THE EFFECT OF INTERLEUKIN 17 ON OLIGODENDROCYTE PROLIFERATION AND DIFFERENTIATION

by

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A Dissertation submitted to the

Graduate School-Newark

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Biology

written under the direction of

Dr. Haesun A. Kim

and approved by

Newark, New Jersey

October, 2012
ABSTRACT OF THE DISSERTATION
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Interleukin-17 (IL-17) is a cytokine that may cross the blood-brain barrier to promote inflammation and participate in the formation of lesions in patients with multiple sclerosis, where recurrent inflammatory attacks lead to oligodendrocyte loss, loss of axons, and ensuing neurological infirmities. Studies have indicated that the levels of IL-17 mRNA are elevated in the cerebrospinal fluid of multiple sclerosis (MS) patients, and microarray analysis of MS lesions show elevated levels of IL-17 transcripts. In this study we set up an in vitro system to examine the role of IL-17 on oligodendrocyte precursor cell (OPC) proliferation and their differentiation into mature oligodendrocytes (OLs). OPCs were treated with increasing doses of IL-17 (0.03 nM, 0.1 nM, 0.3 nM, 0.9 nM) in the presence of PDGF and FGF, and the effect on proliferation was assessed by BrdU uptake. The results show that while IL-17 alone had nominal mitogenic effect on OPCs it promoted growth factor induced proliferation (p<0.0001). Interestingly, the effect on proliferation was only seen at low doses of IL-17, whereas at high doses, the cytokine inhibited OPC proliferation. IL-17 did not have any significant effect on OPC
differentiation in culture. All together these results show a dose-dependent effect by IL-17 on OPC proliferation. Additional experiments will be important to further elucidate the role of IL-17 on the oligodendrocyte lineage formation.
Acknowledgments

First and foremost, I would like to thank my parents and family for their encouragement and help. They are the key to my optimism. I would like to express my gratitude to Dr. Haesun Kim. She encouraged and supported me throughout this thesis. I would also like to thank Dr. Mill Jonakait, who provided helpful observations and commentary. I would like to thank the members of the Kim lab, Dr. Maurel, and his lab. I would like to thank all the people who read over my thesis, Dr. Silva-Vargas, Dr. Ren, and Mrs. Wong. I wish to give a special thanks to Mrs. Pradeepa Gokina, without her this thesis would not have been possible. I have gained a great deal of knowledge while attending Rutgers University.
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INTRODUCTION

Glial cells represent a substantial part of the central nervous system (CNS), where they participate in various aspects of development, regulation, and maintenance (Baumann and Pham-Dinh 2001). Oligodendrocytes are the myelinating glial cells in the CNS that wrap the axons with laminar processes of the plasma membrane to form myelin sheaths (Nave and Trapp 2008). The multi-spiraling myelin formations protect axons and aide in the conduction of action potentials along the axonal projections of a nerve cell (Nave and Trapp 2008; Emery, 2010). Disruptions of this complex system and subsequent loss of the myelin sheath around the axons in demyelinating diseases such as MS can cause various complications and disorders (Siffrin et al., 2010). Thus, it is critical to get further insights on the regulatory mechanisms of these processes.

Heterogeneity of Multiple Sclerosis

Multiple Sclerosis (MS) is a disease where recurrent inflammatory attacks lead to myelin loss in the CNS (Siffrin et al., 2010). Several genetic investigations have indicated the role of a number of factors whose combined effects may result in a higher susceptibility to the pathogenesis of MS (Lock et al., 2002; Sadovnick et al., 1996; Bomprezzi et al., 2003). Analyses using a spectrum of immunological as well as neurobiological markers of the demyelinating MS lesions, have reported variegated structural as well as immunological changes that may give insights into mechanisms contributing to the development of MS (Lucchinetti et al., 2000). It has been found that, in the early stages of MS, demyelination occurs in conjunction with the destruction of
other lesional tissues such as axons, OLs, and astrocytes (Ozawa et al., 1994; Lucchinetti et al., 1996)

