Immune Mediators of Central Nervous System Demyelination and Remyelination

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The principal goal of an immune response is to protect the host from pathogens, maintain tissue homeostasis and/or to repair damaged tissue. However, an inappropriate or prolonged immune response may be detrimental. Neuroinflammation is an integral component of demyelinating disease, such as multiple sclerosis, and often leads to oligodendrocyte damage and depletion. Here, we report on two immune mediators, interleukin-1 (IL-1) and lymphocyte activation gene-3 (LAG-3, CD223), and their role in demyelination and recovery.

IL-1 can be clinically beneficial or detrimental in human diseases. In this study, we examined the individual roles of IL-1α and IL-1β and their effects on myelination and cellular populations during neurodegeneration and recovery. After extensive backcrossing of $IL-1\alpha^+/+$, $IL-1\beta^+/+$, and $IL-1\alpha^+\beta^+$/ mice onto the C57BL/6 background, we demonstrate that IL-1α and IL-1β do not exacerbate cuprizone-induced demyelination and do not contribute to subsequent remyelination. However, an interesting phenotype emerged in which $IL-1\beta^{-/-}$ and $IL-1\alpha^+\beta^+$/mice show reduced numbers of mature oligodendrocytes in the corpus callosum, before and after cuprizone treatment.

LAG-3 is a membrane-bound glycoprotein that is involved in negative regulation of T cell activity and clonal expansion through its interaction with major histocompatibility complex class II (MHCII). Our study is the first to demonstrate upregulation of LAG-3 gene
and protein expression during demyelination. Furthermore, LAG-3 gene expression coincides with MHCII gene upregulation within the demyelinating lesion. We also demonstrate LAG-3 expression is localized to immature oligodendrocyte progenitor cells (OPCs) and astrocytes. Analyses of LAG-3−/− mice exposed to cuprizone showed an increased depletion of mature oligodendrocytes and an accelerated rate of demyelination. However, OPCs and astrocytes, as well as microglia/macrophages, responded robustly, and during repair, there was a greater number of differentiating mature oligodendrocytes even though remyelination appeared normal. Thus, we have found two novel observations: LAG-3 is expressed on OPCs and astrocytes, and LAG-3 appears to negatively regulate OPCs during remyelination.

These studies suggest that IL-1β and LAG-3 may have significant regulatory effects on glial populations in the central nervous system. Furthermore, the presence of LAG-3 and MHCII in the demyelinating lesion suggests that a direct interaction of OPCs and microglia is plausible.
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<tbody>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BMT</td>
<td>bone marrow transplant</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CD200R</td>
<td>CD200 receptor</td>
</tr>
<tr>
<td>CNPase</td>
<td>2’3’-cyclic nucleotide 3’-phosphohydrolase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalitis</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GSTpi</td>
<td>glutathione-S transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>I-Aβ</td>
<td>major histocompatibility complex class II</td>
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ICAM  intercellular adhesion molecule
IFN  interferon
IGF-1  insulin-like growth factor-1
IgSF  immunoglobulin superfamily
IL  interleukin
IL-18R  interleukin-18 receptor
IL-1R  interleukin-1 receptor
IL-1Ra  interleukin-1 receptor antagonist
iNOS  inducible nitric oxide synthase
LAG-3  lymphocyte activation gene-1
LFA-1  lymphocyte function-associated antigen-1
LFB-PAS  luxol fast blue-periodic acid Schiff’s
LPS  lipopolysaccharide
LT  lymphotoxin
LTP  long term potentiation
LTβR  lymphotoxin-β receptor
MAG  myelin-associated glycoprotein
MAPK  mitogen-activated protein kinase
MBP  myelin basic protein
MCP-1  macrophage chemotactic protein-1
MHC  major histocompatibility complex
MHV  mouse hepatitis virus
MMP  matrix metalloproteinase
<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor κ B</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NT</td>
<td>neurotrophin</td>
</tr>
<tr>
<td>OPC</td>
<td>oligodendrocyte progenitor cell</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
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<td>PGE₂</td>
<td>prostaglandin E₂</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PrP</td>
<td>prion protein</td>
</tr>
<tr>
<td>RCA-1</td>
<td><em>Ricinus communis agglutin</em>-1</td>
</tr>
<tr>
<td>sLAG-3</td>
<td>soluble lymphocyte activation gene-3</td>
</tr>
<tr>
<td>SOD1</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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xii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>TGFβ</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler’s murine encephalitis virus</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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CHAPTER 1

INTRODUCTION
1.1 Immune Responses in the Central Nervous System

Neuroinflammation is often implicated in the progressive nature of neurodegeneration in diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis (MS), prion diseases, and HIV-associated dementia. Immune responses in the central nervous system (CNS) are uniquely dissimilar to that in the peripheral nervous system (PNS) and other physiological systems when challenged by injury and infection. Because neuronal maintenance is essential to CNS integrity, the CNS has developed anatomical and physiological mechanisms that limit vulnerability to irreversible damage caused by immune reactions. Early observations led to characterizing the CNS as "immune-privileged": allograft survival within the brain parenchyma, the absence of typical lymphatic drainage, the presence of a blood-brain barrier (BBB), and the reduced expression of major histocompatibility complex class I (MHCI) and MHCII antigens [1]. Furthermore, the CNS is protected physically by the dura mater that restricts blunt trauma and invasive injuries, as well as limits swelling and edema, and by cerebrospinal fluid, which reduces injury from gravitational, rotational, and acceleratory forces [2]. Since these initial findings, the last few decades of research have challenged the concept of the CNS as an immune-privileged site.

It is now accepted that immune T cells, B cells, and monocyte-derived cells monitor the CNS in the absence of neuropathology and mount an immune response in the face of insult [3]. In relevance to this dissertation, key cellular players of the CNS immune response and their participation in homeostasis, neuroinflammation, and repair will be discussed below.
Microglia/macrophages. In the milieu of infection, injury, or disease in any tissue, resident macrophage activation is one of the first observable immune responses. This is no exception in the CNS. The largest population of macrophage-like cells in the brain, comprising 10-20% of total glial cells, is parenchymal microglia, otherwise known as resident microglia [4-6]. Studies during fetal development demonstrate that microglial “precursors” migrate in a single influx, largely guided by intercellular adhesion molecule-1 (ICAM-1, CD54) and/or ICAM-2 expression on cerebral endothelium and lymphocyte function-associated antigen-1 (LFA-1, CD11a) expression on the colonizing monocytes, to set up residence in the brain [7, 8]. These cells are exceptionally long-lived and are replaced from the bone marrow in less than a few percent per year [9-11]. In a normal physiological state, microglia initially take on an ameboid form during migration into the CNS and transition into a ramified form in the grey matter, a more longitudinally branched form in the white matter, and a compact form that resembles classical macrophages in the periventricular regions [4, 5]. Resident microglia remain downregulated in the absence of insult, partly due to constitutive transforming growth factor-β (TGFβ) expression in the brain, and are characterized by lack of endocytic and phagocytic activity, low expression of the leukocyte common antigen (CD45) and CD18/CD11b (Mac-1), and undetectable levels of MHCI and MHCII (reviewed in [4, 12]).

Aside from immune surveillance, auxiliary functions of microglia in the normal CNS remain elusive. They may play a role in maintaining homeostasis and repair in response to localized physiological and stress stimuli.

In the context of CNS insult, activated microglia are tightly regulated spatially and temporally in order to preserve an otherwise immunologically silent environment. However, in many aspects, they resemble other macrophages and differentiated monocytes in
phenotype and function. Upon activation, microglia acquire macrophage differentiation markers and effector properties, such as cytokine and chemokine receptors, scavenger receptor, death receptors/ligands (i.e. Fas/FasL), Fc receptors (i.e. Fcγ RI, RII, RIII), complement receptors (i.e. CR1, CR3, CR4, C1qRp), pattern recognition receptors (i.e. CR3, mannose receptor, CD14, or TLR4), and OX2R/CD200R (reviewed in [4, 12, 13]). Moreover, they release many effector and regulatory immune mediators. In vitro and in vivo systems have demonstrated microglial production of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-12, IL-15, and IL-18, and anti-inflammatory cytokines, such as IL-10, TGFβ, and IL-1 receptor antagonist (IL-1Ra). Matrix metalloproteinases (MMPs), chemokines, reactive oxygen species, inducible nitric oxide synthase (iNOS), and prostanoids have also been localized to activated microglia. Functionally, microglia are competent phagocytic cells that are able to clear myelin debris and apoptotic cells in a number of neurodegenerative diseases [13-20].

In addition to expressing the before-mentioned receptors upon activation, microglia are able to alter their immunophenotype with upregulation of MHCI, MHCII, CD45, and adhesion/costimulatory molecules, such as LFA-1, LFA-3 (CD58), ICAM-1, CD40, B7-1 (CD80), and B7-2 (CD86) (reviewed in [4, 12]). Despite the activated immunophenotype, the capacity to present antigen by activated microglia is debatable. Again, in the normal, physiological state of the parenchyma, MHC expression is minimal or absent, and when upregulated, it is restricted to a subset of microglia and astrocytes, the latter of which will be discussed in a later section [21-24]. Ample in vitro studies demonstrate that microglia from fetal or neonatal rodent or human brain are able to take up, process, and present antigen to naïve, memory, and differentiated T cells, leading to T cell proliferation and/or effector
functions (for examples, see [25-32]; reviewed in [4, 33]). In contrast, several studies challenge the capacity of microglial antigen presentation [24, 34, 35]. Of these studies, a notable one by Ford and colleagues demonstrate the diminished ability of microglia purified from adult rat brain to present myelin basic protein (MBP) to CD4+ MBP-specific T cell line [24]. However, microglia were able to induce some T cell proliferation but they did not effectively stimulate IL-2 secretion when compared to macrophages.

Aside from microglia, there are several other blood-derived macrophages that circulate and reside in the CNS. One type, in particular, is perivascular macrophages. These cells are believed to have the greatest immunological importance in the brain. Studies in early neonatal development demonstrate that perivascular microglia/macrophages leave the bone marrow and penetrate the BBB to colonize the CNS [6, 36, 37]. Perivascular microglia/macrophages are continuously replaced from the bone marrow, as they are usually found near small- and medium-sized blood vessels in or around the BBB [37]. They function in general maintenance of BBB integrity but are also key participants in immune surveillance [9, 38]. Unlike resident microglia, perivascular macrophages constitutively express MHCII [36, 39]. Because of their location, these cells rapidly sense immune-related alterations in the serum and are able to transduce proinflammaratory signals from the periphery across the BBB and into the CNS parenchyma [9, 40]. Meningeal and choroid plexus macrophages are located tactically to guard the ventricular/subarachnoid compartment, have a high turnover rate, and have similar functions to perivascular macrophages [12, 41].

Microglia and macrophages in the CNS are a highly responsive population of cells. Their distribution throughout the parenchyma and sites of CNS entry makes them indispensable participants in immune surveillance and response. Their role in
immunoregulation includes the following: expression of immune-related receptors, secretion of regulatory factors, antigen sampling and presentation, and effector functions, such as phagocytosis. Their role in demyelination and remyelination, in regards to IL-1 and lymphocyte activation gene-3 (LAG-3, CD223), will be discussed in this dissertation.

Astrocytes. Astrocytes comprise a dynamic and, likely, heterogeneous cell population that is involved in physical structuring of the CNS, metabolism, synaptic functions, and response to infection, injury, or disease. Physiologically, astrocytes aid in the maintenance of the extracellular ionic environment, uptake of extracellular glutamate that would otherwise negatively affect oligodendrocyte homeostasis, and aid neurons in development, function, and repair [42-44]. Additionally, these cells play an integral role, in conjunction with endothelial cells, in BBB formation and maintenance, limiting entry of leukocytes into the CNS. In a forebrain stab injury study by Bush and colleagues, astrocytes were ablated by treating *gfap* promoter-driven HSV-thymidine kinase transgenic mice with gancyclovir [45]. Injured tissue exhibited a prolonged 25-fold increase in leukocyte infiltration into the CNS and an inability to repair the BBB.

In the context of CNS insult, the hallmark of astrocyte responsiveness is reactive gliosis, or astrogliosis, characterized by proliferation, hypertrophy of cellular processes, upregulation of glial fibrillary acidic protein (GFAP) and vimentin, and re-expression of nestin [44, 46, 47]. Activation is accompanied by expression of adhesion molecules [e.g. vascular cell adhesion molecule-1 (VCAM-1)], cytokines [e.g. IL-1, TNF-α, IL-6, lymphotoxin-α (LTα), granulocyte macrophage-colony stimulating factor (GM-CSF), TGFβ], chemokines [e.g. macrophage chemotactic protein-1 (MCP-1), fractalkine (CXC3CL1),
CXCL12 (SDF-1), MMPs, and growth factors [e.g. fibroblast growth factor-2 (FGF-2), insulin-like growth factor (IGF-1)] (for examples, see [48-53]; reviewed in [44, 54, 55]). Although not considered professional antigen presenting cells (APCs) that constitutively express MHCII and costimulatory molecules, reactive astrocytes have been shown to express MHCII, inducible by interferon-γ (IFNγ) \textit{in vitro} [56]. However, their role in antigen presentation is unclear due to conflicting studies about their costimulatory molecules, namely B7-1 and B7-2 [43]. Furthermore, \textit{in vivo} studies are not consistent in MHCII localization to astrocytes. MHCII localization to astrocytes has not been demonstrated in mice constitutively expressing IFNγ in the CNS or in experimental autoimmune encephalomyelitis (EAE) rodents [43]. In contrast, MHCII has been localized to astrocytes during cuprizone-demyelination; however, this is rather rare or occasional [23].

Astrocytes can be classified as the “jack of all trades, master of none” in the CNS. These cells make up nearly half the brain parenchyma and appear to play supportive roles in maintaining CNS homeostasis, immune responsiveness to CNS insult and subsequent repair. In terms of demyelination, astrocytes have been shown to express LTα, which has been demonstrated to exacerbate demyelination but not affect remyelination [50]. LTα/β complexes can form between LTα and multiple subunits of LTβ and then induce signaling through LTβ receptor (LTβR), which is primarily expressed on microglia and also exacerbates demyelination [57]. Thus, LTα produced by astrocytes may exacerbate demyelination by associating with LTβ to form a LTα/β ligand that can then stimulate LTβR+ microglia.
Neurons. Neurons are often overlooked as having significant immune contributions in response to CNS insult, mainly due to the lack of abundant MHCII expression inducible by IFNγ [58]. These cells, however, do play an important role in regulating glial cells, in particularly microglia in the physiological and diseased states (for an in-depth review, see [59]). Recent studies have demonstrated constitutive expression of CD200 at the neuronal membrane surface. These molecules are members of the immunoglobulin superfamily (IgSF) known to downregulate myeloid cell function [60, 61]. Moreover, in the CNS, microglia have been shown to express the receptor to CD200 (CD200R) [60, 62]. Wright and colleagues demonstrated that CD200R antibodies augment clinical signs of EAE, which was then accompanied by an increased macrophage influx [62]. Furthermore, these finding were corroborated by the same group in CD200−/− mice, which displayed aggravated symptoms and enhanced microglial activity during EAE [60].

Other molecules expressed by neurons may have effects on microglia. CD45 is expressed at low levels on microglia and is upregulated upon activation [4, 12]. This receptor has been shown to inhibit microglial activity [63]. CD22, the corresponding ligand, is released by neurons upon neuronal damage. Moreover, it has been shown that MHCII induction in microglia can be restricted by neurotrophin release from electrically active neurons [64]. Of the downregulatory functions of neurons, a novel finding by Liu et al. has been pivotal in the re-examination of T-cell mediated multiple sclerosis by demonstrating that neurons are able to convert encephalitogenic T cells to regulatory T cells that dampen the immune response and inhibit EAE [65]. This phenomenon essentially turns their enemies into friends.
While neurons play this seemingly important role in downregulation of an active immune response in a typically immunologically silent environment, they can induce the opposite effect and upregulate microglia and other APCs when damage occurs. Once injured, neurons release purines, such as ATP and UDP, which correspond to receptors, such as P2X4, P2Y12, and P2Y6, on microglia [59]. In vitro and in vivo studies demonstrate dynamic changes in microglial motility via P2X4 and P2Y12 receptor interactions with ATP [66]. Moreover, P2Y6 receptor activated by UDP stimulates the phagocytic effector function of microglia [67]. Thus, neurons may stimulate an immune response when the CNS is under duress.

Neurons are also involved in the critical relationship with oligodendrocytes at the axo-glial junctions ([68]; reviewed in [69]). Several molecules and factors such as neuregulins, neurotrophins, contactin and neutrophil chemotactic protein-1 (NCP-1) on axons communicate with neurofascin 155 (NF155) on oligodendrocytes to promote myelination and formation of paranodes and Nodes of Ranvier. The proper formation of the axo-glial structures is important for the saltatory conduction and function of nerves. When there are perturbations to either the neurons or oligodendrocytes, degradation of the myelin ensues, leading to the degeneration of axons. This appears to be significant in cuprizone-induced demyelination and multiple sclerosis, as axonal fallout occurs during episodes of demyelination [70]. This perturbation to myelin and axons results in responses by microglia that may be detrimental or reparative.

Neurons play a significant role in the strict regulation of immune responses in the brain. They serve to protect local microenvironments of brain tissue with intact neurons, in
spite of upregulation of neuroinflammation in nearby areas. However, upon damage, they are capable of calling in professional APCs to clear infection and/or repair injury.

**Oligodendrocytes.** Oligodendrocytes are responsible for myelin sheath formation by extending and wrapping membrane processes around axons of many neurons in the CNS. Unlike Schwann cells, their counterparts in the PNS, oligodendrocytes are able to myelinate multiple axons. Myelin is a phospholipid layer that electrically insulates axons and allows efficient transduction of nerve impulses along the axon. Nodes of Ranvier are the gaps between the myelin sheets on which action potentials travel from one location along an axon to the next. White matter tracts, such as the corpus callosum, are comprised of myelinated axon bundles. Astrocytes and microglia are present within the white matter, as well as a small population of oligodendrocyte precursor cells (OPCs) [71].

OPCs are an endogenous pool of cells in the CNS capable of maturing into myelinating oligodendrocytes (reviewed in [72]). They are present in high numbers throughout the embryonic brain, with a denser population in the ventral forebrain [73]. OPCs proliferate in germinal zones during embryogenesis and continue to proliferate as they migrate to their terminal destination, where they begin to differentiate and express myelin genes [74, 75]. In adult brain, OPCs are largely found in the subventricular zone (SVZ) and are distributed elsewhere in the CNS in low numbers [72]. Several factors have been demonstrated to promote proliferation and differentiation of OPCs, such as platelet-derived growth factor (PDGF), FGF2, and IGF-1 [76]. OPCs, as demonstrated in this dissertation and several publications, are recruited to demyelinating lesions and are responsible for remyelination.
Oligodendrocyte differentiation has been well-studied using stage-specific antibodies in dissociated cell cultures, tissue sections, and *in vivo*. Common markers that characterize their pattern of differentiation include the following: A2B5 and LB1 for early OPCs that proliferate in germinal centers during embryogenesis [77, 78], NG2 and PDGFRα for later OPCs that proliferate and migrate to areas for terminal differentiation [79], O4 for pro-oligodendrocytes and immature oligodendrocytes [78, 80], and galactocerebroside [78], O1 [80], and GSTpi [81] for mature oligodendrocytes. Moreover, mature oligodendrocytes are often immunolabeled for myelin proteins, such as PLP, MBP, MOG, and MAG [82]. It should be noted that PLP has been demonstrated to be expressed by OPCs that are NG2+ [79].

**T cells.** T cells have been characterized to play a transient, immunosurveillance role in CNS immunity, passing through the brain parenchyma in search of their specific antigen. Most studies indicate T cells may cross the BBB only in an activated state without regards to their antigen specificity [9]. However, it has been demonstrated that naïve cells can cross the BBB [83]. Interestingly, the number of T cells that traffic the CNS are in relatively low concentrations compared to other organs in the body, likely due to selectivity of the BBB [84, 85]. Once across the BBB, activated T cells are met with the anti-inflammatory environment of the CNS, partly due to constitutive expression of TGFβ. Studies demonstrate that TGFβ suppresses T cell proliferation and the chemoattractants that permit leukocyte migration across the BBB (reviewed in [3]). Moreover, T cells are also met by a hostile environment in which cell death via apoptosis is inherent. Studies by Bauer *et al.* demonstrate that, in various models of autoimmune encephalitis, T cells in the CNS
parenchyma undergo apoptosis, a phenomenon that appears to be antigen recognition-independent [86]. Similarly studies by Moalem et al. demonstrate extensive T cell apoptosis after CNS nerve injury that is not apparent after PNS nerve injury [1]. Both studies may be a result of Fas/FasL mechanisms. Microglia, astrocytes, and neurons are capable of expressing FasL, and in the latter study, Fas was significantly upregulated in the CNS compared to the PNS [1, 87]. Despite this unfavorable environment, T cells readily cross the BBB and are able to engage in normal immune functions in the context of disease [9]. However, in T cell-dependent demyelinating disease of the CNS, both autoimmune T cells and viral-specific T cells have been extensively studied and amply reviewed elsewhere [88-91]. In this dissertation, the cuprizone intoxication model will be used. The BBB remains intact in C57BL/6 mice during demyelination, and there is no role for T cells in demyelination or remyelination [49, 92, 93].

