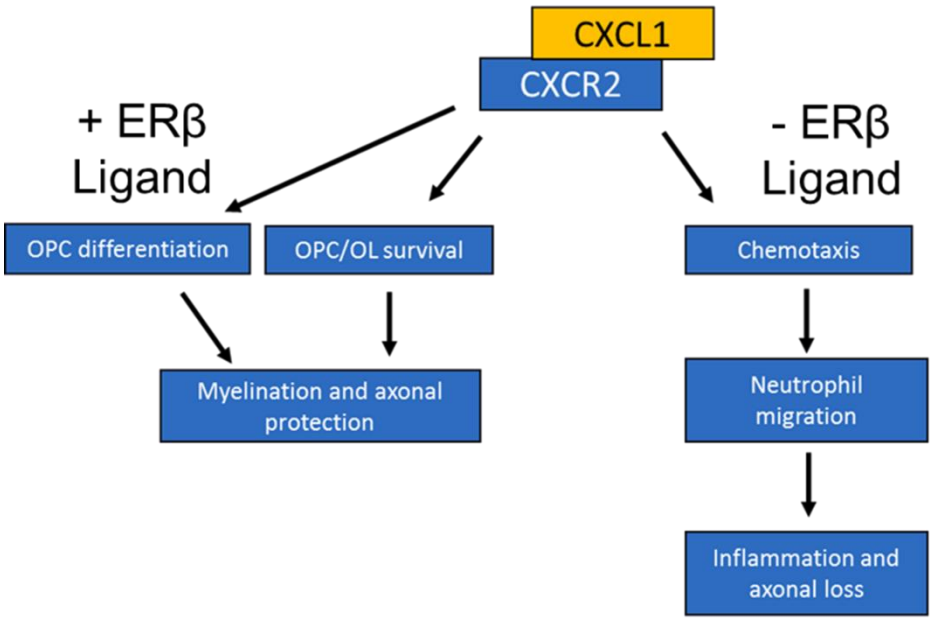

Figure 33. CXCL1/CXCR2 activity in the presence and absence of ERβ ligands. CXCL1 acting on CXCR2 is involved in chemotaxis, and neutrophil migration – this leads to release of pro-inflammatory mediators and activation of other immune cells which causes cell death, axonal loss and neurodegeneration. Howver, in the presence of ERβ ligands, we hypothesize that CXCL1 and CXCR2 signaling protects OLs and allows them to differentiate to aid in myelination and axonal protection.

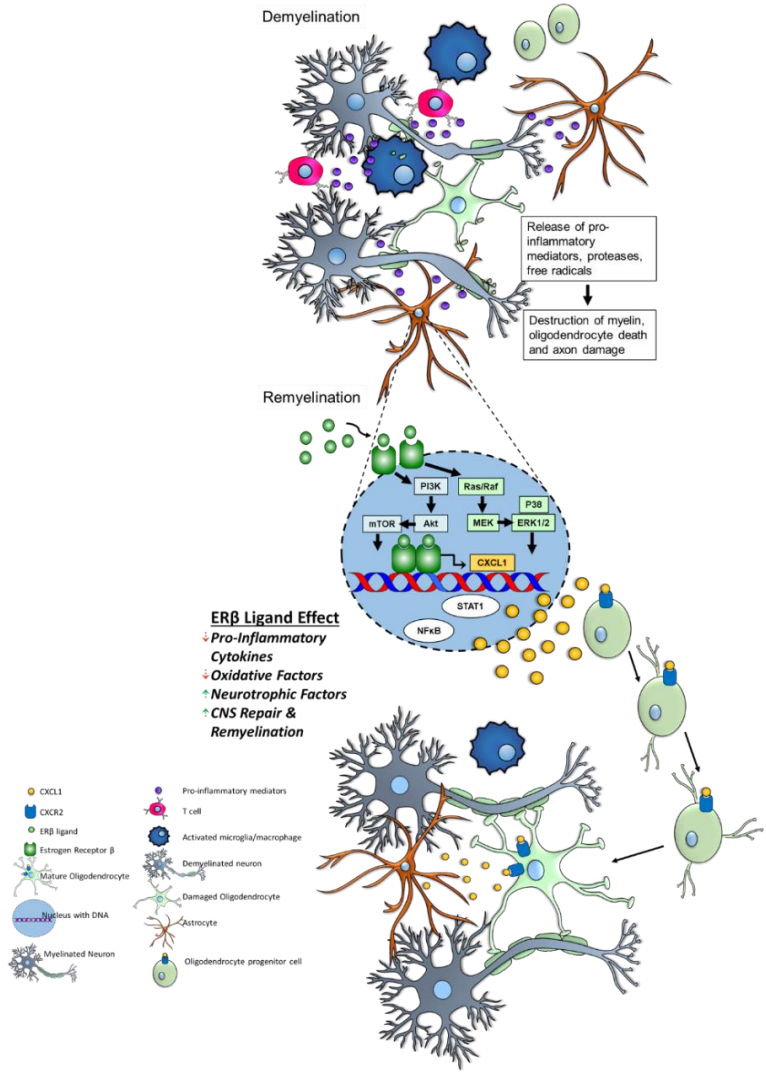

Figure 34. Model of ERβ ligand-treatment effects during EAE. Immune cells such as macrophages and T helper lymphocytes become activated in the periphery and cross the broken blood brain barrier into the CNS. These cells, along with activated microglia, and astrocytes secrete pro-inflammatory mediators such as reactive oxygen species, IFNγ, TNFα, and IL- 17, that promote destruction of OL and the myelin sheath, leading to demyelination, axonal damage and ultimately neurodegeneration. Treatment with ERβ ligands have been shown to decrease pro-inflammatory IFNγ, IL-23, and IL-6 cytokines while promoting OL survival and functional remyelination. ERβ ligand treatment increases BDNF and phosphorylated Akt and mTOR levels. The PI3K/Akt/mTOR pathway plays a role in OL differentiation and myelination. Based on our results, we hypothesize that ERβ ligand treatment decreases OL-toxic IFNγ levels and IFNγ-inducible chemokine CXCL10 while enhancing chemokine CXCL1 (implicated in the proliferation and differentiation of OLs) levels on astrocytes. Thus, hinting at a possible mechanism for ERβ-mediated remyelination/neuroprotection that involves changing CNS populations' response to inflammatory cytokines and chemokines released during EAE.

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