This is consistent with profiles of MS lesions showing variation and heterogeneity in lesions among MS patients, as well as within the same patient (Lucchinetti et al., 2000; Soldan and Rodriguez, 2002). For instance, demyelination can primarily occur with little or minor loss of OLs; or it may occur simultaneously with a major loss of OLs or be secondary to a prior degeneration or cessation of OLs (Lucchinetti et al., 1996). Some episodes of demyelination occur in the early stages (>1 year of MS onset) of MS; some occur in the late and chronic stages (<1 year of onset of MS and relapses) of MS (Lucchinetti et al., 1996; Ozawa et al., 1994). MS lesions are also characterized by their state of demyelination (Ozawa et al., 1994). Lesions that have active demyelination are termed “active lesions” and those that have a cessation of demyelination are called silent or inactive lesions (demyelination has already occurred) (Ozawa et al., 1994; Mews et al., 1998). It has been shown that these lesional patterns of MS could possibly occur as a result of activated macrophages (Lassmann et al., 2000) and microglia (Sriram and Rodriguez, 1997), or by interference from viruses or exposure to toxins (Itoyama et al., 1980). Reactive oxygen species or nitrogen species (Bagasra et al., 1995) as well as cytokines (Hofman et al., 1989; Selmaj et al., 1991) have also been implied in the development of MS. However, it is not known which pathway contributes to which lesional pattern or the exact mechanisms by which these factors contribute to the pathogenesis of MS.

Despite the heterogeneity of MS lesions, a loss in OLs in patients with a long term progression of the disease is common (Lassmann et al., 1997). Furthermore, MS lesions
have varying degrees of OL loss, and subsequently, varying degrees of remyelination. In lesions with remyelination some of the functional deficits caused by demyelination are restored depending on their OL density (Lassmann et al., 1997). However, the myelin sheaths formed during instances of spontaneous remyelination in MS lesions tend to be thinner than average (Franklin and ffrench-Constant, 2008; Figure 1).

**OPC development in CNS**

Oligodendrocyte precursors cells (OPCs) are generated during early embryonic development from neuroepithelial cells in the ventricular zone (Hardy and Reynolds 1991; Reynolds and Wilkin 1988; Anderson et al., 2001; Levinson and Goldman 1993). These cells have the ability to proliferate, and migrate throughout the CNS before they lose motility and differentiate into mature OLs (Baumann and Pham-Dinh 2001). OPCs are immature cells characterized by distinct neuronal cell bodies with bipolar morphology that can be induced to proliferate and mature into postmitotic OLs extending laminar processes (Figure 2A). Different repertoires of growth factors are amplified in the CNS depending on whether the OPCs are induced to proliferate or differentiate (Baumann and Pham-Dinh 2001; Noble et al., 2003; Barres et al., 1994). For instance platelet-derived growth factor (PDGF), an OPC survival factor, and fibroblast growth factor (FGF) have been identified as mitogens for inducing a restricted number of OPC cell divisions (McKinnon et al., 1990; Grinspan and Franceschini 1995; Eccleston and Silberberg 1984). In addition, it has been found that the differentiation of OPCs can be triggered by thyroid hormone, retinoic acid, or mitogen withdrawal, like PDGF (Tokumoto et al., 1999)
The cell identity of oligodendrocytes is associated with a correlative change in the expression of antigens present on the cell surface, as well as phase marker proteins, indicating shifts in proliferative and migratory abilities and stages of maturation (Baumann and Pham-Dinh 2001) (Figure 2B). Classic markers of the OPC stage include platelet derived growth factor receptor α (PDGFR-α) and monoclonal antibodies such as O4, A2B5, all of which are down-regulated as the cell initiates differentiation (Baumann and Pham-Dinh 2001) (Figure 2B). Typical markers of OL maturation include the expression of the proteolipid protein (PLP) gene, myelin basic protein (MBP) and, galactocerebrosides (GalC) (Figure 2B) (Baumann and Pham-Dinh 2001). All of these markers can be detected on mature OLs prior to their initiation of myelination and during myelination (Baumann and Pham-Dinh 2001). In addition, myelin oligodendrocyte glycoprotein (MOG) is used as a marker for the late stages of OL maturation (Baumann and Pham-Dinh 2001). This is located on the surface of OLs and on the plasma membrane of their multilaminar extensions (Brunner et al. 1989; Solly et al., 1996). Mature OLs are vital for preventing or eradicating the lesions produced in the early stages of MS, given that they remyelinate axons that have been initially demyelinated (Siffrin et al., 2010). Conversely, the ability to generate new myelin forming OLs is greatly reduced in cases of MS and contributes to further degeneration for the duration of the disease (Siffrin et al., 2010).