**Neuroinflammation in CNS Repair.** The principal goal of any immune response is to protect the tissue from pathogens and/or to repair damaged tissue. However, when the immune response is directed against self, insufficient, or prolonged, the repercussions can be devastating. Recent studies have exploited the protective activities of the CNS in an inadequate or overactive immune response in order to promote repair and remyelination. Using the acute model of demyelination through administration of ethidium bromide, a recent study enhanced the innate immune response by a single intracerebral lipopolysaccharide (LPS) bolus and demonstrated increased accumulation of Olig1+ and Olig2+ OPCs, a cell type that later matures into myelin-producing cells [94]. Furthermore, the same study demonstrates dexamethasone, an anti-inflammatory agent, enlarged the size
of a lesion caused by Tween-20, a surfactant that provokes localized damage to cerebral tissue, thus demonstrating a protective role of innate immunity in acute brain injury.

Inflammatory cytokines conventionally believed to contribute to oligodendrocyte death and myelin destruction may also contribute to CNS repair by recruitment and differentiation of oligodendrocyte progenitors. TNF-α has been demonstrated to be neuroprotective by signaling through TNF receptor 2 (TNFR2) to upregulate the Akt-1/protein kinase B (PKB)-dependent pathway [95]. Akt-1 stabilizes the mitochondrial membrane potential and prevents the release of cytochrome c and subsequent apoptosis [96]. Another study suggests TNF-α-N-methyl-D-aspartate (NMDA)-dependent NFκB activation through TNFR2 protects neurons from glutamate neurotoxicity [97]. Furthermore, Arnett and colleagues demonstrated TNF-α/TNFR2 may be instrumental in oligodendrocyte proliferation and remyelination [49].

Growth factors and other neurotrophins are upregulated during CNS insult and have been shown to promote CNS repair and remyelination. IGF-1 is a growth factor that directly affect oligodendrocytes and myelination and is required for normal oligodendrocyte development and myelination in vivo [98]. Moreover, IGF-1 has been shown to protect oligodendrocytes from TNF-α-induced death in vitro, and constitutive IGF-1 expression in mice (IGF-1Tg/o) has been demonstrated to protect mature oligodendrocytes from depletion during cuprizone treatment, resulting in rapid recovery from the demyelinating insult. When IGF-1Tg/o are crossed with mice expressing the TNF-α transgene, there are twice the number of oligodendrocytes [99, 100]. As for other neurotrophins upregulated during CNS insult, organotypic slice cultures of adjacently placed brain cortex and spinal cord from neonatal rats with transplanted neural progenitor cells demonstrated that axon growth from the brain
cortex into the spinal cord was promoted by brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and nerve growth factor (NGF) released by the neural progenitor cells [101].

The subtle balance between neuroinflammation and neuroregeneration warrants careful elucidation of mechanisms discussed in this section in order to determine appropriate therapeutic approaches.

1.2 Interleukin-1: The Prototypical Cytokine

IL-1 is one of the most widely studied pro-inflammatory cytokines in the CNS. There is extensive evidence implicating IL-1 as a key contributor to a diverse range of biological activities associated with infection, inflammation, and autoimmune processes, particularly in acute and chronic neurological disorders. The canonical interleukin family is comprised of three proteins closely related in ancestry and structure: two functional agonists, IL-1α and IL-1β, and one antagonist, IL-1 receptor antagonist (IL-1Ra) (reviewed in [102]). Despite being encoded by different genes, IL-1α and IL-1β share high sequence homology and similar intron-exon organization. Furthermore, both isoforms are synthesized as 31 kDa pro-forms lacking leader sequences and are cleaved to produce 17 kDa mature forms. Despite similar biological effects of IL-1α and IL-1β, the two agonists differ in the cellular compartments in which they are primarily active.

IL-1α is biologically active in its pro-form and can be cleaved by membrane-associated cysteine protease calpains to the active form. Both forms largely remain intracellular, and
the mature form is only efficiently released by activated macrophages. Other cell types express IL-1α; however, IL-1α is not released unless substantial cell injury occurs, such as ischemic brain damage [103]. In contrast, IL-1β is not biologically active in its pro-form and is cleaved by caspase-1 (IL-1β-converting enzyme, ICE) to form its mature counterpart [104]. IL-1β is mostly secreted by macrophages but can be secreted by other cells, such as T cells. Keller et al. recently described that activation of caspase-1 by inflammasome complexes is directly linked to a non-classical secretion pathway by which both agonists, despite IL-1α not being processed by caspase-1 and in addition to other leaderless proteins, are secreted [105]. IL-1α and IL-1β are believed to induce similar cellular responses, as they both bind a common receptor, IL-1 type 1 receptor (IL-1R1), which recruits the IL-1 receptor accessory protein (IL-1RAcP) and activates intracellular signaling pathways including nuclear factor κ B (NFκB) and mitogen-activated protein kinases (MAPKs) [106, 107]. IL-1R1 signals through the Toll/IL-1 receptor (TIR) domain, a conserved intracellular region, which recruits adaptors, such as myeloid differentiation factor 88 (MyD88) and TNF receptor-associated factor 6 (TRAF6) [108]. Signaling may lead to the induction of other immunomodulators, such as IL-6, TNF-α, and prostaglandin E_2 (PGE_2). Additionally, IL-1α and IL-1β are able to bind a second receptor, IL-1R2, which lacks an intracellular domain. Acting as a decoy receptor, downstream signaling through IL-1R2 does not occur. Interestingly, a study using mixed glial cultures from IL-1R1−/− mice demonstrates an upregulation of several genes that are independent of the classical NFκB and MAPK pathway, suggesting against IL-1R1 as the sole functional receptor for IL-1 [109].

IL-1Ra is unique in being an endogenous competitive antagonist in a cytokine signaling system. It is synthesized in three intracellular forms and one secreted form by the same cells
that produce IL-1. While the roles of the intracellular forms of IL-1Ra remain poorly defined, the secreted form binds IL-1R1 to competitively inhibit binding of the agonists and, thus, effectively block IL-1 signaling.

In addition to IL-18, several new IL-1 family members have been identified based on gene homology (sequence similarity of 20-52% with IL-1α, IL-1β, and IL-1Ra), gene location, and receptor binding: IL-1F5 (IL-1 family, member 5), IL-1F6, IL-1F7, IL-1F8, IL-1F9, and IL-1F10 (reviewed in [110-113]). In relevance to this dissertation, key family members and their role in neurological disorders will be discussed.

*Regulation of IL-1 production and activity.* IL-1 is expressed at low constitutive levels in the healthy CNS [114]. Regulation of IL-1 is tightly regulated and is multi-tiered: transcription, translation, cleavage, cellular release, and bioactivity. However, IL-1 appears to be dispensable for the CNS development, as the brains of *IL-1*^{-/-} mice appear normal [115, 116].

The expression of IL-1α and IL-1β may differ in correlation to their promoter elements. The promoter for the *IL-1α* gene does not contain sequences that correspond to the TATA box, a typical motif for inducible genes [102]. Inducible gene expression is controlled by a 4.2 kb upstream region and a proximal promoter region of 200 bp [102]. Seemingly, downstream sequences can be deleted without consequence to gene expression. In contrast, the *IL-1β* gene possesses a TATA box in its promoter region, as well as a CAAT box, which controls the efficiency of transcription initiation [117]. *IL-1β* gene transcription is complexly controlled. Unlike most cytokine promoters, the regulatory regions of *IL-1β* are distributed over several thousand basepairs downstream from the transcriptional start site [102].
Notably, there are two enhancer regions that act cooperatively: one that contains a cyclic AMP (cAMP) response element and a NFκB-like site and another that is a composite cAMP response element-NF-IL6 and is responsive to LPS [102, 118]. Activating protein-1 (AP-1) also participates in LPS-induced IL-1β expression. Moreover, there are several binding regions for transcription factors, such as nuclear factor-κB (NFκB) and SP1, which impart tissue specificity. Undeniably, IL-1β gene regulation is under sophisticated transcriptional control and is selectively triggered by a number of immune insults, such as LPS or, more relevant to the CNS, β-amyloid.

IL-1β protein production is regulated in several ways. Characteristic of IL-1β, and also to TNF-α, there appears to be a disconnection between transcription and translation [119]. For example, during hypoxia, pro-IL-1β mRNA transcripts are synthesized in large amounts in monocytes; however, this is not always accompanied with translation into the pro-IL-1β protein. In this case, the signal for translation may not be sufficient. Contrastingly, LPS has been shown to increase IL-1β protein synthesis by stabilization of the transcript. However, cellular release of IL-1β requires additional signals [120]. In the case of LPS-primed macrophages, subsequent activation of P2X7 by ATP is required for cleavage of pro-IL-1β by caspase-1 and release, which is dependent on marked changes in ionic composition [121]. Stimuli other than ATP may serve as the signal that induces cleavage and subsequent release but common to these stimuli is the capability of stimulating ionic flux. A small antimicrobial peptide LL37 (CAMP), released by neutrophils and epithelial cells, has been identified to induce P2X7 activation and subsequent IL-1β release [122]. As with most processes, there are counter-pathways. IL-1β release has been demonstrated to be blocked by
a family of cytokine-release inhibitory drugs (CRIDs) (for characterization, see [123]). This family does not appear to function through P2X7.

Lastly, IL-1β activity is regulated upon cellular release by several mechanisms including competitive binding for receptors and expression of the receptors [124]. As mentioned previously, IL-1Ra serves as an endogenous competitor for IL-R1. However, it has been demonstrated that IL-1Ra concentrations need to be in 100-fold excess relative to IL-1 in order to block IL-1 signaling. Thus, during inflammation, upregulation of IL-1Ra may not be adequate in countering IL-1-mediated activities. Additionally, IL-1R1, IL-1R2, and IL-1RAcP can be shed from the membrane. IL-1R1 and IL-1R2 function in the sequestering of the IL-1 agonists, and IL-1RAcP sequesters IL-1R1. Furthermore, IL-1R2 can bind IL-1RAcP, preventing recruitment to IL-1R1. Again, IL-1, a potent immunoregulator, needs only a few receptors to induce immune-related biological activities. Thus, these regulatory mechanisms may be more functional in maintaining physiological homeostasis rather than taming IL-1 mediated activities during inflammation. The extent to which IL-1 is mitigated demonstrates the potential biological importance of IL-1.

**IL-1 in the CNS.** All members of the canonical IL-1 family, namely IL-1α, IL-1β, IL-1Ra, IL-1R1, IL-1R2, and IL-1RAcP, are expressed in the CNS [114]. However, the cellular source of IL-1 remains elusive, as expression is very low to undetectable in normal brains and many studies for the cellular expression of IL-1 are performed *in vitro* and in the context of CNS injury. During injury or disease, however, microglia and astrocytes have been shown to express IL-1 and this is discussed in greater detail below. Furthermore, the function of
these IL-1 family members, even the well-characterized IL-1β, in the normal CNS also remains somewhat elusive. Genetic knockout mice lacking IL-1α and/or IL-1β, as well as IL-1RI, develop normally with no obvious differences from wild type mice. However, in Chapter 2 of this dissertation, we characterize a novel finding in mature oligodendrocyte populations in mice lacking IL-1β.

One of the few known physiologically important roles for IL-1 is its function in sleep patterns. A study in 1991 administered IL-1Ra to rabbits and observed reduced non-rapid eye movement sleep [125]. It also has been reported that IL-1RI−/− mice sleep less than wild type control mice [126]. Moreover, IL-1 concentrations are higher in the brain during sleep [114]. A second role of IL-1 has been associated with synaptic plasticity. Increased IL-1 expression has been observed after induction of long-term potentiation (LTP) in the hippocampus [127]. Moreover, IL-Ra impairs the maintenance phase of LTP [127]. Thus, IL-1 appears to affect subtleties in neuronal function; however, there are no reports yet of other roles for IL-1 in the physiologically normal CNS.

Microglia are the early primary source of IL-1 following CNS injury, infection, or inflammation, which is not surprising considering that monocytes and macrophages are the primary source in the periphery [128]. Astrocytes also express IL-1 and usually follow microglia in expression after CNS insult. Furthermore, oligodendrocytes, neurons, and cerebrovascular cells have been reported to express IL-1 [129]. IL-Ra and the IL-1 receptors are usually induced by the same stimuli that upregulated IL-1 and, thus, are expressed on the same cell types [129-133]. In cuprizone-induced demyelination, nearly all microglia and about 50% of astrocytes express IL-1 [51]. Of interest to this dissertation is how IL-1 affects astrocytes, oligodendrocytes and neurons rather than which cells express IL-1.
Microglia express IL-1R1 to which IL-1 can directly bind and induce expression of several immune mediators: reactive oxygen species, reactive nitrogen species, PGE2, proinflammatory cytokines, and chemokines (see Section 1.1: Microglia/macrophages; reviewed in [134]). Rat microglia also can be stimulated to produce several anti-inflammatory cytokines: such as IL-10, TGFβ, and IL-1Ra. There is, however, a species-specific difference in IL-1 signaling, as mouse microglia express low levels of IL-1R1 and, thus, fail to induce signaling in vitro [133]. Effectively, they behave similarly to peripheral macrophages.

Of the CNS cell types, astrocytes appears to be most affected by IL-1 (reviewed in [135]). In addition to stimulating proliferation, or rather astrogliosis, IL-1 signaling in astrocytes plays a key role in leukocyte migration [135, 136]. As previously mentioned, astrocytes are an integral part of regulating cell entry through the BBB. Studies demonstrate that IL-1 induces sustained expression of the chemoattractant cytokine IL-8 and adhesion molecules, VCAM-1 and ICAM-1, in astrocytes [137, 138]. Interestingly, this may play a key role in MS and Alzheimer’s disease, where expression of leukocyte adhesion molecules in astrocytes and leukocyte entry is high [139, 140]. Additionally, like microglia, IL-1 signaling can induce production of pro- and anti-inflammatory immune mediators. Microarray analysis reports that IL-1 upregulates neurotoxic mediators, such as chemokines, MMPs, proinflammatory cytokines (IL-6, TNF-α), and survival mediators, such as NGF [141].

Despite expression of IL-1 and its functional receptor, oligodendrocytes, the cell type responsible for myelin sheath formation, have been poorly studied in terms of IL-1 effects. Interestingly, Vela and colleagues demonstrated that IL-1 inhibits proliferation and promotes
differentiation of OPCs, as well as promoting maturation and survival of differentiating oligodendrocytes *in vitro* [142]. This study could translate to profound effects *in vivo*, suggesting IL-1 may promote remyelination. In line with this notion, IL-1β has been shown to be important for remyelination in cuprizone-induced demyelination and for stimulation of oligodendrocytes in response to Fcμ that promotes CNS repair [51, 143]. In contrast, IL-1Ra over-expression has been shown to ameliorate EAE, suggesting that IL-1 is detrimental [144]. Thus, IL-1 can affect astrocytes and oligodendrocytes in harmful or beneficial ways.

**IL-1 in CNS Disease.** IL-1 expression is increased in several neurological disorders with varied etiology and presentation: multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer’s disease, Parkinson’s disease, prion diseases, HIV-associated dementia, and epilepsy. However, it is unknown whether IL-1 is a contributing factor to disease or rather it is part of the immune response that triggers resolution of inflammation and initiates reparative processes. Several studies of IL-1 in CNS disease are discussed below.

IL-1β is indirectly implicated in ALS, a motor neuron degenerative disorder in which 25-30% cases are associated with mutations in the superoxide dismutase gene (SOD1) [145]. A study by Pasinelli and colleagues demonstrates that mutant SOD1 promotes apoptosis in an oxidatively stressed neuroblastoma cell line by activating caspase-1, which subsequently cleaves pro-IL-1β and promotes release of the mature form [145]. Furthermore, activation of caspase-1 and subsequent cleavage of pro-IL-1β was confirmed in two transgenic strains of mice expressing mutant SOD1 proteins. Interestingly, an independent study demonstrates inhibition of caspase-1 retards clinical symptoms of ALS-like symptoms in mutant *SOD-1*
transgenic mice [146]. This study suggests that IL-1 may exacerbate ALS-like symptoms and inhibition of IL-1 may be an effective therapeutic strategy to explore further.

More recently, human post mortem spinal cord specimens from MS and ALS patients were examined for cyclooxygenase-2 (COX-2) and P2X7 expression in activated microglia/macrophages [147]. There is significantly more COX-2+ microglia/macrophages in MS spinal cord compared to controls, while P2X7+ microglia/macrophages were more populous in both MS and ALS spinal cords. This study suggests that activation of P2X7-induced IL-1β secretion stimulates COX-2 and downstream pathogenic mediators, thus giving value to P2X7 and COX-2 inhibitors as possible therapeutic agents in MS and ALS. It should be noted that COX-2 inhibition prolongs survival in animal models of ALS and thus, IL-1 appears to be detrimental to these diseases.

Prion diseases are fatal neurodegenerative disorders that are caused by the conformational change of a ubiquitously-expressed and noninfectious prion protein (PrP\textsuperscript{C}) to a disease-associated isoform (PrP\textsuperscript{Sc}) [148]. Previous experiments demonstrate that any gene product involved in PrP\textsuperscript{Sc} formation affects the onset of prion disease if knocked out or overexpressed [148]. Interestingly, Tamguney et al. demonstrated prolonged onset of disease in IL-1R\textsuperscript{1-/-} mice. Thus, this study implicates a possible role of IL-1R1, and thus IL-1, in the conformational change of PrP\textsuperscript{C} to its disease-associated isoform.

IL-18. IL-18 should be noted because of its emergence as a prominent cytokine in neuroinflammation and neurodegeneration. IL-18, originally identified as IFNγ-inducing factor, is similar to IL-β in several ways [reviewed in [149, 150]]. It is synthesized in a 24
kDa, biologically inactive pro-form that lacks a signal sequence. Cleavage by caspase-1 forms the 18 kDa, biologically active counterpart. IL-18 binds IL-18 receptor (IL-18R; IL-1-receptor-related protein), which recruits IL-18 receptor accessory protein (IL-18AcP) to initiate signal transduction. Like IL-1/IL-1R1 signaling, IL-18/IL-18R signaling leads to NFκB activation and subsequent expression of pro-inflammatory cytokines, such as IL-1β and TNF-α, chemokines, iNOS, and cell adhesion molecules [151]. Additionally, IL-18 is able to induce FasL. Unlike IL-1, IL-18 able to induce IFNγ but is unable to induce COX-2, which results in upregulation of PGE2. Interestingly, IL-18 also has an endogenous antagonist, IL-18 binding protein. However, it functions not by binding IL-18R but, instead, binds and sequesters IL-18. IL-18BP has a high affinity only for the mature form.

Microglia, astrocytes, neurons, and ependymal cells constitutively express IL-18, IL-18R, and caspase-1 [149]. IL-18BP has been demonstrated to be constitutively expressed on primary microglia in vitro and in normal rat brain in vivo. Interestingly, IL-18 expression in the brain is higher in postnatal stages and is downregulated to low, almost undetectable levels in the adult, suggesting a role in brain development. Unfortunately, few studies have examined IL-18 in development. IL-18−/− mice develop with no observable abnormalities [152].

IL-18 and caspase-1 have been implicated in playing a role in neurodegeneration, particularly in MS and EAE. In the periphery, IL-18 has been demonstrated to induce Th1 and Th2 helper T cell responses [153]. Clinical symptoms of EAE were mitigated upon administration of caspase-1 inhibitors, as well as in caspase-1−/− mice. This has been attributed to the capability of the IL-18/caspase-1 pathway to modulate Th1 responses. However, the IL-1/caspase-1 pathway cannot be dismissed. In support of the former
hypothesis, clinical symptoms of EAE were exacerbated in wild type mice administered exogenous IL-18 [154]. Furthermore, *IL-18−/−* mice appeared resistant to EAE.

1.3 LAG-3: A Two-Way Signaling Molecule

LAG-3 is a membrane-bound glycoprotein that shares many structural similarities to CD4. It is expressed in all subsets of T cells and a subset of natural killer (NK) cells following cellular activation [155-157]. Recently it has also been identified on B cells [158]. Deemed as a “two-way signaling” molecule, LAG-3 is expressed on activated T cells and binds MHCII, its only known ligand [156, 159], on APCs and induces two cellular functions: (1) downregulation of T cell receptor (TCR) signaling, leading to cell inactivation and [160, 161]; (2) activation of monocytes and dendritic cells, leading to optimal MHCI and MHCII T cell responses [162-164]. Although LAG-3 mRNA expression has been demonstrated in brain tissue, there is a lack of literature addressing the CNS cell type that expresses LAG3 and the role it may play in the physiological and diseased states [157]. However, there is no reason to assume that the same processes LAG-3 carries out in the peripheral immune cells cannot be applied to the CNS, particularly because the relevant regulatory molecules are expressed there.