**Oligodendrocytes in MS**

Relying on DNA fragmentation experiments and utilizing immunocytochemistry for markers of MOG, Ozawa et al demonstrated that in the early onset of MS there was a
lack of fragmented DNA in the OL nuclei in the active demyelinating lesions, when compared with the normal white matter, indicating a prevalence of OLs despite demyelination (Ozawa et al., 1994). Using in situ hybridization they also found a prevalence of the MOG expressing myelinating OLs over that of the non-myelinating OLs with the PLP expression (Figure 2B). This finding demonstrated that in the early stages of MS remyelination had not yet begun in the active demyelinating lesions (Ozawa et al., 1994). On the other hand, in the late stages of MS there are significant amounts of fragmented DNA in the nuclei of OL located near the lesional borders of the active demyelinating lesions (Ozawa et al., 1994). Furthermore, in the late stages of MS there is a predominance of non-myelinating OLs containing the PLP mRNA over that of MOG positive myelinating OLs within the demyelinating lesions, which indicates an initiation of remyelination (Ozawa et al., 1994) (Figure 2B).

Several studies have demonstrated the presence of OPCs that were O4 positive and GalC negative within the lesions of patients with chronic MS. In addition, very few lesions contained GalC positive mature OLs, in accordance with the studies of Ozawa et al (Wolswijk, 1998; Ozawa et al., 1994). Other studies have also suggested an abundance of precursor oligodendrocytes in the early stages of MS (Raine et al., 1981). However, Wolswijk determined that the proliferative capabilities of OPCs within these lesions were halted since very few OPCs expressed the Ki-67 antigen (Wolswijk 1998). Taken together the studies indicate that in the late stages of MS there is an absence of mature OLs within lesions, while OPCs remain with diminished proliferative capabilities.

The presence of an assortment of pathological and immunological qualities of MS lesions may contribute to the inhibition of remyelination. Various reports implicate
the role of macrophages in clearing myelin debris formed after demyelination (Kotter et al., 2005). Moreover, studies indicate that an accumulation of macrophages may render OLs susceptible to the toxins released by macrophages, such as reactive oxygen species (ROS) (Griot et al., 1990; Bagasra et al., 1995). Other studies have suggested that over-expression of myelin-related proteins as a result of infections or virus could be culprits in demyelination, hindering the formation and function of OLs (Inoue et al., 1996b; Kagawa et al., 1994). It has been noted that over-expression of myelin proteins such as proteolipid proteins in transgenic mice cause OL apoptosis during their active phase of myelination (Kagawa et al., 1994; Inoue et al., 1996b; Readhead et al., 1994).

**Cytokines role in Demyelination and Myelination Processes**

Several cytokines have been shown to have atypical expression in cases of MS, suggesting that they may also play a role in regulating the oligodendrocyte lineage (Kadi et al., 2006; Hofman et al., 1989; Selmaj et al., 1991; Lund et al., 2004; Calderon et al., 2006; Filipovic et al., 2003). The studies of Kadi et al using immunolabeling and ELISA demonstrated that chemokines such as growth regulated oncogene alpha (GRO-α), interleukin-8 (IL-8), and alpha chemokine stromal cell-derived factor (SDF-1α) increase the synthesis of MBP in a dose-dependent fashion in OLs, subsequently increasing myelin formation in cultures, while also inducing the proliferation of mouse oligodendrocyte precursor-like cell lines (Kadi et al., 2006). In addition to the studies performed by Kadi et al, Robison et al demonstrated that GRO-α promotes the proliferation of spinal cord OPCs (Robinson et al., 1998).
Selma et al and Navikas et al have noted an increased expression of lymphotoxin and tumor necrosis factor alpha (TNF-α) in brain lesions and in the mononuclear cells of MS patients (Selmaj et al., 1991; Canella et al., 1994; Navikas et al., 1996).

Morphological studies by Selmaj et al have shown that lymphotoxin affects OL cell processes as well as induce nuclear degeneration in OL cultures (Selmaj et al., 1991). Additional T–cell products such as perforin and TNF have been shown to induce damage to OLs (Paintlia et al., 2011; Selmaj and Raine, 1988; Scolding et al., 1990), diminishing the ability of these cells to form myelin. Pantlia et al describes the synergistic effect of TNF-α and IL-17 on inducing oxidative stress-induced cell death in OPC-like cells, while Selmaj and Raine describe the TNF-α induced morphological damage in mouse spinal cord OLs (Paintlia et al., 2011; Selmaj and Raine, 1988). In contrast to the effect of TNF-α described above, Arnett et al has shown that TNF-α promotes the proliferation of OPCS and subsequent remyelination of axons (Arnett et al., 2001). Supporting this Agresti et al found that TNF-α alone did not inhibit OPC proliferation (Agresti et al., 1996).

Interferon-γ (IFN-γ) is another major cytokine known to have elevated levels of mRNA in MS lesions (Woodroofe and Cuzner 1993). It has been shown that IFN-γ has the capacity to reversibly hamper the proliferation of rat OPCs, with these effects being potentiated by TNF-α (Agresti et al., 1996). IFN-γ has also been noted to inhibit OL differentiation (Agresti et al., 1996), and more importantly inhibit OL remyelination in MS, thus impairing the conduction of impulses along the axon causing further neurological symptoms. Understanding the factors that regulate OPC proliferation and subsequent differentiation is crucial in understanding their inability to remyelinate and the steps required to repair and renew myelination after extensive demyelination (Siffrin
et al., 2010). Our present study focuses on determining the role of Interleukin-17 on OPC proliferation and subsequent differentiation.

**Interleukin-17 and Multiple Sclerosis**

Notably, in MS patients IL-17 mRNA has been found in excess in mononuclear cells in blood and cerebrospinal fluid (Matusevicius et al., 1999), and significantly expressed in MS lesions (Tzartos et al., 2008), suggesting that it may cross the blood brain barrier to induce the formation of lesions in cases of MS (Matusevicius et al., 1999). In a recent study using primary OPC cultures, immunocytochemistry, and antibodies for IL-17R, Paintlia et al demonstrated the expression of the IL-17R receptor on OLs. This excess of IL-17 in MS lesions may play a role in the subsequent loss of OLs as the disease progresses.

**IL-17 Cytokine**

IL-17, a pro-inflammatory cytokine was first cloned in 1993 by Rouvier et al and was originally called cytotoxic T lymphocyte-associated antigen-8 (CTLA-8) (Rouvier et al., 1993). Furthermore, sequencing of the human genome resulted in the discovery of more members of this 30-35 kDa polypeptide, which were appropriately named IL-17B to IL-17F (Chabaud et al., 2001; Paradowska et al., 2007). These proteins are potentially secreted as a mix of unglycosylated or glycolysated heterodimeric polypeptides (Fossiez et al., 1996). The members of the IL-17 family each have varying patterns of expression suggesting that their biological roles are distinct (Paradowska et al., 2007; Table1). Our
focus is on IL-17 A (also known as IL-17), which is known to be produced by natural killer cells and CD4+ T cells (Th17-T helper cells) originally discovered in mouse models (Miossec et al., 2009; Paradowska et al., 2007).

Studies have shown that TGF-β as well as IL-6 are required for CD4+ T cells to differentiate (McGeachy et al., 2007; Korn et al., 2009). IL-17 may in turn induce the secretion of other soluble cytokines such as IL-6, or IL-8, in stromal cells and human keratinocytes (Fossiez F, et al., 1996; Teunissen et al., 2010), as well as upregulate the secretion of several other cytokines such as, granulocyte colony-stimulating factor (G-CSF), IL-1β, and TNF-α from stromal cells, epithelial cells and macrophages (Fossiez F et al., 1996; Kotake et al., 1999; Paradowska et al., 2007; Lubberts et al., 2001; Jovanovic et al., 1998). Furthermore, IL-17 is regulated by other cytokines, including IL-15 and IL-23 which may work to promote the survival and subsequent production of IL-17 (Ziolkowska et al., 2000; Iwakura and Ishigame 2006). IL-17 may also act synergistically with TNF-α to induce inflammatory responses in the joints and the destruction of cartilage (Paradowska et al., 2007; Zwerina et al., 2007).

**IL-17(IL-17A) Receptor**

The type I transmembrane protein receptor for IL-17 (IL-17R) has an extracellular domain of 293 amino acids in length, a transmembrane domain of 21 amino acids, and a cytoplasmic tail that is 525 amino acids in length (Paradowska et al., 2007; Kolls and Linden, 2004). Studies have shown that the IL-17R mRNA can be detected in several tissue types, including B and T cells, fibroblasts, and stromal cells (Silva et al., 2003). In addition others studies have detected the IL-17R protein directly, in human vascular
endothelial cells and peripheral blood T cells (Moseley et al., 2008). Chromosome mapping and genetic studies have identified an additional four receptors, showing varying expression (Paradowska et al., 2007).