Structure. Human LAG-3 was first characterized in 1990 by Triebel and colleagues who focused on genomic organization and structure [155]. The *LAG-3* gene includes eight exons and encodes a 498-amino acid membrane protein with four extracellular IgSF domains. The
first domain (D1) belongs to the V-SET, which is an Ig-like domain resembling the antibody variable domain. Interestingly, D1 contains a proline-rich 30 amino acid loop in the middle of the domain and an unusual disulphide bridge (Figure 1.1). As a side note, an unusual disulphide bond of a different kind has been reported in the V-like domain of the CD8α chain [165]. Mutagenesis studies show that the V-type Ig-like D1 domain includes the MHCII binding site on LAG-3 [159]. Additionally, a prospective second binding site is located on D1 and is believed to be involved in LAG-3 oligomerization. Huard et al. postulates that LAG-3 oligomerization induces structural changes in the MHCII binding site than enhances complementation at the interface between LAG-3 and MHCII [159]. Supportive of this hypothesis, LAG-3 has a 100-fold stronger binding affinity to MHCII compared to soluble CD4, which has a binding affinity < $10^4$ [159, 166-168]. Also, LAG-3 binds MHCII on a site that is distinctive from CD4, as MHCII antibodies that are able to block CD4 interaction are unable to block LAG-3 [166]. D2, D3, and D4 belong to the C2-SET, which is a protein structural domain that aids in targeting proteins to the cell membrane [155]. Despite <20% amino acid sequence identity with CD4, LAG-3 is strikingly similar in structure to CD4: both genes contain an intron within the first IgSF domain and the positions of their introns are almost analogous. Both genes are located on the distal part of the short arm of chromosome 12 and, because both genes have internal sequence homologies between D1 and D3 and between D2 and D4, have been proposed to share a common evolutionary ancestor.

Of note, in cloning of murine LAG-3, Mastrangeli and colleagues describe interesting motifs in the cytoplasmic domain, which is distinctly different from CD4 [169]. There are three conserved regions between murine LAG-3 and human LAG-3: (1) a potential serine phosphorylation site, which may be analogous to the CD4 protein kinase C binding site, (2) a
conserved KIEELE motif with no homology to other proteins, (3) an unusual glutamic acid-proline (EP) repetitive sequence. The latest motif is postulated to mediate interactions with a broad range of molecules or ubiquitous proteins that perform a common function. Signaling molecules, such as platelet-derived growth factor receptor (PDGFR), contain an EP domain, and thus the cytoplasmic tail is implicated in a potential role in intracellular signaling and molecular aggregation [170]. Interestingly, deletion of this cytoplasmic tail has been demonstrated to result in the loss of LAG-3 inhibitory effects on CD4+ T cell function [170].

Tissue Distribution. Initial studies of LAG-3 expression were performed in healthy human tissues, which exhibited little to no LAG-3+ cells in peripheral blood mononuclear cells (PBMCs), primary lymphoid tissues, such as thymus and bone marrow, and secondary lymphoid tissues, such as spleen, mucosa-associated lymphoid tissue, and lymph nodes [171]. However, in hyperplastic lymph nodes, germinal centers and interfollicular areas consistent with T cell populations immunostained positively for LAG-3. Moreover, cancer patients with high-dose IL-2 infusions had elevated numbers of LAG-3+ PBMCs. It should be noted that only a fraction of the LAG-3+ cells were CD25+, a marker on activated T and B cells.

The distribution of murine LAG-3 expression by Western blot exhibits strong signal in the thymus, spleen, and areas of the brain outside of the cerebellum [157]. Interestingly, in situ hybridization revealed LAG-3 gene expression is restricted to sparse cells throughout the cortex and confirmed the absence of LAG-3 in the cerebellum. In contrast, day 7 postnatal brain exhibited LAG-3 gene expression in defined tracts at the base of the cerebellum, in the
developing white matter, and in the choroid plexus. A similar study in the rat revealed a slightly expanded distribution with LAG-3 message also appearing in the lung, liver and small intestine, and again, there was also strong message in the brain tissue [172]. In rodent tissue, the expression pattern of LAG-3 mRNA largely correlates with tissues that contain active T cell and NK cell tissue, with the exception of the brain.

LAG-3: A Negative Regulator of T Cells. The initial observation that LAG-3 had an inhibitory effect was suggested by the upregulation of CD4+ T cell clone activation. Soluble anti-LAG-3 antibodies that effectively blocked MHCII interaction increased thymidine incorporation in CD4+ T cells, as well as enhanced CD25+ expression and production of IFNγ or IL-4 [160]. This study was followed up by Hannier and colleagues, who demonstrated that binding of LAG-3 antibody to LAG-3 followed by crosslinking (LAG-3XL) led to inhibition of cell proliferation and cytokine secretion by pre-activated T cells [161]. Importantly, the physical interaction of LAG-3 and TCR/CD3 was demonstrated by LAG-3-XL down-modulation of CD3/TCR, inhibition of calcium response to CD3 stimulation upon LAG-3 and CD3 co-engagement, and capping of CD3 resulted in co-capping of LAG-3 molecules. Ultimately, this study demonstrated the inhibitory effects of LAG-3 signal on TCR-induced activation.

It has been postulated that “supramolecular assemblies” between LAG-3, CD3, CD8, and MHCII may result from the organization within lipid raft microdomains [173, 174]. These microdomains provide clustering sites for signaling molecules and are required for efficient T cell activation. Clustering of rafts brings the TCR and MHC molecules within the
vicinity of each other, forming an immunological synapse, for the purpose of TCR antigen recognition. Hannier and colleagues demonstrated that engagement of CD3/TCR complexes with anti-TCR-specific antibodies or MHC-peptide complex recognition in T cell-B cell conjugates results in LAG-3 being co-clustered with the CD3/TCR complexes [174]. Moreover, following CD8 or MHCII engagement, LAG-3 also was found co-clustered with the respective molecules. A follow-up study by the same group found LAG-3 and MHCII in the cell fractions of these detergent-insoluble lipid rafts after centrifugation of Triton X-100-treated activated T cells on sucrose gradients [173]. These studies propose a means by which LAG-3 can physically engage respective immune molecules. The most telling experiments of the negative regulatory capacity of LAG-3 are done in the LAG-3−/− mouse. In 2004, Workman et al. demonstrated (1) LAG-3−/− T cells exhibited a delay in cell cycle arrest upon in vivo stimulation with staphylococcal enterotoxin B, thus leading to increased T cell expansion and splenomegaly, (2) increased T cell expansion in adoptive recipients of LAG-3−/− OT-II TCR transgenic T cells following antigen stimulation in vivo, (3) Sendai-viral infection in LAG-3−/− mice increased memory CD4+ and CD8+ T cells, and (4) LAG-3−/− mice infected with murine gammaherpesvirus-68 exhibited a delayed CD4+ T cell expansion [175]. Ultimately, these studies confirmed the role of LAG-3 in regulating expansion of activated T cells and the development of the memory T cell pool.

Recent studies have focused on LAG-3 and regulatory T cells (Tregs), a cell type that functions in the inhibition of effector T cells and in maintenance of immune homeostasis. A pivotal study in 2004 revealed that LAG-3 is selectively upregulated on Tregs [176]. Moreover, this study demonstrates that LAG-3 inhibits the suppressive functions of Tregs in vitro and in vivo and that ectopic expression of LAG-3 on CD4+ T cells is sufficient for
reducing proliferation. Interestingly, studies in \( LAG-3^{-/-} \) demonstrate that LAG-3 regulates T cell homeostasis by Treg-dependent and independent mechanisms [177]. In the absence of LAG-3, Tregs were unable to sufficiently maintain optimal control of T cell homeostasis \textit{in vivo}. Furthermore, dysregulation of T cell homeostasis led to expansion of multiple cell types, such as B cells, macrophages, granulocytes, and dendritic cells.

The evidence of negative regulatory function of LAG-3 on T cells is ever-expanding [178]. Interestingly, despite LAG-3 expression in the brain, very few T cells are present in the physiological state. It can be inferred that LAG-3 may be expressed on a different cell type in the CNS, as will be addressed in Chapter 3.

\textit{LAG-3: An APC Activator.} MHCII signal transduction has been demonstrated on several cell types including B cells, T cells, and monocytes using primarily antibodies to MHCII. Although the interaction of MHCII signal transduction is presumed to be engagement of TCR, recent evidence indicates that LAG-3 may also activate monocytes and dendritic cells [164]. LAG-3 in conjunction with CD40L was shown to activate TNF-\( \alpha \) and IL-12 secretion from dendritic cells. Blocking the LAG-3/MHCII engagement prevented signaling from MHCII\(^+\) cells and also inhibited T cell responsiveness to IL-12. However, this study was conducted with human T cells stimulated with IL-2, and it is plausible that interaction could be different with Tregs interacting with dendritic cells to achieve immunosuppression [178].

\textit{LAG-3 in multiple sclerosis.} Due to the lack of research on LAG-3 in the CNS, there has been only one reported link to CNS disease. In 2005, LAG-3 was flagged as a potential
susceptibility marker for MS [179]. However, since this initial study, LAG-3 association with risk of multiple sclerosis (MS) has become debatable. The LAG-3 gene was studied in two independent sample sets of MS patients and healthy controls [179, 180]. Although the gene was reported initially to be associated with risk of multiple sclerosis (MS) [179], it failed to be replicated in an independent case-control study using a larger patient pool [180]. It must be noted that the lack of genetic evidence for a role in determining risk of disease does not preclude the potential importance for LAG-3 in MS pathogenesis.

1.4 The Cuprizone Intoxication Model of Demyelination and Remyelination

Cuprizone (bis-cyclohexanone oxalylhydrazone) was characterized in the late 1960s as a CNS demyelinating agent when fed in low doses to mice [181, 182]. This toxic insult resulted in spongy degeneration and oligodendrocyte cell death [182-184]. By the early 1970s, Blakemore comprehensively characterized cuprizone-induced demyelination and remyelination after the removal of cuprizone from the diet [185, 186]. Importantly, Blakemore’s characterization established reproducibility in myelination patterns, as well as activation of glial cells and their recruitment during temporally-defined stages of cuprizone treatment. The majority of these earlier studies in understanding of demyelination and remyelination relied heavily on morphology through electron micrographs, and there was only a limited immunohistochemical analyses. With the availability of genetic knockout and transgenic mice, the cuprizone intoxication model has since been adapted to the C57BL/6 background [187]. Moreover, our laboratory has re-characterized a lower dose of cuprizone treatment, compared to the dosing in previous strains of Swiss Webster, Swiss albino, or ICI
mice, that allows efficient and reproducible demyelination of the corpus callosum without systemic toxicity [187]. More importantly, a different pattern of demyelination and remyelination has been described for C57BL/6 mice that suggest strain variability with regards to disease presentation [188].

Mechanism of Action. Cuprizone is a copper chelator and when bound to copper forms a highly chromogenic complex that possesses very intense and unusual absorption band in the visible region, centered at 595 nm [189]. Thus, it is often exploited in clinical chemistry for spectrophotometric determinations of copper [189]. However, in mice dosed with cuprizone, the mechanism by which cuprizone induces CNS demyelination is unknown. Copper is an essential trace element and is a cofactor in many metalloenzymes [190]. Cuprizone exposure results in a copper deficiency in the brain, as well as a decrease in copper-dependent enzymes, such as monamine oxidase and cytochrome oxidase [191, 192]. Chelation of cuprizone prior to administration prevented these symptoms [191]. However, administration of dietary copper does not alleviate cuprizone-induced neurotoxic effects [182].

It is hypothesized that cuprizone may inhibit mitochondrial enzymes, such as monamine oxidase, resulting in metabolic perturbation and cell injury [191]. Interestingly, oligodendrocytes appear to be preferentially susceptible to cuprizone effects, as several oligodendrocyte-associated proteins are down regulated upon treatment before histological demyelination is evident: glutathione-S transferase (GSTpi), 2’3’-cyclic nucleotide 3’-phosphohydrolase (CNPase), carbonic anhydrase II (CAII), and myelin proteins, such as myelin-associated glycoprotein (MAG) and myelin basic protein (MBP) [193-196]. Minor
irregularities have been observed in the mitochondria of oligodendrocytes [183, 197]. Thus, this preferential susceptibility to cuprizone may be a result of the inability of the oligodendrocyte to meet the high metabolic demand of maintaining the myelin sheath.

Oligodendrocytes have been demonstrated to undergo apoptosis during cuprizone intoxication [198]. A recent study demonstrated *in vivo* inhibition of microglial activation with minocycline prevented cuprizone-induced demyelination in Swiss mice [199]. Based on *in vitro* experiments in primary rat oligodendroglial cultures, this group attributed oligodendrocyte protection to the absence of proinflammatory cytokines, such as TNF-α and IFNγ, produced by the otherwise present microglia [199]. This suggests that microglial factors may contribute to the cuprizone insult to tip oligodendrocytes towards apoptosis. Regardless of the exact mechanism, cuprizone administration triggers oligodendrocyte cell loss with subsequent CNS demyelination.

**Historical perspective.** Initial characterization of cuprizone intoxication was reported in the late 1960s to cause widespread spongiosis and oligodendrocyte cell death in the brain, often accompanied by hepatic disturbance [181-184]. These observations were made in various mouse strains: Swiss-Webster, Swiss albino, and ICI. Interestingly, this spongy degeneration in cuprizone-fed mice was similar to that in found in scrapie [200, 201].

In 1973, Blakemore published the first comprehensive studies characterizing glial responses and myelination in the superior cerebellar penducle of ICI mice during 0.5% cuprizone-induced demyelination and remyelination after the removal of cuprizone from the diet [185, 186]. As early as 1 week of cuprizone treatment, pyknotic oligodendrocytes were
observed prior to the presence of demyelinated axons. By 2 weeks of cuprizone treatment, myelin sheaths displayed signs of disintegration, partially attributed to microglial stripping of myelin lamellae and phagocytosis of vacuolated myelin [184]. Glial populations, namely microglia and astrocytes, accumulated dramatically during demyelination and were found in direct proximity to naked axons. Upon removal of cuprizone administration, these glial populations diminished. As mentioned previously, Blakemore’s characterization established reproducibility in myelination patterns, as well as activation of glial cells and their recruitment during temporally-defined stages of cuprizone treatment. Moreover, he demonstrated remyelination could be studied in a model that was highly reproducible.

Swiss mice studies with 0.6% cuprizone administration displayed a similar temporal ability to demyelinate upon intoxication and remyelinate upon cuprizone removal [197]. In these studies, Ludwin observed myelin wrapping during remyelination was comparable to that in development. Moreover, he suggests that the population of remyelinating oligodendrocytes are comprised of residual surviving oligodendrocytes, as well as newly proliferating and differentiating OPCs. It should be re-noted that in C57BL/6 mice, Mason et al. demonstrated the apoptosis of oligodendrocytes, suggesting repopulation is from new population of proliferating and differentiating OPCs [198].

In addition to demyelination and remyelination, axonal integrity has been examined thoroughly in cuprizone-induced chronic demyelination. Axons in Swiss mice subjected to cuprizone treatment for 6 to 7 months retained their capacity to remyelinate [202]. Moreover, axonal diameter was only slightly decreased. Since these studies, Mason et al. characterized chronic cuprizone treatment in C57BL/6 mice and found that the regenerative capacity of axons were exhausted by 16 weeks, when the mice were near death [188].
Moreover, this study demonstrated a significant reduction in axonal diameter during acute demyelination that returned to near-normal upon remyelination. Furthermore, in mice chronically-treated for 16 weeks, axons were reduced to 60% of normal axonal diameter. Recent studies by Blakemore suggest aged mice (6-7 months), compared to young adult mice (7-8 week old), respond to cuprizone with greater incidence of axon transaction, greater axonal loss in the medial corpus callosum, and a more robust glial response [203]. Retrospectively, these studies demonstrate a genetic- and age-susceptibility to axonal degeneration associated with demyelination.

There are several features of the cuprizone intoxication model that distinguish it from others models of demyelination, such as EAE, Mouse Hepatitis Virus (MHV) or Theiler’s murine encephalitis virus (TMEV). The cuprizone model, despite various dose concentrations and mouse strains, displays temporally and spatially consistent demyelinated lesions throughout the brain, such as the superior cerebellar penducle and corpus callosum. The lack of anatomical reproducibility in EAE or viral models hampers the ability to make reliable assessments of changes in cellular populations, myelination quantifications, and biochemical analyses. Additionally, the BBB in the cuprizone model remains intact, as demonstrated by horse radish peroxidase (HRP) tracing and immunohistochemical staining for extravasated serum proteins [92, 204, 205]. Likely due to this intact BBB, the cuprizone intoxication model also lacks a significant role in T cell immune responses [92, 184, 197]. RAG-1−/− mice display a phenotypically similar response to cuprizone as wild type mice during demyelination and remyelination [49, 93]. Moreover, the infiltration of CD3+ into a 5-week cuprizone-treated corpus callosum is less than 2 cells/mm² [92]. In general, the only immune cells present in the demyelinated area appear to be microglia/macrophages and
astrocytes [190]. Lastly, the temporal and spatial predictability of the cuprizone models allows consistent and reliable assessment of remyelination and underlying processes.

_Cuprizone-induced Demyelination and Remyelination Time Course in C57BL/6._ As mentioned previously, our laboratory has adapted the cuprizone model to the C57BL/6 mouse [187]. The dose of cuprizone has been titrated to 0.2%, which allows efficient and reproducible demyelination of the corpus callosum without systemic toxicity, evidenced by liver toxicology [187]. By light microscopy, demyelination can be observed by 3 weeks of cuprizone treatment with progression to near-complete demyelination by 5 or 6 weeks; however, subsequent experiments suggest full demyelination is reached by week 5 and remyelination begins at week 6 [49, 188, 206]. By electron microscopy, observable demyelination occurs after 2 weeks of treatment, when oligodendrocyte apoptosis is also evident [188, 198]. Similar to previously cuprizone studies, microglia/macrophage and astrocyte accumulation was present [185, 187]. Microglia/macrophage accumulation is observed as early as 1 week of cuprizone treatment and dramatically increases by 3 weeks, with a continual rise through 6 weeks of treatment. Although 90% of the recruited peripheral cells were macrophages, they were far outnumbered by resident microglia, comprising approximately 2-4% of the myeloid cells in the corpus callosum [92]. Astrocyte accumulation begins by 3 weeks of cuprizone treatment and also continually rises through 6 weeks [187]. As noted before, although some T cells are observed in brains of cuprizone-treated mice, the number is negligible compared to the other cellular populations [92]. Furthermore, studies in _RAG-1^{-/-}_ demonstrate that the absence of T cells does not affect cuprizone-induced demyelination or remyelination [49, 93].
In addition to demyelination, studies from our laboratory and others also have defined cellular events in the C57BL/6 mice during remyelination upon the removal of cuprizone administration. Oligodendrocyte mRNA patterns, i.e. MAG and myelin oligodendrocyte glycoprotein (MOG), in C57BL/6 mice were consistent with previous cuprizone studies [193, 195, 207]. OPCs could be identified in increased numbers as early as 1 week of cuprizone exposure and continually increased and peaked at 4 weeks, when morphological changes were observed and were attributed to maturation [198]. Many of these cells were actively dividing, as demonstrated by BrdU (5’-bromo-2’-deoxyuridine) incorporation [198]. Mature oligodendrocytes re-appear in the demyelinating lesion at 6 weeks, despite continued cuprizone administration [198]. Upon removal of cuprizone, the oligodendrocyte population continued to repopulate the demyelinated lesion, which was accompanied by increasing myelination [198]. Thus, this pattern permits the study of acute demyelination and full remyelination or, with continuous administration of cuprizone, a spontaneous but limited remyelination first observed at week 6, followed by a second demyelination and then second but feeble remyelination observed at week 12 [188]. Afterwards, demyelination ensues and the lesion remains permanently demyelinated. This is presumably due to a lack of OPCs as adoptive transfer of OPCs injected into the lesions restores myelination to many of the demyelinated fibers (Mason et al, 2004).

Cuprizone Intoxication: A Model for MS-like Lesions. Multiple sclerosis is a chronic inflammatory disease of the CNS that is characterized by large focal lesions or plaques of primary demyelination with relative sparing of axons (50-70% reduction of neurite density in chronic plaques) [208]. Relapsing-remitting functional impairments in patients are caused
mainly from inflammation and demyelination, while the accumulation of irreversible neurological deficit is caused mainly by axonal destruction and loss.