**IL-17 in disease**

Cells of the innate immune system are the initial route of resistance against pathogens, and cytokines produced by this system help regulate the differentiation of helper T cells (Miossec et al., 2009). IL-17, in particular, helps in regulating both immune response and inflammatory response (Paradowska et al., 2007). Despite this benefit, it is also thought to be a key player in the progression of several autoimmune diseases, such as inflammatory bowel diseases (Fujino et al., 2003), psoriasis (Lowes et al., 2008), and rheumatoid arthritis (Hwang and Kim 2005). In cases of rheumatoid arthritis, a autoimmune disease characterized by cartilage destruction and the subsequent inflammation of joints, IL-17 has been found in excess in synovial fluids and in synovium (Ziolkowaska et al., 2000), and thought to induce the production of MMP-1 and MMP-13 collagenases associated with matrix and bone destruction (Cai et al., 2001). Based on the role of IL-17 in disease and the findings that the expression is increased in MS lesions, in this study we examine the effect IL-17 has on OPC proliferation and differentiation.
Figure 1

A

Franklin and ffrench-Constant 2008

B

Franklin and ffrench-Constant 2008
Figure 1. Demyelination in MS.
A, Taken from Franklin and ffrench-Constant 2008. Representative schematic of demyelination in MS, showing the thinner myelin sheaths in remyelination B, Taken from Franklin and ffrench-Constant 2008. Thin sections of embedded fixed tissue from adult rat brain white matter in resin examined by light microscopy, showing normal myelinated axons, demyelinated axons, and the thinner myelin sheaths of remyelinated axons.
Figure 2

Oligodendrocyte Precursor Cell

A

Differentiated Oligodendrocyte

B

Baumann and Pham-Dinh 2001
Figure 2. Oligodendrocyte Morphology and cell identity.
Oligodendrocyte precursor cell (OPC). A, Taken from O’Meara et al., 2011. Representative schematic of an OPC, a glial cell in the CNS while in the progenitor or precursor stage- prior to and after differentiation into the myelin producing mature Oligodendrocytes(OL), Taken from Lyssiotis et al., 2007. Phase bright image of OPC showing its simple bipolar morphology, immunohistochemical image of differentiated OLs with extend laminar projections. Immunohistochemistry was performed using MBP (Alexa546)/DAPI) of Oligodendrocyte precursor cells (OPCs) differentiated for 5 days and cultured in N2B2 +T3 (Triiodothyronine). OPC stages of development B, Taken from Baumann and Pham-Dinh 2001. Representative schematic showing morphological and antigen progression during the developmental stages of OPCs with boxes surrounding the stage specific markers, RNA is italicized.
<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Other names</th>
<th>Chromosomal location</th>
<th>Homology of murine to human(%)</th>
<th>Major Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17</td>
<td>IL-17A, CTLA-8</td>
<td>6p12</td>
<td>62</td>
<td>Stimulated epithelial, endothelial, and fibroblastic stromal cells, inflammation, bone metabolism</td>
</tr>
<tr>
<td>IL-17B</td>
<td>CX1</td>
<td>5q32-34</td>
<td>88</td>
<td>Induce IL-6 in human foreskin fibroblast, cytokine secretion, induce TNF-α and IL-1β from a monocyte line</td>
</tr>
<tr>
<td>IL-17C</td>
<td>CX2</td>
<td>16q24</td>
<td>83</td>
<td>Induce TNF-α and IL-1β from a monocyte line, induce IL-6 in human foreskin fibroblasts, regulation of Th1 cytokines</td>
</tr>
<tr>
<td>IL-17D</td>
<td>IL-27</td>
<td>13q11</td>
<td>78</td>
<td>Cytokine secretion</td>
</tr>
<tr>
<td>IL-17E</td>
<td>IL-25</td>
<td>14q11.1</td>
<td>81</td>
<td>Induce production of IL-8, regulator of Th2 response</td>
</tr>
<tr>
<td>IL-17F</td>
<td>ML-1</td>
<td>6p12</td>
<td>77</td>
<td>Regulation of Th1 cytokines</td>
</tr>
</tbody>
</table>

Table 1
IL-17 family of cytokines and their major functions (Paradowska et al., 2007)
AIMS OF THESIS

Cytokines are a large part of the innate immune system, forming precise networks that not only regulate the immune response but can act as paracrine and autocrine mediators regulating the function of cells in the CNS (Mizuno et al., 1994). Recently, several studies sampling the products of inflammatory cells have indicated that cytokines may play an important role the pathogenesis of inflammatory diseases (Selmaj et al., 1991; Fujino et al., 2003; Lowes et al., 2007; Hwang and Kim 2005). In particular an abundance of IL-17 mRNA has been found in the active lesions of patients with multiple sclerosis: a demyelinating disease characterized by chronic inflammation (Tzartos et al., 2008). IL-17 may have a direct impact on the OLs in patients with MS, and disturb their normal function. The goal of this thesis project was to elucidate the role of the IL-17 cytokine on regulation of cortical oligodendrocyte lineage.

Aims of this thesis project were to:

a) Determine the effects that progressive doses of IL-17 have on the proliferation of OPCs. To this end, we used OPCs prepared from rat cortical neurons. We assessed proliferation by BrdU incorporation. It was found that IL-17 promoted a growth factor mediated induction of OPC proliferation (p<0.0001), in a possibly does-dependent manner.

b) Examine the role of IL-17 on the differentiation of OPCs into mature OLs. The effect on differentiation was assessed by immunohistochemistry by detecting the expression of MBP. Our experiments showed that OPC differentiation was not affected in the presence of IL-17. Altogether the results from this study show that
IL-17 induced OPC proliferation in a dose-dependent manner yet had no effect on OPC differentiation into mature OLs
MATERIALS AND METHODS

Materials

Purchases from Invitrogen-BRL included media for cell culture (MEM, F12), trypsin 0.25% with EDTA, insulin-selenium-transferrin (ITS). Triiodothyronine (T3), laminin, poly-D-lysine, glucose, Penicilin- Streptomycin, D-biotin, apo-transferin, progesterone were purchased from Sigma. DMEM was purchased from Mediatech. Recombinant FGF-2 was purchased from R&D Systems. FBS was purchased from Atlas Biologicals. Coverslips were obtained from Deckglaser. Primary antibodies for myelin basic protein (MBP-SMI94) were purchased from Millipore Bioscience Research Agents, BrdU antibody, Brd-U Clone BU-33 mouse was purchased from Sigma, and Olig2 rabbit DF308, a gift from Dr. John Alberta (Dana-Farber Cancer Center). Secondary antibodies Alexa Fluor 488 conjugated goat anti-mouse IgG, were purchased from Jackson ImmunoResearch, while Alexa Fluor 546 conjugated goat anti-mouse IgG, and Alexa Fluor 546 goat anti-rabbit IgG was purchased from Molecular Probes. Interleukin -17A was purchased from Prospec.

Preparation of primary oligodendrocyte precursor cultures procedures.

Protocols for the animal experiments using the Sprague Dawley rat pups were performed according to guidelines for laboratory animals provided by the National Institutes of Health. The protocols were approved by the Rutgers Institutional Animal Care and Use Committees (IACUC). OPCs were purified from mixed cortical glial cells using already established methods (McCarthy and de Vellis, 1980). Briefly, cortical pieces from the
Sprague Dawley rat pups of postnatal day 0-2 were dissected and placed in a 60 mm petri dish on ice in a solution of PBS supplemented with 1 mM MgCl₂ and 0.6% glucose. After removal of the cortical meninges the cells were minced and gently dissociated. 15-20 ml of MEM-C (minimal essential media supplemented with 10% FBS, 1% L-glutamine, 1% Pen-strep), was added to the cells. The cells were then filtered and centrifuged at 1800 rpm for 10 minutes. The mixed cortical cells were re-suspended in MEM-C and plated on T75 flasks. The mixed glial cells were maintained for 10-15 days; the media in the flasks were changed every 2 to 3 days. The differential shake method (McCarthy and de Vellis, 1980), was used to obtain purified OPCs, which were re-suspended in N2S (N2B2 at 66% containing DMEM/F12 w 15mM HEPES which was supplemented with D-Biotin(1 mg/ml), BSA (66 ng/ml), ITS (10%), apo-transferin (4 mg/ml), putrescine (1.6 mg/ml), progesterone (20 nM) and B104 condition media at 34%, plus additional FGF (5 ng/ml), and 0.5% FBS). The purified OPCs were then plated on the T75 flasks coated with poly-D-lysine at a density of 2x10⁴ cells/cm². The cells were maintained in N2S for 3-7 days, passaged once and then plated for further experiments.

**Differentiation Assay**

A well-established protocol (Tokumoto et al., 1999) for mitogen withdrawal was utilized to induce differentiation of OPCs. The OPCs were plated at a density of 4x10⁴ cm⁻² or 5x10⁴ cells/cm² onto12 mm glass coverslips coated with poly-D-lysine-laminin. The next day the N2S media was switched with N2B2 (mitogen free supplemented with triiodothyronine (T3-30 ng/ml), to initiate differentiation in the absence or in the presence of IL-17 for 5 days. For the IL-17 treatments a stock solution of IL-17A at 1 nM was re-
suspended in N2B2 supplemented with T3 to obtain the various treatments concentrations. In this assay the control received N2B2+T3 alone. Following the initial treatments the media and treatments were replenished every 48 hours.