Although MS is widely accepted as an autoimmune disorder, this concept has been challenged due to the heterogeneity of MS lesions. In 2000, Lucchinetti and colleagues categorized MS lesions into four patterns based on myelin protein loss, geography and extension of plaques, patterns of oligodendrocyte destruction, and immunopathological evidence of complement activation (Table 1.1) [209]. In Patterns I and II, lesions are similar to those found in EAE with active demyelination mainly associated with T-cell- and macrophage-mediated inflammation. Pattern II is distinctive from Pattern I with immunoglobulin and complement deposition (C9neo antigen) at the site of active myelin destruction. Pattern III and IV also contain inflammatory T cells and macrophages in the active lesion but are mainly characterized by features that suggest primary loss of oligodendrocytes rather than autoimmunity. Moreover, Pattern III oligodendrocyte loss is attributed to apoptosis. Similar to Pattern III, newly forming lesions in relapsing-remitting MS patients exhibit common early structural changes, being extensive oligodendrocyte apoptosis and microglial activation [210]. Interestingly, these newly forming lesions had few to no T cells.

Cuprizone intoxication produces lesions that appear similar to Pattern III lesions and newly forming lesions with extensive oligodendrocyte cell death, microglial activation, and lack of T cell involvement [49, 93, 187, 190]. Although cuprizone is an artificial compound and should not be considered a cause of MS, it should be mentioned that other copper chelators are present in the environment [190]. Moreover, the cuprizone intoxication model
allows the investigation of microglia/macrophage function in demyelination outside the context of a T cell immune response.

_Cuprizone Studies in Genetic Knockout and Transgenic Mice._ Since adapting the cuprizone intoxication model to the C57BL/6 background, a tremendous effort has been made to understand underlying processes and mechanisms involved in demyelination and remyelination _in vivo_. The use of genetic knockout and transgenic mice has been invaluable in isolating the contribution of immune molecules, such as cytokines and surface receptors, and growth factors to specific roles in a demyelinating lesion and/or a lesion undergoing repair. Several of these studies from our lab and others are summarized below.

MHCII has been demonstrated to exacerbate demyelination [93]. Moreover, the role of MHCII is dependent on an intact cytoplasmic tail. Mice deficient in MHCII (_I-A\(^{\beta}\)\(^{-/-}\)) or mice with a truncated I-A\(_{\beta}\) (_I-A\(^{\beta\text{tr}}\)), which have intact extracellular and transmembrane sequences but lack the cytoplasmic domain, exhibited a delayed demyelination and reduced oligodendrocyte apoptosis during demyelination compared to wild type mice. Furthermore, microglia/macrophage accumulation in the corpus callosum of cuprizone-treated mice was decreased significantly. Diminished IL-1\(\beta\) and TNF-\(\alpha\) production in the brains of cuprizone-treated _I-A\(_{\beta}\)\(^{-/-}\) and _I-A\(_{\beta\text{tr}}\) mice indicated an attenuated immune response. Interestingly, MHCII also has been demonstrated to be involved in the regeneration of oligodendrocytes and subsequent remyelination [23]. _I-A\(_{\beta}\)\(^{-/-}\) mice exhibit a delay in mature oligodendrocyte population that is accompanied by a delay in remyelination. Thus, as with many immune
molecules, MHCII appears to play conflicting roles in the demyelinating and repair processes.

Like $I-A_\beta^{\Delta}^+$ mice, $TNF-\alpha^{\Delta}^+$ mice exhibit a delay in demyelination and oligodendrocyte apoptosis yet also exhibit a delay in remyelination and oligodendrocyte repopulation [49]. This is likely attributed to the two distinctive signaling pathways mediated through TNFR1 (p55), which mediates cell death, and TNFR2 (p75), which can either enhance cell death or promote cell activation, growth, or proliferation. Notably, TNF-α is upregulated in MS and, thus, was flagged as a potential therapeutic target. However, upon inhibition of TNF-α, MS patients experienced increased relapses and CNS inflammation (The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1999). This is likely due to the hampering of the beneficial effects of TNF-α.

LTα is structurally similar to TNF-α and belongs to the TNF superfamily. $LT\alpha^{\Delta}^+$ mice exhibit a delay in demyelination that far exceeds that of TNF-α and is accompanied by a delay in oligodendrocyte loss [50]. In contrast to $TNF-\alpha^{\Delta}^+$ mice, $LT\alpha^{\Delta}^+$ mice exhibited remyelination that was similar to wild type. Upon examination of LTβR, the receptor to which LTαβ heterotrimer binds, studies demonstrated deletion of the gene resulted in delayed demyelination with a slight delay in remyelination [57]. Perplexingly, inhibition of LTβR signaling by an LTβR-Ig fusion decoy demonstrated successfully delaying demyelination and accelerating remyelination [57]. These studies bode well for targeting LTα as a therapeutic agent and for the use of LTβR-Ig as a therapeutic agent in demyelinating disorders.

Transgenic mice also have shown the protective effects of immune modulators against cuprizone intoxication. $IGF-I^{Tg/o}$ mice demyelinated with less severity and exhibited
a shortened cuprizone-induced demyelination with near-complete recovery by 5 weeks of cuprizone treatment [206]. Interestingly, this rapid recovery was attributed to the survival of mature oligodendrocyte upon initial cuprizone insult, thus enabling them to restore myelination earlier. This study suggests that IGF-1 is important in prevent oligodendrocyte depletion during demyelination.

The use of genetic knockout and transgenic mice in the cuprizone model has elaborated the function of several other genes: iNOS [211], neuronal nitric oxide synthase (nNOS) [212], FGF [213, 214], growth arrest-specific 6 (GAS6) [215], CXCR2 [216], macrophage inflammatory protein-1α (MIP-1α) [217], leukemia inhibitory factor (LIF) [218], TNF-like weak inducer of apoptosis (TWEAK, TNFSF12, Apo3L) [219], PDGF [220, 221], IFNβ [222], IFNγ [223, 224], acyl-CoA synthetase lipidosin [225], OLIG1/2 [226], p8 [227], p75NTR [228], Notch1/Jagged1 [229], and osteopontin [230]. Furthermore, this model has utility in assessing hormones, as well as drugs and growth factors on prevention of demyelination or promotion of remyelination. Interestingly, a cocktail of PDGF, bFGF, NT-3 and IGF-1 was injected intracerebroventricularly into cuprizone-treated mice and showed increased OPC in the corpus callosum that resulted in greater myelinated fibers [231]. Thus, there is an enormous potential of examining currently known factors and yet to be identified genes and compounds that may provide insights to basic demyelination and remyelination processes, and this model will continue to be invaluable in identifying potential therapeutic agents.
TABLE 1.1. Structural and Immunological Features of Different Patterns of Active Multiple Sclerosis Lesions.

<table>
<thead>
<tr>
<th>FEATURE</th>
<th>PATTERN I</th>
<th>PATTERN II</th>
<th>PATTERN III</th>
<th>PATTERN IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammatory infiltrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3⁺ T cells</td>
<td>197 ± 68</td>
<td>133 ± 18</td>
<td>145 ± 23</td>
<td>134 ± 71</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>5.9 ± 1.9</td>
<td>9.3 ± 2.1</td>
<td>5.4 ± 1.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Microglia/macrophages</td>
<td>1158 ± 105</td>
<td>931 ± 71</td>
<td>842 ± 91</td>
<td>1650 ± 30</td>
</tr>
<tr>
<td>C9neo</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Demyelination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perivenous pattern</td>
<td>±</td>
<td>+</td>
<td>–</td>
<td>±</td>
</tr>
<tr>
<td>Lesion edge</td>
<td>Sharp</td>
<td>Sharp</td>
<td>ill-defined</td>
<td>Sharp</td>
</tr>
<tr>
<td>Concentric patter</td>
<td>0/10</td>
<td>0/45</td>
<td>8/25</td>
<td>0/3</td>
</tr>
<tr>
<td><strong>Oligodendrocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># Ols in inactive plaques</td>
<td>295 ± 73</td>
<td>249 ± 30</td>
<td>51 ± 24</td>
<td>55 ± 55</td>
</tr>
<tr>
<td>OL nuclear DNA fragmentation</td>
<td>±</td>
<td>–</td>
<td>++</td>
<td>++ (periplaques)</td>
</tr>
<tr>
<td>OL apoptosis in active plaques</td>
<td>–</td>
<td>–</td>
<td>14-37%</td>
<td>–</td>
</tr>
<tr>
<td>Myelin protein loss</td>
<td>Even</td>
<td>Even</td>
<td>MAG &gt;&gt; others</td>
<td>Even</td>
</tr>
<tr>
<td><strong>Remyelination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shadow plaques</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Values given in cells/mm².

Modified from [209].
CHAPTER 2

IL-1α and IL-1β DIFFERENTIALLY AFFECT MATURE OLIGODENDROCYTES

Modified version submitted as: Vivian S. Chen, David D. Chaplin, Yoichiro Iwakura, Glenn K. Matsushima. IL-1α and IL-1β Differentially Affect Mature Oligodendrocytes. *Journal of Neuroscience Research.*
2.1 ABSTRACT

IL-1 is the archetypical proinflammatory cytokine and can be clinically beneficial or detrimental in human diseases. It has been reported to affect many cell types and is frequently studied in the context of inflammation. The IL-1 gene family contains two established functional agonists that are closely related in ancestry and structure: IL-1α and IL-1β. IL-1 has been implicated in the pathology of many neurological diseases, such as multiple sclerosis. In the brain, the activation of microglia/macrophages and astrocytes results in the production of inflammatory mediators, such as IL-1. In this study, we examined the individual roles of IL-1α and IL-1β and their effects on myelination and cellular populations during neurodegeneration and recovery. After extensive backcrossing of IL-1α−/−, IL-1β−/−, and IL-1α/+β−/− mice onto the C57BL/6 background, we demonstrate that, while neither family member affects demyelination or remyelination, an interesting phenotype emerged in which IL-1β−/− and IL-1α/+β−/− mice show reduced numbers of mature oligodendrocytes in the corpus callosum, before and after cuprizone treatment.

2.2 INTRODUCTION

Neuroinflammation is an integral component of demyelinating disease, such as multiple sclerosis, and often leads to oligodendrocyte damage and depletion [210]. Inflammatory cytokines conventionally are believed to contribute to oligodendrocyte death
and myelin destruction [232-235]; however, they may also contribute to CNS repair by recruitment and differentiation of oligodendrocyte progenitors [49, 57, 236, 237]. Thus, there appears to be a subtle balance between inflammation and neuroregeneration.

The IL-1 gene family contains two functional agonists: IL-1α and IL-1β (reviewed in [102]). Both molecules are synthesized in pro-forms. IL-1α can be active in its pro-form and can be cleaved by membrane-associated cysteine protease calpains to an active 17 kDa form. ProIL-1β is cleaved by caspase-1 and is only active in its 17 kDa secreted form. Despite no significant differences in the range of activities induced by recombinant IL-1α and IL-1β, the two agonists differ in the cellular compartments in which they primarily are active. IL-1β is secreted mainly by monocytes and macrophages but can be secreted by other cell types. IL-1α is less abundant in the body, and although it is synthesized in large quantities in cells, such as keratinocytes, it appears to be efficiently released only by activated macrophages. Although other cell types express cell-associated IL-1α, they do not secrete the 17 kDa form unless substantial cell injury occurs. IL-1α and IL-1β bind a common receptor, IL-1 type I receptor, which recruits the IL-1 receptor accessory protein to initiate signal transduction, resulting in similar but not completely overlapping responses to the two agonists [102, 238, 239].

In the healthy CNS, IL-1 is expressed at low constitutive levels [114]. Whether this endogenous level of IL-1 participates in the physiology of a healthy brain is unknown, as IL-1α^{−/−} or IL-1β^{−/−} mice appear to develop normally [115, 116]. Upon experimental insult, such as ischemia or neurotoxic stimuli, IL-1 expression is quickly upregulated [51, 103, 240-242]. IL-1 is expressed first by microglia and is followed by astrocytes, infiltrating cells, oligodendrocytes, and neurons [128, 129, 243]. It remains debatable whether this IL-1
response is involved in neurodegeneration or as part of an inflammatory response to resolve
the insult. The differential roles of IL-1α and IL-1β in the context of disease has become
more prominent with the development of IL-1 knockout mice: \( IL-1\alpha^{-/-}, \) \( IL-1\beta^{-/-} \) and \( IL-1\alpha^{-/-}\beta^{-/-} \) mice [115, 116]. To address the roles of IL-1α and IL-1β in demyelination and
remyelination, we applied the cuprizone intoxication model, which allows the analysis of
neuroinflammation, demyelination, and remyelination in a predictable time course and
pattern, to these knockout mice [183, 184, 187, 190, 195]. Previously, our lab published that
\( IL-1\beta^{-/-} \) mice display a delay in remyelination, resulting from a delay in mature
oligodendrocyte repopulation [51]. Since our initial report, we have backcrossed the \( IL-1\beta^{-/-} \) strain further onto the C57BL/6 background. In this study, we report a phenotypic change in
these backcrossed mice in which there are inherently fewer mature oligodendrocytes.
Additionally, we characterize the role of IL-1α in cuprizone-induced demyelination and
remyelination.

2.3 MATERIALS AND METHODS

Mice. All mice were housed in a pathogen-free facility and were maintained in accordance
with NIH guidelines and approved protocols by the University of North Carolina Institutional
Animal Care and Use Committee and the University of North Carolina Division of
Laboratory Animal Medicine. C57BL/6 mice were either purchased from Jackson
Laboratories (Bar Harbor, ME) or bred in-house at the UNC animal facility. \( IL-1\beta^{-/-} \) mice on
the C57BL/6 background [116] were obtained from Dr. David D. Chaplin (University of
Alabama at Birmingham) and were backcrossed further onto our in-house C57BL/6 background (N>6), originally obtained from Jackson Laboratories. *IL-1α−/−* and *IL-1α−/−β−/−* mice [115] were obtained from Dr. Yoichiro Iwakura (The Institute of Medical Science, The University of Tokyo) and bred onto our in-house C57BL/6 background (N>6). C57BL/6 mice hemizygous for the green fluorescent protein (GFP) gene under the control of the chicken β-actin promoter and cytomegalovirus (CMV) enhancer were bred in-house with wild type C57BL/6 mice (C57BL/6 *GFP* Tg/o, N>12). These GFP transgenic mice were originally obtained from Jackson Laboratories, and their generation and breeding have been previously described [244, 245].

*Bone Marrow Transplant and Flow Cytometric Analysis of Bone Marrow Engraftment.*

Methods for bone marrow transplants have been previously modified and described [92]. Briefly, C57BL/6 *GFP* Tg/o and IL-1β−/− *GFP* Tg/o chimeras were generated by lethal irradiation of 4-5 week old C57BL/6 or IL-1β−/− male mice with a total of 800 cGy at a dose rate of 50 cGy/minute using a 60Co γ irradiator (Atomic Energy of Canada, Kanata, Ontario). Following overnight recovery, the irradiated mice were injected intravenously through the tail vein with 3-5 x 10^7 bone marrow cells flushed and isolated from the femurs and tibias of donor C57BL/6 *GFP* Tg/o male mice as previously described [92, 246]. No antibiotic treatment was given and animal mortality from bacterial infections after irradiation did not occur. Animals were allowed to reconstitute for 4 weeks. PBMCs were isolated as described previously for flow cytometric analysis to check for near to complete reconstitution before beginning cuprizone treatment [92, 246].
**Cuprizone Treatment.** 8-10 week old male mice that were approximately 25 grams were fed 0.2% (w/w) cuprizone (Sigma, St. Louis, MO) mixed in ground rodent chow (ProLab IsoPro RMH 3000, LabDiet, Brentwood, MO) for up to 6 weeks to induce demyelination [187]. At 6 weeks of treatment when remyelination begins, the mice were returned to a normal diet. Untreated male mice were maintained on normal diet for the duration of the experiment.

**Tissue Preparation.** Mice were perfused intracardially with 0.15 M phosphate buffer, pH 7.4, [247] for 1 minute followed by 4% paraformaldehyde (PFA) in phosphate buffer for 8 minutes. Brains were removed and postfixed in 4% PFA for a minimum of 24 hours at 4°C before processing and embedding by the UNC Lineberger Comprehensive Cancer Center Histopathology Core. Five-micron coronal sections of the corpus callosum at midline between region 220 and 260 of the Atlas of the Mouse Brain and Spinal Cord were used for histology and immunohistochemistry [248].

**Luxol Fast Blue-Periodic Acid Schiff’s Staining.** Paraffin sections were stained with a Luxol fast blue-periodic acid Schiff (LFB-PAS) stain as previously described [187]. Myelination was scored on a scale of 0 to 3. For myelination, a score of 0 was equivalent to no myelinated axons in the demyelinated region, and a score of 3 was indicative of full myelination.
Transmission Electron Microscopy. Mice were perfused intracardially with 0.15 M phosphate buffer [247] for 1 minute followed by 4% PFA and 2.5% glutaraldehyde in phosphate buffer for 10 minutes. Mice were postfixed in 4% PFA and 2.5% glutaraldehyde for a minimum of 2 weeks. The region of the corpus callosum corresponding to the region in sections 220-260 of the Sidman mouse brain atlas was cross-sectioned and stained in partial collaboration with Clarita Langaman and the UNC Microscopy Services Laboratory. Corpora callosa were analyzed using Image-Pro Plus v.5.0.2.9 (Media Cybernetics, Inc., Bethesda, MD). A minimum of five micrographs per mouse were quantified for the number of myelinated and unmyelinated axons. Axons below 0.3 μm in diameter are rarely myelinated and were excluded. Each treatment group contained a minimum of three mice.

Immunohistochemistry and Lectin Staining. Paraffin sections were deparaffinized with Histo-Clear (National Diagnostics, Atlanta, GA) and rehydrated through a series of graded ethanols. GSTpi immunohistochemistry was performed to identify mature oligodendrocytes as described previously [51]. Briefly, sections were blocked and permeabilized in 2% normal goat serum (NGS) and 0.1% Triton X-100 in PBS for 30 minutes at room temperature and then unmasked with 0.1% trypsin in a 50 mM Tris solution (pH 7.4) for 15 minutes at 37°C. Sections were then incubated with rabbit anti-GSTpi antibody (1:1000, Biotrin, USA) for overnight at 4°C. Tissues were rinsed and incubated with Alexa Fluor goat anti-rabbit IgG (1:400, Invitrogen/Molecular Probes). Apoptosis was determined by In Situ Cell Death Detection, Fluorescein (Roche Applied Science, Indianapolis, IN). Ricinus communis agglutin-1 (RCA-1) lectin stain was performed to identify microglia/macrophages as described previously [187]. Briefly, sections were unmasked with 0.025% protease, type
XIV (Sigma) for 2 min at 43°C, blocked and permeabilized with 5% bovine serum albumin (BSA) and 0.1% Triton X-100 in phosphate buffered saline (PBS), and incubated with biotinylated RCA-1 (1:500, Vector, Burlingame, CA) for overnight at 4°C. Slides were washed and incubated with Alexa Fluor Strepavidin (Invitrogen/Molecular Probes). GFAP immunohistochemistry was performed to identify astrocytes as previously described [187]. Briefly, sections were blocked and permeabilized in 2% NGS and 0.1% Triton X-100 in PBS for 30 minutes. Sections were incubated in rat anti-GFAP antibody (1:400, EMD/Calbiochem, San Diego, CA) for overnight at 4°C. Tissues were then washed and incubated with Alexa Fluor goat anti-rat IgG (1:400 Invitrogen/Molecular Probes). All slides were mounted in Vectashield HardSet with DAPI (Vector) in order to visualize nuclei.

Image-Pro Plus was used to count and calculate cells per mm² from digital images. Quantification included cells in which a nucleus was colocalized with immunohistochemical staining.

Statistics. Data are expressed as mean ± SEM. Multiple comparisons were evaluated using a two-tailed Student’s *t*-test. Differences were considered statistically significant if *p* ≤ 0.05.

2.4 RESULTS

*IL-1β*⁻⁻⁻ mice lost their previously characterized myelination phenotype after extensive backcrossing to *C57BL/6*. *IL-1β*⁻⁻⁻ mice were previously characterized to display a
demyelination phenotype similar to wild type mice during cuprizone-intoxication yet a
delayed or impaired ability to remyelinate upon discontinuation of cuprizone treatment [51].
Moreover, our laboratory has demonstrated that 90% of recruited myeloid cells to the corpus
callosum in cuprizone-treated mice are microglia and approximately 4% are peripheral
macrophages [92]. Because cuprizone-induced demyelination is characterized by a robust
microglia/macrophage response, our objective at first was to determine whether
microglia/macrophages, in terms of IL-1β production, play an important role in promoting
remyelination of the CNS [92, 187]. Four- to five-week old IL-1β−/− mice and wild type
controls were lethally irradiated and subsequently received bone marrow transfers from
GFPTg/o mice. Four weeks later, bone marrow engraftment was measured by flow cytometry
of PBMCs. While background fluorescence was minimal in C57BL/6 mice (1%), 80% of the
PBMCs were GFP+ in the GFPTg/o mice. In the chimeric mice, 83% of the PBMCs were
GFP+ (data not shown). With cuprizone treatment C57BL/6 GFPTg/o and IL-1β−/− GFPTg/o
chimeras demyelinated almost completely by 5 weeks, 4% and 3% respectively, assessed by
transmission electron microscopy (Figure 2.1A). Likewise, C57BL/6 and IL-1β−/− mice that
did not receive bone marrow transfers demyelinated almost completely, to levels of 5% and 4
% respectively (Figure 2.1A). After 2 weeks of discontinued cuprizone treatment, C57BL/6
and IL-1β−/− mice that did not receive bone marrow transfers appeared to recover fully and
equally (Figure 2.1A). These mice alone suggest a loss of the remyelination phenotype
found originally in the IL-1β−/− mice (Mason, 2001b). Thus, it was not a surprise that, despite
a delayed remyelination compared to mice that did not receive bone marrow transfers, both
chimeras also remyelinated equally (Figures 2.1A-C). It should be noted that these IL-1β−/−
mice used here were further backcrossed onto the C57BL6/J background and genes that may
have contributed to the original phenotype appear to have been removed by recombination
events. Whether macrophages/microglia production of IL-1β is important to the promotion
of remyelination in the CNS remains inconclusive.

**IL-1 does not affect cuprizone-induced demyelination and remyelination.** In addition to the
IL-1β⁻/⁻ mice, two other strains of mice were obtained from Dr. Yoichiro Iwakura: IL-1α⁻/⁻
mice and IL-1α⁻/⁻β⁻/⁻ mice. The role of IL-1α was examined in the cuprizone model, in
addition to the re-examination of IL-1β. 8-10 week old mice were placed on the cuprizone
diet for up to 6 weeks. Maximum demyelination is known to occur after 5 weeks of
treatment, during which time remyelination spontaneously begins to occur and continues
upon removal of cuprizone from the diet. Myelination of the corpus callosum at midline was
examined by LFB-PAS stain. Myelinated fibers stained blue, and unmyelinated fibers
stained pink. The sections were blind scored on a scale from 0 to 3, in which 0 is
representative of complete demyelination and 3 is reflective of full myelination. All strains
appeared to demyelinate fully and remyelinate at a similar rate (Figure 2.2).

**IL-1 does not affect mature oligodendrocyte apoptosis.** The absence of IL-1 did not alter the
number of apoptotic GST-pi⁺ mature oligodendrocytes during demyelination. The death of
mature oligodendrocytes peak at week 3 and results in the ongoing demyelination until week
5. IL-1 has been implicated in the death of oligodendrocytes in vitro (Merrill 1991); however
as shown in Figure 2.3, IL-1α⁻/⁻, IL-1β⁻/⁻, IL-1α⁻/⁻β⁻/⁻ mice showed similar amounts of
apoptotic mature oligodendrocytes as wild type animals at week 3. A slight amount of
apoptotic mature oligodendrocytes were detected at week 4 and at week 5; however, these were not statistically significant. Thus, it appears that IL-1 does not contribute importantly to the apoptosis of mature oligodendrocytes. It is possible that other mediators, such as TNF-α or NO, may compensate for the loss of IL-1 [49, 211].

IL-1 does not affect the accumulation or the resolution of the microglia/macrophage population in the corpus callosum during cuprizone-induced demyelination or remyelination. With the exposure to cuprizone and the death of mature oligodendrocytes, microglia accumulate within the corpus callosum presumably to clear dying cells and debris. In addition, microglia are important for providing growth factors and cytokines, including IGF-1 and TNF-α [49, 198]. To determine whether the absence of IL-1 affected the microglial populations, we quantified microglia accumulation at key time points during demyelination and remyelination. RCA-1 is a lectin that is used to identify microglia/macrophages by histochemical analysis. RCA-1+ cells in the midline of the corpus callosum were enumerated for individual mice and averaged for each treatment group. The results do not indicate a significant difference in the accumulation of this cell population in absence of IL-1α and/or IL-1β (Figure 2.4) at any time point. During remyelination the microglia/macrophage population diminishes similarly in all of the knockout strains, as well as wild type mice (Figure 2.4).

IL-1 does not affect astrogliosis or resolution of the astrocyte population in the corpus callosum during cuprizone-induced demyelination or remyelination. A second prominent
source of IL-1 during demyelination is astrocytes, which accumulate in moderate numbers and are present throughout remyelination [51, 187, 198]. GFAP is the astrocyte marker used for immunohistochemical analysis in this report. GFAP+ cells were counted in the midline of the corpus callosum for individual mice and averaged for each treatment group. The data show no significant differences in the accumulation of astrocytes in the corpus callosum in the absence of IL-1β and/or IL-1α (Figure 2.5). Moreover, there are no significant differences in the diminishing population during remyelination (Figure 2.5).

IL-1β affects the mature oligodendrocyte population in untreated and remyelinated corpus callosum. Although we did not observe differences in demyelination or remyelination among the IL-1 genotypes (Figure 2.1), we quantified the mature oligodendrocyte population as done previously in the published characterization of the IL-1β−/− mice, which showed a delay in the repopulation of the demyelinated corpus callosum [51]. GSTpi immunohistochemistry was performed to identify mature oligodendrocytes in wild type, IL-1β−/−, IL-1α−/−, and IL-1α−/−β−/− mice during cuprizone-induced demyelination and remyelination. GSTpi+ cells were enumerated at the midline of the corpus callosum for individual mice and averaged for each treatment group. Interestingly, in untreated mice, IL-1β−/− displayed a significant deficit in the number of mature oligodendrocytes compared to wild type, while the absence of IL-1α appeared to have no effect (Figures 2.6A, B). Although more subtle, the IL-1α−/−β−/− mice also displayed a significant deficit in the number of mature oligodendrocytes and showed an intermediate phenotype between the IL-1α−/− mice and IL-1β−/− mice (Figures 2.6A, B). During demyelination the number of mature oligodendrocytes dying by apoptosis, as demonstrated by co-staining with terminal
deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL), is similar in all strains, indicating a comparable insult by cuprizone (Figure 2.3). At 5 weeks, which coincides with full demyelination, few mature oligodendrocytes are found in the lesion. Upon remyelination, mature oligodendrocytes in \( IL-1\beta^-\) mice repopulate to untreated levels after 1 week of discontinued cuprizone treatment. Furthermore, 4 weeks after cuprizone removal, mature oligodendrocyte populations in all strains reflect similar numbers in untreated mice (Figures 2.6A, B). Inherently, \( IL-1\beta^-\) mice have fewer mature oligodendrocytes before cuprizone insult and, upon remyelination, repopulate to their original numbers that is lower than wild type mice and \( IL-1\alpha^-\) mice. Moreover, \( IL-1\alpha^-/\beta^-\) mice displayed an intermediate phenotype between \( IL-1\alpha^-\) mice and \( IL-1\beta^-\) mice. NG2+ immature oligodendrocytes were examined during demyelination at 5 weeks, and the number of immature oligodendrocytes did not differ significantly (data not shown), indicating that infiltration of NG2+ cells is not affected by IL-1\(\beta\). Thus, IL-1\(\beta\) appears to affect the number of oligodendrocytes within the corpus callosum despite myelination of axons appearing normal (Figure 2.1C compared to Figure 2.1B).

2.5 DISCUSSION

The results from our study indicate that IL-1\(\alpha\) and IL-1\(\beta\) are not important mediators of cuprizone-induced demyelination or remyelination (Figures 2.1, 2.2). This supports our previously published assertion that IL-1, in its proinflammatory role, does not exacerbate demyelination [51]. Although the initial insult to oligodendrocytes in our model is toxicity,
we demonstrate that IL-1α and/or IL-1β neither enhances nor reduces oligodendrocyte apoptosis, as these cells are similarly affected in wild type, IL-1α^+/−, IL-1β^+/−, and IL-1α^−/−β^−/− mice (Figure 2.3). Because IL-1 does not appear to be enhancing oligodendrocyte apoptosis, it may play a protective role for oligodendrocytes during insult, which is contrary to in vitro data suggesting IL-1 is detrimental to oligodendrocytes [249]. We, however, have found a novel and inherent difference in the mature oligodendrocyte population in the IL-1β^+/− and IL-1α^+/−β^+/− mice compared to wild type mice. IL-1β^+/− mice have significantly fewer mature oligodendrocytes in the corpus callosum compared to wild type mice prior to cuprizone treatment, and the effect that the lack of IL-1β has on this population can also be seen in the IL-1α^+/−β^+/− mice, albeit more subtly (Figure 2.6). Moreover, this inherent difference in number is restored upon remyelination, with IL-1β^+/− mice fully repopulating to untreated levels after 1 week of cuprizone removal and IL-1α^+/−β^+/− mice fully repopulating to untreated levels after 4 weeks of cuprizone removal. The significance of this oligodendrocyte effect is underscored by the fact that the IL-1β^−/− and the IL-1α^−/−β^−/− strains, the two IL-1 targeted strains that show this phenotype, were originally generated in laboratories at Washington University in the USA and at the University of Tokyo in Japan, respectively, and only became apparent after extensive backcrossing to the C57BL/6 strain. The fact that this phenotype was not observed in IL-1α^+/− mice that were subjected to the same backcrossing effectively makes the possibility that the IL-1β effect is due to confounding strain genetic background issues less likely. Our observations may suggest IL-1β is needed for the establishment of the oligodendrocyte population and is consistent with in vitro studies that report IL-1β promotes maturation and survival of differentiating oligodendrocytes [142]. Thus, in the absence of IL-1β, fewer oligodendrocytes mature. Although both the IL-1β^+/−
and $IL-1\alpha$/$\beta$−/− mice have this deficit in oligodendrocyte numbers, myelination appears to be consistent with both wild type and $IL-1\alpha$−/− mice (Figures 2.6A, 2.2). This may be explained by more complex arborization and myelinating processes per oligodendrocyte in the mice lacking IL-1β. This hypothesis was tested by examining mature proteolipid protein (PLP)-expressing oligodendrocytes in the corpus callosum of untreated wild type PLP-EGFP mice and PLP-EGFP mice backcrossed onto the $IL-1\beta$−/− background (data not shown). Although there appeared to be fewer PLP-expressing oligodendrocytes in the corpus callosum, examination of the arborization was inconclusive due to the compact nature of a fully myelinated corpus callosum that prevented quantification.

Studies indicate that upon CNS insult, immune mediators, such as IL-1, activate microglia and result in proliferation and/or infiltration [249]. Surprisingly but consistent with previously published data from our lab, accumulation of microglia/macrophages in $IL-1\beta$−/− mice during cuprizone intoxication was not significantly different from wild type mice upon exposure to cuprizone (Figure 2.4). Additionally, the $IL-1\alpha$−/− and $IL-1\alpha$/$\beta$−/− mice behaved similarly, indicating that the microglia/macrophage proliferation and/or infiltration were neither IL-1α-dependent nor IL-1β-dependent (Figure 2.4). As demonstrated for microglia/macrophages, it has been shown that astrocytes produce IL-1β in our cuprizone-intoxication model [51]. Similar to the microglia/macrophage population and despite their role in IL-1 production, their migration and proliferation in the demyelinating lesion is not dependent on IL-1α or IL-1β (Figure 2.5).

Despite signaling through the same receptor, namely IL-1RI, IL-1β is often studied for its proinflammatory effects, while IL-1α is just as often overlooked. Admittedly, the two molecules are differentially expressed on a subcellular level. IL-1β is only active in its
mature, 17 kDa form and is released from phagocytic cells in association with activation of caspase-1 and the newly defined inflammasome [105]. In contrast, IL-1α can be active in both its pro-form and its mature, 17kDa form, and for the most part remains intracellular or cell-associated. However, although it is clear that in the process of cell death, IL-1α is readily released from the cell [102], there is emerging evidence that IL-1α can also be released from cells as a consequence of non-lethal cell injury, also by a caspase-1-dependent mechanism [105]. In cases involving a CNS insult, whether by damage or toxin, it is feasible that IL-1α contributes to the inflammatory processes. In our cuprizone model, IL-1 is upregulated upon intoxication [198]. The data we report here demonstrates that, while neither isoform of this cytokine appears to be essential for either the demyelination or the remyelination process, IL-1β, as shown through analysis of IL-1β−/− and IL-1α−/−β−/− mice, does play a contributory role in the establishment of mature oligodendrocyte populations.
FIGURE 2.1. Bone marrow transfers from donor $GFP^{Tg/0}$ (C57BL6) mice into recipient $IL-1\beta^{-/-}$ mice does not rescue a lacking phenotype. (A) Quantification of percent myelination by transmission electron microscopy reveals a loss of a previously characterized myelination phenotype. Wild type (C57BL6) and $IL-1\beta^{-/-}$ mice demyelinated and remyelinated similarly upon delivery and removal of cuprizone, respectively. Wild type and $IL-1\beta^{-/-}$ mice that were irradiated and reconstituted with wild type EGFP also remyelinated similarly. (B) Representative micrograph of EGFP/C57BL6 chimeras at (6+2) weeks. Average myelination for n=4 was 41.5%. (C) Representative micrograph of $EGFP/IL-1\beta^{-/-}$ chimeras at (6+2) weeks. Average myelination for n=3 was 39.7%. Scale bar is 2 μM.
FIGURE 2.1

A

% Myelination

0 5 6+2

Treatment + Recovery (weeks)

C57BL6
<br>
IL-1β−/−
<br>
EGFP / C57BL6 CHIMERA
<br>
EGFP / IL-1β−/− CHIMERA

B  EGFP / C57BL6 CHIMERA
<br>
C  EGFP / IL-1β−/− CHIMERA
FIGURE 2.2. IL-1 does not affect cuprizone-induced demyelination or remyelination.

Tissues were stained by LFB-PAS and scored on a scale from 0 to 3 in which a score of 0 represents full demyelination and 3 represents full myelination. The midline of the corpus callosum from a minimum of eight mice for each treatment group were scored and averaged. Bars represent average ± SEM.
FIGURE 2.3. Exposure to cuprizone does not differentially affect mature oligodendrocyte death in $IL-1^{-/-}$ mice and wild type mice. Apoptotic GSTpi$^+$ cells at the midline of the corpus callosum were counted at time points during demyelination and averaged. No statistical differences were observed. Bars represent average ± SEM.
FIGURE 2.4. Microglia/macrophage infiltration and exit are not affected by IL-1 during cuprizone-induced demyelination and remyelination. RCA-1<sup>+</sup> cells were counted at the midline of the corpus callosum from a minimum of eight mice for each treatment group and averaged. Bars represent average ± SEM.
FIGURE 2.4
FIGURE 2.5. Astrogliosis and astrocyte resolution are not affected by IL-1 during cuprizone-induced demyelination and remyelination. GFAP⁺ cells were counted at the midline of the corpus callosum from a minimum of eight mice for each treatment group and averaged. Bars represent average ± SEM.
FIGURE 2.5

![Bar chart showing GFAP* cells/mm² over treatment and recovery periods in weeks for different genotypes](image)

- C57BL6
- IL-1β⁻/⁻
- IL-1α⁻/⁻
- IL-1α⁻/⁻ β⁻/⁻
FIGURE 2.6. IL-1β affects the number of mature oligodendrocytes before cuprizone treatment and after remyelination. (A) GSTpi⁺ cells were counted at the midline of the corpus callosum from a minimum of eight mice for each treatment group and averaged. Bars represent average ± SEM. *p ≤ 0.05 (B) Representative images of GSTpi⁺ cells at the midline of the corpus callosum of C57BL/6, IL-1β⁻/⁻, IL-1α⁻/⁻, and IL-1α⁺β⁺/⁻ mice at the (0+0) and (6+4) time points. Scale bar is 20 μm.
FIGURE 2.6
CHAPTER 3

THE ROLE OF LAG-3 IN CUPRIZONE INDUCED DEMYELINATION AND REMYELINATION
LAG-3 is a membrane-bound glycoprotein that shares many structural similarities to CD4. It was originally identified on the surface of activated T cells where it has been shown to be involved in negative regulation of activity and clonal expansion through its interaction with MHCII. Despite an apparent insignificant role of T cells in the cuprizone intoxication model, we demonstrate elevated LAG-3 gene and protein expression in response to cuprizone insult. Moreover, we demonstrate a previously undescribed location of LAG-3 expression on OPCs and astrocytes. To examine the function of LAG-3, we expose \textit{LAG-3}^{-/-} mice to cuprizone and assessed demyelination and remyelination. We find that demyelination is accelerated in \textit{LAG-3}^{-/-} mice and that a greater number of OPCs and astrocytes are present in the corpus callosum at week 3 of cuprizone treatment. However, a greater proliferation of OPCs was observed in the SVZ of \textit{LAG-3}^{-/-} mice and not the corpus callosum, suggesting that the majority of OPCs divide first and then migrate to the corpus callosum in greater numbers. We did not observe, however, a significant difference in remyelination. Although LAG-3 was not found on microglia, their numbers were increased in \textit{LAG-3}^{-/-} mice at week 3 of cuprizone treatment, and this may be attributed to the rapid demyelination observed. We also examined OPCs in mixed glial cultures and found that LAG3\textsuperscript{+} OPCs engaged MHCII tetramers, suggesting OPCs may engage MHCII directly and presumably limit expansion and trigger differentiation of the OPCs. Thus, we are the first to demonstrate LAG-3 expression on glial cells and deduce that the function of LAG-3 is to regulate OPC and astrocyte populations prior to repair of demyelinating lesions.
LAG-3 is an activation-induced TCR coreceptor that binds MHCII [155, 156, 159, 250]. It was first identified on activated human NK cells and T lymphocytes; more recently, it has been identified on activated B cells [155, 156, 158]. LAG-3 has been shown to be closely related to CD4 in genomic organization and structure [155, 251, 252]. Much of the initial characterization of LAG-3 was focused on the human protein. However, with the development of LAG-3−/− mice, in vitro findings can be validated in a physiological setting [253]. Murine LAG-3 displays 69.9% protein sequence homology to the human homologue and possesses the residues that are putatively important to structure [169]. It can be found in three forms: 70kD full length, and a 54kD and a 16kD fragment that is soluble [254].

LAG-3 serves as a two-way signaling molecule, affecting the cell that expresses LAG-3 and the APC that it engages. As mentioned before, LAG-3 binds MHCII, and it does this with much higher avidity than CD4, suggesting a role as a natural competitor to CD4 [159]. On T lymphocytes, LAG-3 associates with the CD3/TCR complex and negatively regulates signal transduction and antigen-driven T cell expansion [160, 161, 170, 175, 255]. While LAG-3 seemingly has a negative regulatory effect on the cell that expresses it, LAG-3 has the capacity to activate APCs via MHCII. Experimental studies demonstrate that LAG-3 and MHCII are strongly upregulated during inflammation [164]. In response to ligation of soluble LAG-3 (sLAG-3)-Ig fusion protein to MHCII, monocytes and dendritic cells are stimulated for the production of TNF-α and IL-12 [164]. Moreover, T cell-activation of monocytes is suppressed effectively by LAG-3-specific antibodies [164]. sLAG-3-Ig fusion
protein also has been shown to induce dendritic cell maturation and migration to secondary lymphoid organs to prime CD4⁺ and CD8⁺ T cells [162, 164, 256]. In contrast, another study suggests sLAG-3 may play a role in negatively regulating monocyte differentiation into macrophages or fully competent antigen-presenting dendritic cells in vitro [257]. Lastly, a role of LAG-3 in regulatory T cells has been suggested. A recent study shows regulatory T cells expressing LAG-3 suppress dendritic cell maturation and immunostimulatory capacity via engagement with MHCII, resulting in ITAM-mediated inhibitory signaling that involves FcγRγ and Erk-mediated recruitment of SHP-1 [258].

The distribution of murine LAG-3 mRNA expression shows strong expression in the thymus and spleen [157]. Interestingly, there was also a significant amount of message in the brain, which appeared to differ with the maturational state of the animal. In postnatal day 7 mouse brain, LAG-3 mRNA was detected in defined tracts at the base of the cerebellum and in the choroids plexus. In contrast, in the adult brain, LAG-3 mRNA was detected, but only in areas outside of the cerebellum. A similar study in the rat revealed a slightly expanded distribution with LAG-3 message also appearing in the lung, liver and small intestine, and again, there was also strong message in the brain tissue [172]. Most of the tissues in which LAG-3 message was identified in mouse and rat contain either large numbers of circulating lymphocytes, which easily explains the presence of LAG-3 message. The healthy brain, however, contains negligible numbers of these cells and so the expression of LAG-3 here must be attributed to some other cell type. Despite these reports of LAG-3 in the developing and adult brain, there have been few studies investigating the role of LAG-3 function in the central nervous system (CNS) [157, 172]. Although LAG-3 has thus far been characterized solely as an immune mediator, several molecules that were previously thought to be utilized
exclusively by the immune system, most notably MHC I and CD3ζ, have since been found to be important in development of the CNS [259, 260].