**Proliferation Assay**

Purified OPCs were plated at a density of 2x10⁴/cm or 3x10⁴ cells/cm² onto 12 mm glass coverslips coated with poly-D-lysine-laminin. After plating, medium was replaced with mitogen free N2B2 (mitogen free media) for twelve to fifteen hours, and then the cells were treated with a combination of 5 ng/ml PDGF (platelet derived growth factor) and FGF, along with IL-17 for fifteen hours. Following the treatments, OPCs were pulsed with 10 µM of BrdU for 3 hours.

**Immunocytochemistry for Proliferation and Differentiation Assays**

**Proliferation**

Following the completion of each assay the OPCs and the differentiated OLs were fixed with 4% paraformaldehyde at room temperature for 15-20 minutes followed by two PBS washes. Chilled methanol was added to the cells and placed in the -20 °C for 15-20 minutes. Following this the cells were placed in a humidifying chamber, rehydrated with PBS, and incubated with 2 N HCL for 15 minutes at 37 °C. Cells were then rinsed at room temperature with 0.1 M Boric acid (pH 8.5) three times for 10 minute periods. This was followed by three washes with PBS for 5 to 10 minute periods and a subsequent blocking step. The coverslips were incubated with a blocking buffer (1X PBS containing 0.4% Triton-X 100 and 5% normal goat serum (NGS)) for 1hr.
Olig2, BrdU antigens were detected by incubating the coverslips with blocking buffer containing a 1:1000 dilution of primary antibodies for Brd-U (Clone BU-33 mouse) and Olig 2 (rabbit DF308) for 24 hours at 4 °C. The cells were washed three times with PBS and incubated in blocking buffer containing a 1:1000 dilution of 4′, 6′-diamindino-2-phenylindole dihydrochloride (DAPI), and secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (1:1000) and Alexa Fluor 546 goat anti-rabbit IgG (1:1000), at room temperature for 1 hour.

**Differentiation**

Following the completion of each assay the differentiated OLs were fixed with 4% paraformaldehyde at room temperature for 15-20 minutes followed by two PBS washes. Chilled methanol was added to the cells and placed in the -20 °C freezer for 15-20 minutes. Following this, cells were placed in a humidifying chamber, and rehydrated with PBS. The coverslips were incubated with a blocking buffer (1X PBS containing 0.2% Triton-X 100 and 5% normal goat serum (NGS)) for 1hr.

MBP (SMI-94) antigens (marker for maturation of OLs-Figure 2B) were detected by incubating the coverslips with blocking buffer containing primary antibody SMI-94 for 24 hours at 4 °C. The following day cells were washed three times with PBS and incubated in blocking buffer containing a 1:1000 dilution of (DAPI), and secondary antibody (Alexa Fluor 546 goat anti-mouse IgG (1:1000)) at room temperature for 1 hour. In the final steps of both assays the coverslips were once again washed with PBS and mounted onto SuperFrost slides using fluoromount G. Images of the coverslips were taken using the Nikon Eclipse te 2000 via MetaMorph Imaging Application version
6.1r0. The coverslips were quantified by using a minimum of 25 pictomicrographic fields obtained using a 20X objective. For the proliferative assay, the total number of cells was obtained for the DAPI and the BrdU, and the number of OPCs containing BrdU was expressed as a percentage of BrdU+ cells to the overall DAPI+ cells. For the differentiation, the total number of cells were counted for DAPI and the SMI94, and the number of differentiated OLs containing MBP was expressed as a percentage of MBP+ cells to the overall DAPI+ cells.