In this study, we examined the function of LAG-3 during CNS insult. We used the cuprizone intoxication model, which allows the analysis of neuroinflammation, demyelination, and remyelination in a predictable time course and pattern [183, 184, 187, 190, 195, 198]. With cuprizone treatment of C57BL/6 mice, perturbation and mature oligodendrocyte cell death are followed by microglia/macrophage infiltration, astrogliosis, and substantial demyelination by 5 weeks [190]. Concurrently, oligodendrocyte progenitors begin to populate the demyelinating lesion after 3 weeks of treatment and differentiate into mature oligodendrocytes during remyelination beginning at week 6 [188, 195, 198]. Interestingly, T cells do not play a significant role during cuprizone-intoxication and/or amelioration [49, 92, 93]. RAG-1<sup>-/-</sup> do not display phenotypic differences in demyelination, remyelination, or cellular populations throughout the cuprizone time course [49, 93]. Despite the lack of T cell participation in our model, we demonstrate a substantial increase in LAG-3 gene and protein expression during cuprizone intoxication. Moreover, in this study, we report novel localization of LAG-3 to immature oligodendrocytes and astrocytes, as well as the role of LAG-3 during demyelination and remyelination.

3.3 MATERIALS AND METHODS

Mice. All mice were maintained in a pathogen-free facility in accordance with NIH guidelines and approved protocols by the University of North Carolina Institutional Animal
Care and Use Committee and the University of North Carolina Division of Laboratory Animal Medicine. C57BL/6 mice were either purchased from Jackson Laboratories (Bar Harbor, ME) or bred in-house at the UNC animal facility. LAG-3<sup>-/-</sup> mice on the C57BL/6 background were obtained from Dr. Dario A. Vignali (St. Jude Children's Research Hospital, Memphis, TN). PLP promoter-driver enhanced GFP transgenic mice (PLP-EGFP<sup>Tg/o</sup>) were obtained from Dr. Wendy B. Macklin (Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH) [79].

**Cuprizone Treatment.** 8-10 week old male mice that were approximately 25 grams were fed ad libitum a diet consisting of 0.2% (w/w) cuprizone (bis-cyclohexanone oxaldihydrazone, Sigma-Aldrich, St. Louis, MO) mixed in ground rodent chow (ProLab IsoPro RMH 3000, LabDiet, Brentwood, MO) for up to 6 weeks to induce demyelination (Hiremath et al. 1998). For remyelination, the mice were returned to a normal diet after 6 weeks of treatment. Untreated control mice were maintained on normal diet for the duration of the experiment.

**Microarray analysis.** Affymetrix GeneChip arrays (Affymetrix, Santa Clara, CA) were used to profile gene expression. Corpus callosum was dissected from three mice at 0-6, 8, 10, and 12 weeks from a cuprizone time course. Total RNA was harvested using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer’s instruction and was given to the UNC Neuroscience Center Functional Genomics Core for further processing: cDNA synthesis, in vitro transcription, RNA cleanup, hybridization, washing, and scanning of the microarray with GCOS v1.4 (Affymetrix). The mouse genome U74Av2, containing gene probes for
~6000 genes and ~6000 expressed sequence tags clusters in the Mouse Unigene database, were used for this experiment. Genespring software (GenUs Biosystems, Inc., Northbrook, IL) was used to generate data presented. This experiment was repeated to lend greater fidelity to the data.

*In situ hybridization.* LAG-3 *in situ* hybridization was modified from methods previously described [157] and done in collaboration with Dr. Yongqin Wu of the UNC Neuroscience Center *In Situ* Hybridization Core Facility. Modification of methods replaced sense and antisense [³²P]UTP-labeled riboprobes with digoxigenin-labeled oligonucleotide probes.

*Western blot.* Corpus callosum was dissected from three mice at 0-5, 7, 8, and 10 weeks from a cuprizone time course. Tissue was dissociated with 3 quick pulses at an amplitude of 60% at 4°C using the Sonic Dismembrator (Model 500, Fisher Scientific) in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM PMSF, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 1 μM pepstatin A; Sigma-Aldrich). Lysates were cleared by centrifugation. SDS sample-loading buffer was added to each sample, and the sample was boiled and resolved on a SDS-PAGE gel. Proteins were transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA) and blotted in 5% milk-TBS with 0.1% Tween 20 (TBS-T) for 30 min. Anti-LAG-3 (rabbit polyclonal serum against the D1 domain of LAG-3 gifted by Dr. Dario A. Vignali) was used at 1:500 in 2.5% milk-TBS-T for overnight incubation at 4°C. Blots were washed in TBS-T and incubated in anti-rabbit-HRP antibody (Vector Laboratories, Burlingame, CA) at
1:10,000 in 2.5% milk-TBS-T for 1 h followed by further washing in TBS-T. Blots were incubated with ECL-Plus (GE Healthcare, Piscataway, NJ) and visualized using HyBlot CL autoradiography film (Denville Scientific, Metuchen, NJ).

**Bromodeoxyuridine incorporation.** Oligodendrocyte expansion was determined by injecting 100 mg/kg BrdU (Sigma) in phosphate-buffered saline (PBS) into the peritoneal cavity of experimental mice every 8 hours for two days.

**Tissue Preparation.** Mice were anesthetized with Isoflurane (Vedco, St. Joseph, MO) and perfused intracardially with 0.15 M phosphate buffer, pH 7.4, [247] for 1 minute followed by 4% PFA in phosphate buffer for 8 minutes. For paraffin-embedded tissues, brains were postfixed in 4% PFA for 24 hours at 4°C before processing and embedding by the UNC Lineberger Comprehensive Cancer Center Histopathology Core. Coronal sections, 5 μm in thickness, of the corpus callosum at midline between region 220 and 260 of the Atlas of the Mouse Brain and Spinal Cord [248] were cut and mounted on VWR Superfrost Plus Micro slides (West Chester, PA) for histology and immunohistochemistry. For fixed frozen tissues, brains were postfixed in 4% PFA for 4 hours or overnight before transferring to 30% sucrose for overnight at 4°C. Tissues were then embedded using OCT media (Fisher Scientific, Fairlawn, NJ). Coronal sections, 7 μm in thickness, of the corpus callosum at midline between region 220 and 260 of the Sidman mouse brain atlas were cut and mounted on VWR Superfrost Plus Micro slides for immunohistochemistry.
Luxol Fast Blue-Periodic Acid Schiff’s Staining. Paraffin-embedded sections were histologically stained with LFB-PAS stain as previously described (Hiremath et al. 1998). Myelination and cellularity are blind-scored on a scale ranging from 0 (complete demyelination; normal cellularity) to 3 (no demyelination; heavy accumulation of cells typical of 5-week cuprizone-treated wild type male C57BL/6 mouse).

Immunohistochemistry and Lectin staining. All comparative analyses were restricted to the midline of the corpus callosum.

Fixed-frozen sections were thawed and washed in 0.05 M potassium phosphate buffered saline (KPBS). NG2 is a chondroitin sulfate proteoglycan that is expressed by oligodendroglial precursor cells. Briefly, sections were blocked and permeabilized in 2% NGS and 0.1% Triton X-100 in KPBS for 30 minutes and incubated in rabbit anti-NG2 (1:500, gift from Dr. William Stallcup, Burnham Institute for Medical Research, La Jolla, CA) for overnight at 4°C. Tissues were washed with KPBS and incubated with Alexa Fluor goat anti-rabbit IgG (1:400, Invitrogen/Molecular Probes, Carlsbad, CA). Tissues were washed and mounted in Vectashield Hardset with DAPI (Vector) in order to visualize nuclei. Proliferation was determined by BrdU incorporation (as described above). Briefly, sections were demasked with 2 M HCl treatment for 30 minutes at room temperature and washed with 0.1M sodium tetraborate (pH 8.5, Sigma). Tissues were then incubated with 500 Kunitz units/ml DNAse I (Sigma) for 30 minutes at room temperature and washed with KPBS. Sections were incubated with mouse anti-BrdU (1:500, Zymed) overnight at 4°C and
subsequently followed with Alexa Fluor goat anti-mouse IgG (1:400, Invitrogen/Molecular Probes). Sections were washed and mounted as previously described above.

For LAG-3 immunohistochemistry, fixed-frozen sections were thawed and were blocked in 10% NGS and 1% BSA in PBS for 1 hour. Sections were incubated in rat monoclonal anti-LAG-3 (1:50, C9B7W, gift from Dr. Dario A. Vignali) for overnight at 4°C. Tissues were washed with PBS and incubated with biotinylated anti-rat IgG (1:100, Vector) for 1 hour. Sections were then washed and incubated with Alexa Fluor streptavidin (1:400, Molecular Probes/Invitrogen). Sections were washed, and lightly fixed in 2% PFA for 10 minutes. Again, sections were washed and co-labeled with a cell marker. LAG-3 immunohistochemistry was performed first and followed by the appropriate cell marker immunostaining protocol in this section.

Paraffin sections were deparaffinized with Histo-Clear (National Diagnostics, Atlanta, GA) and rehydrated through a series of graded ethanols. Immunohistochemistry for Glutathione S-Transferase pi (GSTpi) was performed to identify mature oligodendrocytes as described previously (Mason et al. 2000). Briefly, tissue sections were blocked and permeabilized in 2% NGS and 0.1% Triton X-100 in PBS for 30 minutes at room temperature and then unmasked with 0.1% trypsin in a 50 mM Tris solution (pH 7.4) for 15 minutes at 37°C. Sections were then incubated with rabbit anti-GSTpi antibody (1:1000, Biotrin, USA) for overnight at 4°C. Tissues were washed and incubated with Alexa Fluor goat anti-rabbit IgG (1:400, Invitrogen/Molecular Probes). Tissues were washed and mounted in Vectashield HardSet with DAPI (Vector) in order to visualize nuclei. In Situ Cell Death Detection, Fluorescein (Roche Applied Science, Indianapolis, IN) was used to determine apoptosis and used according to manufacturer’s protocol.
RCA-1 lectin stain was performed to identify microglia/macrophages as described previously (Hiremath et al. 1998). Briefly, sections were unmasked with 0.025% protease, type XIV (Sigma) for 2 min at 43°C, blocked and permeabilized with 5% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS, and incubated with biotinylated RCA-1 (1:500, Vector) for overnight at 4°C. Slides were washed and incubated with Alexa Fluor Streptavidin (Invitrogen/Molecular Probes). Tissues were washed and mounted as previous described above.

Immunohistochemistry for GFAP was performed to identify astrocytes as previously described (Hiremath et al. 1998). Briefly, sections were blocked and permeabilized in 2% NGS and 0.1% Triton X-100 in PBS for 30 minutes. Sections were incubated in rat anti-GFAP antibody (1:400, EMD/Calbiochem, San Diego, CA) for overnight at 4°C. Tissues were then washed and incubated with Alexa Fluor goat anti-rat IgG (1:400, Invitrogen/Molecular Probes). Sections were washed and mounted as previously described above.

Image-Pro Plus v.5.0.2.9 (Media Cybernetics, Inc., Bethesda, MD) was used to capture digital images and count cells per mm². Quantification included cells in which a nucleus was associated with immunohistochemical staining.

**Mixed glial cultures.** Methods were modified from [76]. Briefly, brains were removed from day 2-3 old postnatal mice, followed by removal of the meninges and cerebellum. Tissue was mechanically and enzymatically dissociated with 0.08 Wunsch units/ml Liberase Blendzyme I (Roche) in HBSS for 30 minutes at 37°C, agitating every 10 minutes. Tissue
suspension was trititated with a 10 ml pipette and passed through a sterile 70-μm cell
strainer (BD Bioscience). Single cell suspension was brought up to 50 ml in DMEM/F12
(Gibco) +10% FBS (Atlanta Biologicals) and centrifuged at 500 x g for 10 minutes. Cells
were washed with HBSS two more times and resuspended in DMEM/F12 +10 FBS. Cells
were plated onto coverslips in a 24-well plate at 2 x 10^5 cells/well.

For mitogen stimulation, cells were cultured for 6 days. Cells were washed three
times with HBSS and switched to serum-free medium, containing D-biotin (10 ng/ml;
Sigma), insulin (5 μg/ml; Collaborative Research), progesterone (20 nM; Sigma), putrescine
(100 μM; Sigma), selenium (5 ng/ml; Collaborative Research), transferrin (50 μg/ml; Sigma),
glutamine (2 mM; Gibco), HEPES buffer (15 mM; Sigma), 3,3,5-triiodo-L-thyronine (15
nM; Sigma), penicillin/streptomycin (100U/100 μg/ml; Gibco), and BSA (1 mg/ml; Sigma)
in DMEM/F12 (Gibco). Mitogens were added: PDGFAA (5 or 10 ng/ml, Sigma), bFGF (5
or 10 ng/ml, Sigma), or IGF-1 (5 or 10 ng/ml, gift from Dr. A. Joseph D’Ercole, University
of North Carolina at Chapel Hill). Cells were incubated for 24 h before proliferating cell
nuclear antigen (PCNA) and LAG-3 immunostaining.

For LAG-3 expression, cells were cultured for 4 days. Cells were washed three times
with HBSS and switched to DMEM/F12 + 10% FBS + 5 ng/ml PDGF-AA. Cells were
incubated for 3 days, with daily media changes. Cells were then immunostained for LAG-3
and various cell markers.

Mixed glial immunocytochemistry and MHCII tetramer binding. Coverslips were washed
with HBSS and lightly fixed with 2% PFA for 10 minutes. Coverslips were washed 3 times
with HBSS and blocked. LAG-3 and cell marker immunocytochemistry was performed similarly to that listed above, replacing PBS with HBSS. Anti-PCNA (Chemicon) was performed according to manufacturer’s protocol. PE-conjugated MHC tetramer was obtained from the NIH Tetramer Core Facility at Emory University (Atlanta, GA). Tetramer binding was performed according to recommendations by the core facility.

Statistics. Data are expressed as mean ± SEM. Multiple comparisons were evaluated using a two-tailed Student’s t-test.  \( p \leq 0.05 \) were considered statistically significant.

3.4 RESULTS

LAG-3 expression increases during cuprizone treatment and peaks at full demyelination.

Northern blot analysis of the distribution of lag-3 mRNA in mouse revealed strong expression in the thymus and spleen [157]. Surprisingly, there was also a significant amount of message in the brain which appeared to differ with the maturational state of the animal. In postnatal day 7 mouse brains, lag-3 mRNA was detected in defined tracts at the base of the cerebellum and in the choroid plexus. In contrast, lag-3 mRNA was detected only in areas outside of the cerebellum in the adult brain. In this study, microarray data from corpus callosum tissue of a cuprizone time course was analyzed for lag-3 gene expression. During cuprizone insult, lag-3 expression in C57BL/6 wild type mice was upregulated to 10-fold over baseline (untreated) at 5 weeks, when demyelination is nearly complete and cellularity
is highest (Figure 1A). Concurrently with lag-3 expression, MHCII (I-Aβ) is upregulated at week 5 during the height of microglia accumulation while CD4 remains unchanged. lag-3 upregulation was corroborated by in situ hybridization that not only showed a basal level of lag-3 expression in untreated brain compared to lag-3 sense probe (Figure 1C and 1D) but also increased expression of lag-3 that was localized to the corpus callosum after 5 weeks of cuprizone treatment (Figure 1B). Furthermore, Western blot analysis demonstrated two interesting observations: (1) low level LAG-3 protein expression in the untreated corpus callosum of mice that dramatically increased upon cuprizone insult to week 5 and diminished upon cuprizone removal during remyelination, and (2) the presence of truncated LAG-3 (16 kDa) is first observed after 3 weeks of cuprizone treatment that follows the trend with full-length LAG-3 (70 kDa), peaking at full demyelination (5 weeks) and diminishing upon remyelination (Figure 1E). The last observation infers the presence of the 54 kDa soluble fragment would exist in the interstitial fluid if it is not degraded immediately by metalloproteinases [261]. Unfortunately, the Western blot antibody does not recognize soluble LAG-3.

Absence of LAG-3 exacerbates cuprizone-induced demyelination. Regardless of the debate on LAG-3 as a susceptibility marker for multiple sclerosis, the dramatic increase of LAG-3 during cuprizone insult suggests a role in demyelination. LAG-3−/− mice were treated on cuprizone, along with C57BL/6 wild type mice. 8-10 week old mice were treated with a 0.2% cuprizone diet for up to 6 weeks. Maximum demyelination occurs after 5 weeks of insult, and remyelination spontaneously occurs at week 6 and continues upon the removal of cuprizone. Myelination of the corpus callosum was determined by blind-scoring of LFB-
PAS-stained tissue sections at the midline. A scale from 0 to 3 is reflective of the relative ratio of myelinated fibers, which stain blue, to unmyelinated fibers, which stain pink. A score of 0 represents complete demyelination at midline of the corpus callosum; a score of 3 represents full myelination [modified from [187]]. *LAG-3<sup>−/−</sup>* mice displayed accelerated demyelination with a score of 1.30 ± 0.41 after 3 weeks of cuprizone treatment compared to a score of 2.844 ± 0.03 in wild type mice (Figure 2). By week 5 of cuprizone intoxication, demyelination was comparable in *LAG-3<sup>−/−</sup>* mice and wild type mice. Likewise, remyelination progressed similarly in both strains. These results suggest that the absence of LAG-3 has a substantial effect on the pathological processes induced by cuprizone insult.

Absence of LAG-3 alters GST<sub>pi</sub><sup>+</sup> mature oligodendrocyte depletion during demyelination and repopulation during remyelination. Several reports from our lab and collaborators have shown cuprizone-induced inflammation and demyelination are associated with a decrease in the mature oligodendrocyte population in the corpus callosum [51, 190, 211]. GST<sub>pi</sub> immunohistochemistry was used to identify mature oligodendrocytes in *LAG-3<sup>−/−</sup>* and wild type mice during cuprizone-induced demyelination and remyelination. It was not surprising that the number of GST<sub>pi</sub><sup>+</sup> mature oligodendrocytes declined substantially compared to wild type by week 3 of cuprizone treatment (252.76 ± 89.09 vs. 727.09 ± 123.69/mm<sup>2</sup>, Figure 3). These results corroborate the accelerated demyelination displayed in the *LAG-3<sup>−/−</sup>* mice (Figure 2). By week 5, most of the mature oligodendrocytes are depleted in both genotypes. Interestingly, GST<sub>pi</sub><sup>+</sup> mature oligodendrocytes reappear within the demyelinated lesion of *LAG-3<sup>−/−</sup>* mice in significantly higher numbers than wild type mice at week 7 (1015.74 ± 79.63 vs. 722.00 ± 52.27/mm<sup>2</sup>, Figure 3). Presumably, this reappearance of oligodendrocytes
Absence of LAG-3 increases cellularity during demyelination. Cuprizone intoxication results in a significant augmentation of cellular populations, namely microglia/macrophages, astrocytes, and oligodendrocyte progenitors [49-51, 92, 187, 190, 211, 262]. Cellularity was determined by blind-scoring of LFB-PAS stained tissues at the midline of the corpus callosum on a scale from 0 to 3. The score estimates the number of cells that is based on the density of nuclei: 0 is indicative of the number of cells in an untreated corpus callosum, 3 reflects the heavy accumulation of cells at 5 weeks of cuprizone treatment. Compared to wild type mice, LAG-3−/− mice displayed a significant increase in the number of cells after 3 weeks of cuprizone treatment (Figure 4). These results indicate that the recruitment and/or proliferation of cells are affected by LAG-3 and suggest the increase in cellularity in LAG-3−/− mice is not due to mature oligodendrocytes, but likely other cell types.

LAG-3 colocalizes to oligodendrocyte progenitors and astrocytes in vivo. Conventionally, LAG-3 has been shown to be expressed by activated T cells. However, T cells do not play a significant role in the cuprizone model, with CD3+ cells peaking at 2 cells/mm² and RAG-1−/− mice showing no phenotypic differences compared to wild type in response to cuprizone [49, 93, 217]. Due to lack of T cells in the corpus callosum and high LAG-3 expression, cellular
localization of LAG-3 was postulated to be on glial cells. 5-week cuprizone-treated tissues were immunostained for LAG-3 and NG2 or GFAP for colocalization with oligodendrocyte progenitor cells (OPCs) and astrocytes, respectively. LAG-3 (red) colocalized to a subset of NG2\(^+\) OPCs (green) (line arrow heads); however, not all NG2\(^+\) cells were LAG-3\(^+\) (Figure 5A, B, C). Likewise, LAG-3 (red) colocalized to a subset of GFAP\(^+\) astrocytes (green) (solid arrow heads); not all GFAP\(^+\) cells were LAG-3\(^+\) (Figure 5D, E, F). LAG-3 was not colocalized to RCA-1\(^+\) microglia/macrophages (Figure 5G, H, I). GSTpi\(^+\) mature oligodendrocytes were not colocalized with LAG-3 due to their absence at 5 weeks of cuprizone treatment. This is the first report for colocalization of LAG-3 to NG2\(^+\) OPCs and GFAP\(^+\) astrocytes.

LAG-3\(^{-/-}\) mice show an increased accumulation of immature oligodendrocytes into the corpus callosum. An accumulation and maturation of OPCs into the corpus callosum during demyelination results in remyelination upon cuprizone removal. In addition to increased cellularity and colocalization of LAG-3 to OPCs, the rapid reappearance of mature oligodendrocytes into the corpus callosum of LAG-3\(^{-/-}\) mice during remyelination warranted examination of OPCs during cuprizone insult. NG2 immunohistochemistry was performed to identify OPCs at the midline of the corpus callosum during cuprizone insult. NG2\(^+\) OPCs were first detected in the corpus callosum in significantly higher numbers as early as 2 weeks of cuprizone insult and maintain a larger population up to 5 weeks compared to wild type mice (Figure 6A). Interestingly, the number of proliferating OPCs, as measured by BrdU incorporation, was not significantly different in LAG-3\(^{-/-}\) and wild type mice (Figure 6B). Previous studies suggest OPC accumulation likely arose from a migration from the SVZ into
the corpus callosum and from a very small local endogenous population (Mason et al, 2000). Immunohistochemistry of NG2\(^+\) OPCs and BrdU incorporation did not show a significant difference in the number of overall NG2\(^+\) cells or proliferating NG2\(^+\) cells between \(LAG-3^{-/-}\) and wild type mice during demyelination (Figure 6C, D). However, there is a trend towards more proliferating NG2\(^+\) cells in \(LAG-3^{-/-}\) mice at 2 weeks (166.502 ± 48.358 vs. 81 ± 8.18 cells/mm\(^2\)) (Figure 6D). Therefore, LAG3 may be inhibiting OPC numbers in the corpus callosum. This is not due to the proliferation of the endogeneous population of OPCs within the corpus callosum but may be occurring in the SVZ where there is a trend for greater numbers of OPCs.

\textit{Mice lacking LAG-3 show increased astrogliosis during demyelination.} Astrocytes accumulate in moderate numbers during cuprizone-induced demyelination and unlike microglia/macrophages, remain present in modest numbers throughout remyelination. In this report, GFAP is used immunohistochemically as a marker for astrocytes. \(LAG-3^{-/-}\) mice demonstrate a significantly larger accumulation of GFAP\(^+\) cells after 3 weeks of cuprizone treatment compared to wild type (1070.845 ± 103.408 vs. 646.230 ± 49.14 cells/mm\(^2\), Figure 7). By 5 weeks of cuprizone treatment, astrocyte populations peak comparably in \(LAG-3^{-/-}\) and wild type mice (2552.98 ± 137.91 and 2604.02 ± 201.06 cells/mm\(^2\), respectively). During remyelination, astrogliosis begins to resolve but remains in modest and comparable numbers in both strains. Thus, LAG3 on a subpopulation of astrocytes may be regulating astrocytes numbers that accumulate at week 3 during demyelination.
LAG-3<sup>−/−</sup> mice display an elevated number of microglia/macrophages during demyelination. During cuprizone insult, microglia/macrophages presumably accumulate within the demyelinating lesion to clear dying cells, such as oligodendrocytes, and debris. Moreover, LAG-3 is a known ligand of MHCII, expressed by a subset of microglia/macrophages, in the cuprizone model [23, 93]. In this study, RCA-1 is a lectin that we used to identify microglia/macrophages histochemically within the corpus callosum. LAG-3<sup>−/−</sup> mice demonstrate a robust microglia/macrophage response during demyelination. By week 3 of cuprizone treatment, RCA-1<sup>+</sup> microglia/macrophages accumulate to 1519.68 ± 284.52 cells/mm<sup>2</sup> on average compared to 754.07 ± 83.43 cells/mm<sup>2</sup> of wild type mice (Figure 8). RCA-1<sup>+</sup> microglia/macrophage populations are comparable in LAG-3<sup>−/−</sup> and wild type mice by 5 weeks of cuprizone treatment, when demyelination is nearly complete, and during remyelination. Although LAG-3 was not colocalized to microglia (Figure 5G-I), the increased number of microglia at week 3 may be in response to the greater demyelination found in LAG-3<sup>−/−</sup> mice (Figure 2).

LAG-3 expression is stimulated in proliferating cells. LAG-3 expression has been characterized to activated T cells and NK cells [155]. Mixed glial cultures stimulated with PDGF-AA, IGF-1, or bFGF at 5 or 10 ng/ml proliferated to various levels with only 5 ng/ml bFGF being significant over serum-free media (Figure 9A); however, PDGF-AA at 5 and 10 ng/ml induced the most significant amount of LAG-3-expressing cells (Figure 9B). Similarly, when LAG-3 and PCNA were colocalized, mixed glial cultures stimulated with 5 and 10 ng/ml PDGF-AA exhibited the greatest amount of proliferating LAG-3<sup>+</sup> cells (Figure
9C). Subsequent preparations of mixed glial cultures will use PDGF-AA to study the OPC population.

**LAG-3 is expressed on PLP-expressing oligodendrocytes in culture.** As previously shown, LAG-3 is expressed in oligodendrocyte-lineage cells *in vivo* (Figure 5A-C). To corroborate this finding, we examined LAG3 expression in our mixed glial cultures. LAG-3 expression was localized to oligodendrocytes that endogenously expressed EGFP via the PLP promoter (Figure 5D-F). Furthermore, we demonstrated that in our culture system at day 7, the majority of the PLP-expressing cells are NG2+ (Figures 5A-C). Interestingly, LAG-3 expression was restricted to the oligodendrocyte layer of cells (Figures 10D-F); however, the underlying astrocyte bed did not colocalize with LAG-3. This could be due to the level of astrocyte maturity or activation state that may be terminal but would be greater in cuprizone-treated tissues, as in Figures 5D-F. Also, LAG-3 expression was restricted to the cell body and not the processes of the PLP+ oligodendrocyte cells (Figures 10D-F).

**LAG-3 binds MHCII tetramer in vitro.** LAG-3 on activated T cells has been previously demonstrated to interact with MHCII molecules [156]. We demonstrate that LAG-3-expressing cells in mixed glial cultures bind MHCII tetramer (Figures 11A-C). Because we have localized LAG-3 expression in our mixed glial cultures (Figures 5A-C), we postulate oligodendrocytes may bind MHCII through LAG3 on their surface.
This study provides the first evidence demonstrating expression of LAG-3 on two important nonimmune cells of the central nervous system, oligodendrocyte precursor cells and astrocytes. Secondly, we demonstrate LAG-3 may prevent rapid depletion of mature oligodendrocytes, and this is evident at week 3 where demyelination is more severe in LAG-3−/− mice (Figure 2 and Figure 3). Most importantly, LAG-3 may serve to inhibit OPC accumulation in demyelinating lesions as well as inhibit astrocyte accumulation. Our study is the first to demonstrate physiological effects of LAG-3 in the central nervous system during demyelination and repair.

The association of LAG-3 with risk of MS is debatable. The LAG-3 gene was studied in two independent sample sets of MS patients and healthy controls [179, 180]. Although the gene was reported initially to be associated with risk of MS [179], it failed to be replicated in an independent case-control study using a larger patient pool [180]. It must be noted that the lack of genetic evidence for a role in determining risk of disease does not preclude the potential importance for LAG-3 in MS pathogenesis. Hence, our demonstration of LAG3 regulating OPCs is likely relevant to repair processes that may be hampered in patients with MS.

This study evaluated the role of LAG-3 in the cuprizone intoxication model of demyelination and remyelination, which is characteristically a highly reproducible, acute CNS inflammatory model without evidence of T cell involvement [49, 93]. Because T cell involvement during demyelination is lacking, LAG-3 gene and protein upregulation was a
surprising and interesting finding (Figure 1A-E). *In situ* hybridization confirmed the substantial increase in LAG-3 expression was localized to the corpus callosum, the site of acute neuroinflammation during cuprizone intoxication (Figure 1B). Moreover, protein analysis by Western blot revealed that full-length LAG-3 (70 kDa) is detectable at very low levels in normal adult brain, upregulated early upon exposure to cuprizone, and peaks at week 4 through week 5 of cuprizone treatment.

Interestingly, the cleavage product of LAG-3 proteolysis, truncated LAG-3 (16 kDa), was detectable as early as 3 weeks after cuprizone treatment and continued to increase and peak at 5 weeks, when demyelination is nearly complete (Figure 1E). Upon remyelination, both full-length and truncated LAG-3 protein levels returned to baseline untreated levels.

The presence of truncated LAG-3 infers the presence of soluble LAG-3, which would exist in the interstitial fluid if it is not degraded immediately by metalloproteinases [261]. Soluble LAG-3 (54 kDa) is the extracellular domain and does not contain the transmembrane and cytoplasmic domains of the full-length form [254]. LAG-3 is released in a soluble form and has been shown to increase after T cell activation [254]. The function of full-length LAG-3 and sLAG-3 may be important to activation of MHCII-expressing cells [162], such as MHCII⁺ microglia within demyelinating lesions. The presence of sLAG-3 is consistent in our model due to a robust inflammatory response and activation of various cell types during cuprizone insult. However, because the cuprizone intoxication model lacks T cell participation, sLAG-3 must be released from another cell type, likely astrocytes and/or immature oligodendrocytes shown to express LAG-3 (Figure 5A-F). We postulate that LAG-3 and sLAG-3 may be engaging MHCII⁺ microglia, which may be supporting differentiation of OPCs and remyelination based on the critical role MHCII has on
remyelination [23]. The mechanism for MHCII support of remyelination is currently being investigated.

The expression of LAG-3 in wild type mice exposed to cuprizone correlated with active demyelination and mature oligodendrocyte depletion (Figure 2, 3). However, in the absence of LAG-3, both processes were accelerated (Figure 2, 3). Moreover, \( LAG-3^{-/-} \) mice exhibited a significantly larger population of microglia/macrophage during neuroinflammation at 3 weeks (Figure 8). This is consistent with a report that sLAG-3 negatively regulates differentiation of monocytes into macrophages [257]. Buisson and colleagues propose activated LAG-3\(^+\) lymphocytes may reduce differentiation of monocytes in order to limit the magnitude of T-cell immune responses in a localized site of inflammation. Due to absence of LAG-3, there may be a deficiency of this controlled differentiation in our model and, therefore, a large influx of microglia/macrophages into the demyelinating lesion. With greater numbers of these microglia, a larger immune response may be mounted and result in accelerated demyelination. This hypothesis could also be applied to the astrocyte population that have been shown occasionally to express MHCII [23] and is also greater in number in \( LAG-3^{-/-} \) mice compared to wild type during demyelination at 3 weeks (Figure 7).

One of the most important finding in this study is the localization of LAG3 to OPCs both in vivo and in vitro (Figures 5 and 10). Due to this novel finding of LAG-3 expression on OPCs (Figure 5A-C) and because LAG-3 is a known negative regulator of T cell activation and expansion [155, 175], we hypothesized that immature oligodendrocyte proliferation would be less restrained during cuprizone insult in the absence of LAG-3. Furthermore, we demonstrate that it is plausible that MHCII may bind LAG-3 on OPCs as
MHCII tetramers can associate with LAG-3 in vitro (Figure 11). Microarray analysis demonstrated that LAG-3 expression precedes MHCII expression and peaks with nearly complete demyelination (Figure 3.1A). LAG-3 expression by OPCs may be an attempt to slow proliferation and prepare the cell for differentiation. Thus, LAG-3-expressing OPCs may be trying to find a location within the demyelinating lesion flagged by MHCII-expressing microglia in which they can terminally differentiate. We speculate that LAG-3 and other factors induce terminal differentiation of the OPCs to myelinate axons. In support of this, we did characterize a significantly increased number of immature oligodendrocytes in LAG-3\(^{-/-}\) mice throughout demyelination compared to wild type, starting as early as 2 weeks after cuprizone insult (Figure 6A). Confoundingly, this was not due to an increased number of proliferating OPCs. A previous report from our lab suggests many OPCs initially migrate from the SVZ and fornix before accumulating in the corpus callosum during demyelination [198]. Upon examination of the SVZ in LAG-3\(^{-/-}\) and wild type mice, there were no significant differences in the number of OPCs or proliferating OPCs (Figure 6); however, there is a trend of a greater number of proliferating OPCs in the SVZ at week 2 (Figure 6D). Alternatively, plausible explanation may be that a given snap shot, there are no differences in the number of proliferating OPCs; however, the rate at which proliferating cells migrate out of the SVZ and into the corpus callosum may be greater in the absence of LAG-3. Additional analyses on the OPCs within the SVZ is warranted.

Interestingly, LAG-3 was not expressed on the oligodendrocyte processes in vitro (Figures 3.10D-F), suggesting OPCs must be in close cell-to-cell contact with APCs for LAG-3/MHCII signaling to occur. This is consistent with the concept that, as OPCs mature, the extension of processes and complex arborization is indicative of differentiation. If our
hypothesis that LAG-3 negatively regulates the expansion of OPCs is true, LAG-3/MHCII engagement would occur prior to complex arborization. Thus, LAG-3 would most likely be expressed in the cell body rather than the processes.

The astrocytes population may also express LAG-3, at least transiently. Most astrocytes did not express LAG-3 \textit{in vivo} and we did not detect LAG-3 expression in mixed glial cultures. It is possible that astrocytes in culture had already expressed LAG-3 after reaching terminal numbers at the time points in which we were assessing OPCs expression of LAG-3. Alternatively, proper stimulation of astrocytes may be required before LAG-3 expression can be observed. This will be examined in a future study. Nonetheless, the detection of LAG-3 on astrocytes \textit{in vivo} coupled with the increased number of astrocytes at week 3 in \textit{LAG-3}⁻/⁻ mice exposed to cuprizone suggest LAG3 may be functioning to regulate cell numbers.

This study is novel in a couple of aspects: (1) it is the first physiological characterization of LAG-3 in the CNS during demyelination and remyelination, and (2) it is the first localization of LAG-3 to astrocytes and a subset of non-immune cells, immature oligodendrocytes. Furthermore, our results demonstrate that LAG-3 regulates cellular infiltration into the corpus callosum, a site of neuroinflammation, in response to demyelinating insults. Clinical approaches targeting LAG-3 should be approached carefully because of it dichotomous role in balancing immune responses.
FIGURE 3.1. LAG-3 gene and protein expression increases in the corpus callosum during cuprizone-induced demyelination and decreases upon remyelination. (A) Affymetrix gene expression from the corpus callosum of wild type mice during cuprizone-induced demyelination at 0-6 weeks of treatment and upon remyelination at 2, 4, and 6 weeks after cuprizone removal. Corpus callosum was dissected from three mice for each time point. (B-D) LAG-3 in situ hybridization, scale bar = 100mm: (B) 5-week cuprizone-treated corpus callosum, LAG-3 antisense probe, (C) Untreated corpus callosum, LAG-3 antisense probe, (D) 5-week cuprizone-treated corpus callosum, lag-3 sense probe. (E) LAG-3 Western blot of corpus callosum of wild type mice during cuprizone-induced demyelination at 0-5 weeks of treatment and upon remyelination at 1, 2, and 4 weeks after cuprizone removal. Blot was stripped and probed for actin, which served as a loading control. Corpus callosum was dissected from four mice at each time point.
FIGURE 3.1

A

Fold Change

16

14

12

10

8

6

4

2

0

1+0 2+0 3+0 4+0 5+0 6+0 5+2 6+4 6+6

Treatment + Recovery (weeks)

LAG3

I-A\(^b\)

CD4

B

C

D

E

TX + REC 0+0 1+0 2+0 3+0 4+0 5+0 6+0 6+1 6+2 6+4

kDa 130 95 72 55 36 28 17

70 kDa

Full-length LAG-3

16 kDa

Truncated Actin

LAG-3

Actin
FIGURE 3.2. *LAG-3*−/− mice display accelerated demyelination compared to wild type mice during cuprizone insult. Mice were treated for up to six weeks (6+0), which is one week into remyelination. Full demyelination is accomplished at week 5 (5+0). 6+2 means two weeks off cuprizone (but 3 weeks into remyelination). Sections containing the corpus callosum at the level of the fornix were LFB-PAS stained, and the corpus callosum was blind-scored for myelination on a scale of 0 (complete demyelination) to 3 (complete myelination), with blue fibers indicating intact myelin. * p < 0.002.
FIGURE 3.2

![Graph showing myelination levels over treatment and recovery weeks for C67BL6 and LAG3^-/- mice. The graph indicates significant differences with an asterisk (*) representing p < 0.002.]
FIGURE 3.3. *LAG-3*−/− mice display rapid mature oligodendrocyte depletion during demyelination yet accelerated mature oligodendrocyte repopulation during remyelination. GSTpi+ was used as an immunohistochemical marker for mature oligodendrocytes. Positive cells were quantitated per mm² in the corpus callosum. Each bar is representative of an average of the data from a minimum of 7 mice per genotype per time point. * p < 0.02, ** p < 0.005.
FIGURE 3.3

[Diagram showing GSTpi^+ cells/mm^2 across different treatment and recovery weeks for C57BL6 and LAG3^-/- strains. Asterisks indicate significance levels: * p < 0.02, ** p < 0.005]
FIGURE 3.4. *LAG-3*−/− mice display increased cellularity during cuprizone-induced demyelination compared to wild type mice. Sections containing the corpus callosum at the level of the fornix were LFB-PAS stained, and the corpus callosum was blind-scored for cellularity on a scale of 0 (untreated, wild type cell density) to 3 (cell density typical of a 5-week cuprizone-treated wild type mouse), with the number of nuclei indicative of the number of cells present.
FIGURE 3.4

- Bar chart showing cellularity over time for different treatment and recovery periods.
- Comparison between C67BL6 and LAG3−/− groups.
- *p < 0.01 significance marker.
FIGURE 3.5. LAG-3 is localized to immature oligodendrocytes and astrocytes within the corpus callosum of 5-week cuprizone-treated wild type mice. (A-C) LAG-3/immature oligodendrocyte immunohistochemistry: (A) LAG-3 expressing cells were identified with an antibody gifted by Dr. Dario A. Vignali (red), (B) Immature oligodendrocytes were identified by NG2+ staining (green), (C) Colocalization of NG2 and LAG-3 was determined by overlapping immunostaining (lined arrowheads) that associated with a nucleus (DAPI, blue). (D-F) LAG-3/astrocyte immunohistochemistry: (D) LAG-3 expressing cells (red), (E) Astrocytes were identified by GFAP+ staining (green), (F) Colocalization of GFAP and LAG-3 was determined by overlapping immunostaining (solid arrowheads) that associated with a nucleus (DAPI, blue). (G-I) LAG-3/microglia/macrophage immunohistochemistry: (G) LAG-3 expressing cells (red), (H) Microglia/macrophages were identified by RCA+ staining (green), (I) RCA-1 and LAG-3 did not colocalized. Scale bar = 10μm.
FIGURE 3.6. Immature oligodendrocytes infiltrate the corpus callosum in increased numbers in LAG-3$^{-/-}$ mice compared to wild type mice. (A, C) Immature oligodendrocytes were identified during demyelination using an antibody against NG2. Positive cells in the corpus callosum (A) and SVZ (C) were quantitated. Each bar in A is representative of an average of the results from at least 6 mice per genotype per time point. Each bar in C is representative of an average of the results from 4 mice per genotype per time point. * p < 0.05, ** p < 0.005. (B, D) Proliferating immature oligodendrocytes were identified by colocalization of NG2 with BrdU. Positive cells in the corpus callosum (B) and SVZ (D) were quantitated. Each bar in B is representative of an average of the results from at least 6 mice per genotype per time point. Each bar in D is representative of an average of the results from 4 mice per genotype per time point.
FIGURE 3.6
FIGURE 3.7. Astrogliosis occurs more rapidly in $LAG-3^{-/-}$ mice compared to wild type mice; however, resolution of the astrocyte population is similar. Astrocytes were identified during demyelination and remyelination using an antibody against GFAP. Positive cells in the corpus callosum were quantitated. Each bar is representative of an average of the data from at least 7 mice per genotype per time point. * $p < 0.002$. 
FIGURE 3.7

![Graph showing GFAP+ cells/mm² over time for C57BL6 and LAG3−/− strains. The graph indicates a significant difference (*) between the two strains at 6+1 weeks with a p-value less than 0.002.](image-url)
FIGURE 3.8. Microglia/macrophages populations are significantly greater in $LAG-3^{-/-}$ mice compared to wild type during demyelination. Microglia/macrophages were identified during demyelination and remyelination by the cell surface lectin, RCA-1. Positive cells were quantitated in the corpus callosum. Each bar is representative of an average of the results from at least 7 mice per genotype per time point. * $p < 0.02$. 

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FIGURE 3.8

* p < 0.02
FIGURE 3.9. Mixed glial cultures upregulate LAG-3 expression when stimulated to proliferate by PDGF-AA and IGF-1. (A) Proliferating cells were detected by PCNA immunohistochemistry. A minimum of 300 total cells per coverslip were quantitated and the percentage of PCNA$^+$ cells were graphed. n=3. (B) LAG-3$^+$ cells were detected by rabbit $\alpha$-LAG-3 (gift from Dr. Vignali). A minimum of 300 total cells per coverslip were quantitated and the percentage of LAG-3$^+$ cells were graphed. n=3. (C) PCNA$^+$ LAG-3$^+$ cells. A minimum of 300 total cells per coverslip were quantitated and the percentage of double positive cells were graphed. n=3.
FIGURE 3.10. LAG-3 is colocalized to PLP-expressing oligodendrocytes in vitro. (A-C) NG2$^+$ cells (red) were colocalized to PLP-EGFP$^+$ cells (green) to demonstrate the majority, if not all, PLP-EGFP$^+$ cells are immature oligodendrocytes at day 7 in culture. (D-F) LAG-3$^+$ cells are colocalized to PLP-EGFP$^+$-expressing oligodendrocytes (green).
FIGURE 3.11. LAG-3$^+$ cells bind PE-conjugated MHCII tetramer *in vitro*. LAG-3 immunohistochemistry (A, green) colocalizes with PE-conjugated MHCII tetramer (B, red) on day 7 of mixed glial cultures. (C) LAG-3/MHCII tetramer overlay.
The CNS is relatively quiescent immunologically and is a unique compartment in which to study immune responses when challenged by insult. Much effort has been directed at identifying effector molecules that affect demyelination and remyelination for therapeutic utility [reviewed in [91]]. However, this effort is impeded by the fact that many effector molecules are shared in the progression of demyelination and remyelination, two seemingly opposing processes [for example, see Section 1.4: Cuprizone Studies in Genetic Knockout and Transgenic Mice, with particular attention to the TNF-α-/- mouse study [49] and Lenercept clinical trial]. Thus, elucidating the underlying molecular and cellular mechanisms in which these effector molecules are involved will provide insight into their potential as therapeutic targets in neurological diseases. In the studies presented in this dissertation, we examined three effector molecules, IL-1α, IL-1β, and LAG-3, and demonstrate that IL-1β and LAG-3 significantly affect glial populations and that LAG-3 may serve in the negative regulation of OPCs and astrocytes during cuprizone intoxication.

The inherent role of IL-1β in oligodendrocyte maturity. In Chapter 2, we demonstrate that the absence of IL-1β negatively impacts the number of mature oligodendrocytes within the myelinated tract of the corpus callosum without affecting myelination. This finding suggests an inherent difference in oligodendrocyte differentiation and/or maturation that is regulated by IL-1β. Interestingly, progenitor and differentiated rat oligodendrocytes constitutively express IL-1β in vitro and in vivo, suggesting IL-1β may fulfill a physiological role in an autocrine loop [129]. Incubation of differentiating rat oligodendrocytes with IL-1Ra results in cell death, while IL-1β does not negatively affect the survival of oligodendrocytes [142]. Therefore, IL-1β may be a pro-survival factor. Based on these in vitro findings, it is not
surprising that IL-1β−/− mice inherently have fewer mature oligodendrocytes. These cells may not survive as well during development in the absence of IL-1β, or the individual oligodendrocytes that do survive may be less impeded in sending processes to multiple axons for myelination sheath formation. Furthermore, if there are fewer oligodendrocytes, it is plausible that myelinating sheaths may stretch greater internodal distances and that factors that dictate terminal myelination are altered in the absence of IL-1β. The positive impact of IL-1β on oligodendrocyte survival or myelination makes a role for IL-1β in demyelinating disease unlikely, as affecting the repopulation of oligodendrocytes in a demyelinated lesion is not ideal. Future studies to address this issue could be extended to IL-1RI−/− mice.

Our finding that myelination in the absence of IL-1 is similar to that of wild type mice is perplexing, given the decrease in mature oligodendrocytes. We postulate that the mature oligodendrocytes in the IL-1β−/− mice compensate by extending more myelinating processes and more effectively myelinate axons. Preliminarily, our lab has examined mature oligodendrocytes in the corpus callosum of IL-1β−/− mice expressing the PLP-EGFP transgene and wild type controls. We were unable to discern oligodendrocyte processes from the cell body due to the density of fibers and the compact nature of a healthy, fully myelinated corpus callosum. Because this phenotype was restored after cuprizone-induced demyelination, examination of an early time point before remyelination is complete may be more appropriate in future attempts in discerning oligodendrocyte processes because the myelin tract will not be as compact. Alternatively, sophisticated delivery of fluorescent dyes to single or a few mature oligodendrocytes may permit dissociation of single cell bodies and their processes for quantification. Although not ideal, mixed glial cultures also may be utilized for examining oligodendrocyte arborization with the caveat that results may not be
translatable to a physiological setting. Vela et al. demonstrated higher morphological complexity of progenitor oligodendrocytes treated with IL-1β compared to cultures treated with IL-1Ra [142]. This is in contrast to our hypothesis that oligodendrocytes may extend more myelinating processes in the absence of IL-1β and would be interesting to note whether oligodendrocytes from *IL-1β−/−* mice display a similar phenotype *in vitro*. Clearly, the role of IL-1β in arborization needs to be better elucidated. Regardless of the outcomes of the proposed future studies, characterizing the effect of IL-1β on mature oligodendrocytes will elucidate a role for IL-1β in the physiological state, a field that has not yet been explored aside from sleep pattern studies and synaptic plasticity studies.

*IL-1β−/−* mice differ in remyelination phenotype compared to previously published data. A finding that admittedly stands out in Chapter 2 is the loss of a previously characterized phenotype. In 2001, Jeffrey Mason characterized a delay in remyelination following cuprizone-intoxication in *IL-1β−/−* mice [51]. Unfortunately, our original *IL-1β−/−* mouse strain, characterized by Mason, was lost, and a new line of the same mice was re-obtained from Dr. David Chaplin. The new *IL-1β−/−* mice, already on a C57BL/6 background, were backcrossed 6 generations onto our in-house C57BL/6 strain, originally from Jackson Laboratories, for the purpose of reliable phenotypic comparisons to wild type controls. We attribute this backcrossing to the change in the remyelination phenotype based on a couple observations: (1) As previously mentioned in the introduction, cuprizone intoxication can display minor differences in time course depending on dose and, in this case, strain. There have also been observations made that even within a strain, such as C57BL/6 from Jackson Laboratories or Charles River Laboratories, the time course of cuprizone may be altered as
these two sources may be susceptible to subtle genetic drift (personal communication with Glenn Matsushima, UNC-CH). We examined an earlier time point at 7 weeks when remyelination was not complete and saw a slight trend in which $IL-1\beta^{-/-}$ mice displayed slightly lower remyelination (Figure 2.2). However, upon examination by electron microscopy, the number of myelinated axons was similar to wild type ($IL-1\beta^{-/-}$ exhibiting 31.5 ± 3.23 % myelinated axons and C57BL/6 exhibiting 35 ± 2.65 % myelinated axons). A yet earlier time point at 6 weeks may demonstrate differences; however, we do not believe this will be likely. (2) Moreover, further genetic backcrossing of $IL-1\beta^{-/-}$ mice on the C57BL/6 background may have eliminated genes that resulted in the altered phenotype. Each time a mouse with the knockout trait is crossed with a mouse of constant genetic background, the average percentage of the genetic material of the offspring derived from the constant background increases. With sufficient backcrossing, the resulting offspring should differ from the constant background only by the genetic alteration. An example in which a phenotypic change occurred upon backcrossing involves $LT\beta R^{-/-}$ mice. These genetic knockouts originally displayed enlarged lateral ventricles following demyelination and this was attributed to neuronal atrophy and unresolved astrogliosis (unpublished data from Ting lab, UNC-CH). Interestingly, upon further backcrossing onto the C57BL/6 background, this phenotype was attenuated (personal communication with Sheila Plant and Jenny Ting). Similarly, this may be applied to the case of the $IL-1\beta^{-/-}$ mice. Regardless of the grounds to which the phenotype has changed, further backcrossing reveals a truer phenotype that we now report.
LAG-3 in the CNS. In Chapter 3, we demonstrate the first characterization of LAG-3 function in the brain. Of our findings, the most significant is the localization of LAG-3 to NG2⁺ immature oligodendrocytes (OPCs). Moreover, we report that, during CNS insult, LAG-3 on OPCs may interact with MHCII, as MHCII tetramers can associate with LAG-3 in mixed glial cultures, and such an interaction in vivo may negatively regulate OPC proliferation, a function reported in the periphery on LAG-3⁺ activated T cells. Our recent attempts to culture OPCs from LAG-3⁻/⁻ and wild type mice and monitor proliferation or differentiation after stimulating with PDGF and removal of fetal calf serum were unsuccessful due to an inability to detect LAG-3 on wild type mixed glial cultures. Currently, the reagents used in the cultures and the protocol are being reexamined for potential changes that may result in the lack of LAG-3 expression.

To date, we have been unable to co-localize MHCII and LAG-3 in brain tissues during cuprizone treatment, where both molecules have shown to be elevated by mRNA and protein (Figure 3.1, [23]) or in vitro using mixed glial cultures. Results obtained from commercially available oligodendrocyte and monocyte/macrophage cell lines may additionally illustrate actual binding of the two molecules. The caveat of cell lines is that many of them express cell markers that do not appear in vivo and, thus, may be misleading. Unfortunately, the only LAG-3 antibody that has successfully immunostained tissues is the same isotype as our working MHCII antibody. Moreover, double staining has proven difficult, as both antigens provide weak signals and require signal amplification. Therefore, better reagents will be necessary to demonstrate such LAG-3/MHCII interactions in vivo, as well as in vitro. An alternative method to demonstrate LAG-3/MHCII interactions in the brain would be immunoprecipitation of one molecule and Western blot the other. The caveat
of this method is that cellular expression of either molecule would be undeterminable, as LAG-3 is demonstrated on OPCs and astrocytes in Chapter 3 and MHCII has been demonstrated previously on microglia and, to a lesser extent, on astrocytes in the cuprizone model [23].

Additionally, the negative regulation of OPCs should be more intensively studied. The mere presence of a significantly larger population of NG2+ cells in the corpus callosum as early as 2 weeks indicates increased proliferation of these cells within the brain (Figure 3.6A). However, this proliferation does not appear to be occurring in the corpus callosum (Figure 3.6B). Based on previous studies by Jeffrey Mason that suggest many OPCs initially migrate from the SVZ to the fornix before accumulating in the corpus callosum led us to examine the SVZ for proliferating OPCs. Our study suggests a trend of greater proliferating OPCs in the SVZ of LAG-3−/− mice at 2 weeks of cuprizone treatment (166.50 ± 48.36 vs. 81.11 ± 8.18 cells/mm²). However, this study was preliminary and based on counts from 4 mice. Incidentally, significance was lowered from one outlier in the LAG-3−/− mice and, when removed, the significance at 0.13 dramatically lowered to 0.03. Thus, it is feasible that by increasing the number of tissues, a significance in proliferation may surface. Nonetheless, the finding that LAG-3 may regulate OPC is a novel and important finding as few negative regulators of oligodendrocytes have been described. A recent molecule identified to regulate oligodendrocytes is LINGO-1, which activates RhoA and reduces oligodendrocyte differentiation and myelination [263]. LINGO-1−/− mice have not been examined in adult mice during remyelination and it may be interesting to compare phenotypes in the cuprizone model.
Furthermore, the fornix should also be analyzed for OPC numbers in the $LAG-3^{-/-}$ mice. Although from cursory observations in examining the corpus callosum and SVZ, few cells in the fornix appear to be BrdU$^+$. An interesting study that may shed light on the negative regulation of LAG-3 on OPCs due to MHCII interaction would be to examine BrdU incorporation in $LAG-3^{-/-}$ and wild type mixed glial cultures upon the addition of the MHCII tetramer or MHCII-expressing cells (microglia or CIITA-transfected BV2 cells, a microglial cell line). If LAG-3 negatively regulates OPC proliferation, as it does T cells in the periphery, targeting LAG-3 in therapeutic applications could result in priming demyelinating lesions with a greater number of cells that have the capacity to remyelinate upon maturation. This is supported by the significantly larger population of mature oligodendrocytes during remyelination in $LAG-3^{-/-}$ (Figure 3.3). In addition, if one considers propagating OPCs from stem cells or precursor cells, inhibition of LAG-3 may provide a greater yield of OPCs available for transplantation into demyelinated lesions which experimentally is feasible (Mason et al, 2004).

The substantial influx of cells in $LAG-3^{-/-}$ mice during demyelination is only partially attributed to OPCs (Figure 3.4). Microglia and astrocytes, cell types that contribute to the pro-inflammatory response, also accumulate in greater numbers in the absence of LAG-3 at 3 weeks of cuprizone treatment (Figures 3.7 and 3.8). This observation coincides with dramatic demyelination and mature oligodendrocyte depletion observed at the same time point (Figures 3.2, 3.3). LAG-3 is typically associated with activation of APCs (see Section 1.3: LAG-3: An APC Activator), so, in the absence of LAG-3, this observation was surprising. However, a report by Buisson and colleagues demonstrates that sLAG-3, which is present during cuprizone-induced demyelination (Figure 3.1), negatively regulates
monocytes differentiation [257]. They propose that activated lymphocytes that express LAG-3 may reduce differentiation of monocytes in an attempt to limit the magnitude of T cell immune responses in a localized lesion during inflammation. Thus, in the LAG-3-/- mice, there may be a deficiency of this controlled differentiation and, therefore, a large influx of macrophages and astrocytes enter the demyelinating lesion. Moreover, in the periphery, deregulation of in vivo T cell homeostasis by administration of anti-Lag-3 mAb resulted in the expansion of several cell types that include B cells, macrophages, granulocytes, and dendritic cells [177]. Thus, LAG-3 may be playing a similar regulatory role in the CNS, albeit without T cells. Interestingly, we demonstrated LAG-3 expression on astrocytes during demyelination. It is plausible that these LAG-3+ astrocytes may take on an immune regulatory role in the CNS, a role occupied by T cells in the periphery, and in the absence of LAG-3 are unable to control cellular MHCII+ microglial expansion. Targeting LAG-3 for therapy of demyelinating diseases may prove to exacerbate inflammation and demyelination.

Of great interest is reconciling the LAG-3-/- mouse phenotype in which demyelination is exacerbated and remyelination is not affected except for the slight increase in mature oligodendrocytes, and I-Aβ/- mouse phenotype in which demyelination is delayed and remyelination is impaired (Table 4.1) after cuprizone intoxication. Unpublished data from our lab demonstrates that, despite a similar phenotype to I-Aβ/- mice during demyelination, I-Aβtr mice which express I-A but is mutated in the cytoplasmic domain, show no deficit in remyelination and exhibit recovery similar to wild type mice. These studies suggest that, while an intact MHCII molecule promotes remyelination, the absence of a signaling
mechanism does not delay remyelination. The similar remyelination phenotype between 
LAG-3\textsuperscript{-/-} and I-A\textsubscript{\beta}\textsuperscript{-/-} mice suggest that LAG-3/MHCII engagement does not require signaling through the MHCII-expressing microglia for remyelination to occur. Interestingly, the slight increase of mature oligodendrocytes in LAG-3\textsuperscript{-/-} mice at week 7 does not result in accelerated remyelination. This may be a result of LAG-3 independent mechanisms that control the differentiation or maturation of oligodendrocytes or the rate of myelination of axons. Thus, despite a larger population of mature oligodendrocytes in the absence of LAG-3, these cells may not receive proper cues to terminally differentiate into myelin-producing cells. In contrast, I-A\textsubscript{\beta}\textsuperscript{-/-} mice display a deficit in mature oligodendrocyte repopulation up to 2 weeks off cuprizone [23] and I-A\textsubscript{\beta}\textsuperscript{tr} mice exhibit mature oligodendrocyte repopulation similar to wild type mice (unpublished data), suggesting that, while the absence of MHCII signaling does not affect oligodendrocyte repopulation, the absence of the MHCII molecule effectively reduces this population during remyelination. We postulate that the MHCII molecule may engage (1) LAG-3-expressing OPCs to impair OPC migration through the corpus callosum and (2) additional unidentified molecules dependent on engaging MHCII to trigger myelination. Thus, LAG-3 may discriminately regulate OPC population numbers and not mature oligodendrocyte populations. Future directions include examining whether mature oligodendrocytes express LAG-3.

While LAG-3 appears to attenuate demyelination, MHCII exacerbates demyelination [93]. LAG-3 has a 100x stronger binding affinity to MHCII compared to soluble CD4 [159, 167, 168], and LAG-3/MHCII interactions have been postulated to complement other interactions, such as CD40/CD40L in T cell/DC cross talk when conditions are suboptimal (i.e. lack of CD40L expression on poorly activated CD4\textsuperscript{+} or CD8\textsuperscript{+} cells) [250]. We postulate
that in the presence of LAG-3, MHCII engagement induces MHCII signaling and exacerbates demyelination. However, in the absence of LAG-3, MHCII may transiently engage an unknown molecule, inducing weak MHCII signaling and attenuated demyelination. Despite weak signaling through MHCII in \(LAG-3^{-/-}\) mice, with increased microglia/macrophage accumulation, inflammation may be elevated, resulting in exacerbated demyelination. A cytokine and growth factor profile by microarray or ribonuclease protection assay (RPA) in wild type and \(LAG-3^{-/-}\) mice will need to be done. In addition to the negative regulatory function of LAG-3 on the cell that expresses it, we are also interested in the regulatory effects of LAG-3 on APCs. Future aims are focused on \textit{in vitro} studies in characterizing the effects of the addition of LAG-3-expressing cells (e.g. LAG-3 transfected CHO cells) to microglial cultures (i.e. cytokine and growth factor production).

In light of the characterization of \(LAG-3^{-/-}\) mice during cuprizone-induced demyelination and remyelination presented in Chapter 3, future studies can focus now on exploring the functional properties of LAG-3. Because of the two-way signaling involved in LAG-3 function, avenues to explore are (1) the negative regulation of cells expressing LAG-3 and (2) the activation of MHCII-expressing APCs to which LAG-3 binds. In our demyelination and remyelination model, expression of LAG-3 on OPCs may serve to “jump start” remyelination. We hypothesize that LAG-3 inhibits OPC proliferation, allowing OPCs to differentiate into myelin-forming cells. This is not reflected in our results from Chapter 3 that demonstrates \(LAG-3^{-/-}\) mice and wild type mice remyelinate similarly with an increased number of mature oligodendrocytes at 7 weeks in \(LAG-3^{-/-}\) mice. This discrepancy may be due to other unknown inhibitory mediators, such as LINGO-1, or growth factors may initiate remyelination to compensate for the lack of LAG-3 [49, 100, 263, 264]. Furthermore,
negative regulation of astrocytes may be an inherent mechanism to control an overactive immune response, as these cells are able to produce pro-inflammatory mediators, such as TNF-α, LTα, and IFNγ [44, 49, 50]. Astrocytes also function as a supportive cell for oligodendrocytes through production of growth factors, such as IGF-1 and TNF-α [49, 51]. Limiting their expansion may also impair OPC proliferation and influence OPCs toward maturation. Lastly, activation of MHCII+ microglia, possibly through LAG-3 engagement, has been shown by Arnett and colleagues to be important for remyelination processes [23]. Mechanisms to support these hypotheses need to be explored further.

Because LAG-3 is known to be expressed on T cells, our studies also need to be extended to T cell-mediated models of demyelination, such as EAE or viral models of demyelination. Although the cuprizone model allows the study of immune responses, the importance of T cell immunity in diseases, such as multiple sclerosis, should also be examined. We hypothesize that the negative regulation of T cells by LAG-3 during a Th1-mediated autoimmune response would be vital to restrict and contain damage to myelin and oligodendrocytes. Contrastingly, negative regulation of OPC expansion in this scenario may impair remyelination unless compensatory mechanisms exist to propagate this population of cells.

Multiple sclerosis is a heterogeneous disease with unclear causation and pathologic features that in some cases mimic T cell-mediated inflammation but, in other reports, is thought to be primarily an antibody-mediated disease. Other lesions suggest primarily a microglial response, as Barnett and Prineas have described newly forming lesions that have extensive oligodendrocyte apoptosis and microglial activation in the absence of lymphocytes [210]. Our work in the cuprizone model reflects a primarily microglial response and our
studies clearly indicates LAG-3 has significant regulatory roles in the CNS, particularly upon insult. It would be intriguing to examine whether LAG-3 expression in patients with multiple sclerosis may prevent or reduce the OPC population. This could be examined in the cuprizone model under chronic exposure conditions where at week 12, remyelination that is typically feeble in wild type mice may be more robust in \( LAG-3^{-/-} \) mice. Nonetheless, our studies in this dissertation suggest immune mediators, such as IL-1\( \beta \) and LAG-3, may have profound effects in the pathologies of the CNS and may provide insights and considerations to future therapies.
TABLE 4.1. Reconciliation of I-<i>A</i><sup>β</sup>-/- and LAG-3-/- Phenotypes During Cuprizone-induced Demyelination and Remyelination.

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<th>WILD TYPE</th>
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<th>I-&lt;i&gt;A&lt;/i&gt;&lt;sup&gt;β&lt;/sup&gt;&lt;sup&gt;tr&lt;/sup&gt;[93]</th>
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<td>LAG-3 attenuates demyelination</td>
<td>LAG-3/MHCII engagement is not required for remyelination</td>
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<td>Conclusions</td>
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ND = Not determined.

* = Unpublished data.
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