RESULTS

**IL-17 promotes the proliferation of oligodendrocyte precursor cells in a dose-dependent manner**

To examine the role of IL-17 on the proliferation of OPCs, purified OPCs were simulated with varying doses of IL-17 (0.03 nM, 0.1 nM, 0.3 nM, 0.9 nM) in the presence or absence of PDGF+FGF. Fifteen hours later, cultures were treated with bromodeoxyuridine (BrdU) for three hours and then fixed and immunostained for BrdU, DAPI, and Olig2 antibodies (Figure 3). Approximately, all the cells that were DAPI+ expressed Olig2, thus for the subsequent experiments the percentage of BrdU+ OPCs (DAPI+) was determined for 25 fields using two coverslips each (Figure 4D). Representative images of the control and cultures treated with PDGF+FGF are shown in Figure 3. In the first set of experiments (Figure 4A) the cultures in the absence of PDGF+FGF (NT) only a very small percentage of the OPCs (0.42%) incorporate BrdU, indicating that OPC proliferation was not initiated. In the presence of PDGF+FGF the number of BrdU incorporating cells (11.98%) increased as expected. When cultures were
co-treated with IL-17 (0.03 nM, 0.1 nM, 0.3 nM), we observed further increase in OPC proliferation (14.82%, 12.98%, 15.32%, respectively). Interestingly, at 0.9 nM, IL-17 appeared to have an inhibitory effect on growth factor induced OPC proliferation (7.02%). IL-17 alone at 0.9 nM had no effect on OPC proliferation (0.43% BrdU uptake).

In the second set of experiments (Figure 4B) the NT cultures had little to no BrdU incorporation (0.21%), in accordance with the first set of experiments. In the presence of PDGF+FGF the number of BrdU incorporating OPCs increased (21.65%). The cultures co-treated with IL-17 (0.03 nM, 0.1 nM, 0.3 nM) had a BrdU uptake similar to the cultures treated with growth factor alone (18.87%, 19.03%, 19.45%, respectively). Comparatively, the inhibiting effect on OPC proliferation observed at the high doses (0.9 nM) of IL-17 was not detected in the second experiment (18.16%). Interestingly, the cultures treated with the high dose alone had increased OPC proliferation (7.02%) compared to the control. This could be due to the possibility that the cultures in Experiment I were less healthy and more susceptible to a higher concentration of IL-17.

In the third experiment we observed a proliferative promoting effect of IL-17 in the presence of PDGF+FGF (Figure 4C). NT cultures had very little to no OPCs (1.21%) incorporating BrdU. At 0.03 nM, 0.1 nM, and 0.3 nM, there was an approximately 0.9 and 60 fold increase in the %BrdU+ OPCs when compared to the cultures treated with PDGF+FGF alone (12.10%). The OPC proliferation (19.47%, 18.35%, respectively) was most notable at the concentrations of 0.1 nM, 0.3 nM. The cultures co-treated at 0.9 nM of IL-17 did not promote growth factor induced OPC proliferation (11.13%) when compared to PDGF+FGF cultures alone. The cultures treated with IL-17 alone had a slight increase in BrdU+ OPC (3.06%) incorporation when compared to the NT.
The composite of the three experiments is shown in Figure 4D. The result shows that IL-17 at 0.03 nM, 0.1 nM, and 0.3 nM promotes OPC proliferation induced by PDGF+FGF (15.56%, 16.95%, 17.72%, respectively). The increase demonstrated at low doses (0.3 nM) were significant (p<0.0001). At 0.9 nM, IL-17 inhibited growth factor induced OPC proliferation (12.04%; p<0.0001). The cultures treated with IL-17 alone had a significant increase in BrdU+ cells when compared to the NT (3.68%; p<0.0001), indicating that IL-17 had a slight mitogenic effect on OPCs in the absence of growth factors.

**IL-17 does not affect the differentiation of OPCs into mature (OLs)**

To determine if IL-17 had an effect on the differentiation of OLs, purified OPCs were stimulated with varying doses of IL-17 (0.03 nM, 0.1 nM, 0.3 nM, 0.9 nM) in mitogen deficient media (N2B2) supplemented with Triiodothyronine (T3). The cells were treated every two days for 5 days. The differentiation of OPCs was assessed at the end of the culture on the 5th day. A representative image of differentiated OL in culture is shown in Figure 5. To determine the effect of IL-17, the percentage of MBP+ cells in IL-17 treated cultures were compared to that of the control cultures. A total of 25 fields per coverslip and 3 coverslips per condition were used for this study.

In the first set of experiments, the addition of growth factor free N2B2+T3, resulted in a considerable number of MBP+ OLs (73.08%) being expressed (Figure 6A). The cultures treated with IL-17 (0.03 nM, 0.1 nM, 0.3 nM, 0.9 nM) had similar percentage of MBP+ cells (75.54%, 77.49%, 67.90%, 62.50%), indicating that IL-17 did
not affect OPC differentiation into MBP+ OLs. Similar results were observed in experiments II and III (Figure 6B, 6C).

The composite of the three experiments shown in Figure 6D did not show a significant difference in percentage of MBP+ OLs: 83.77%, 84.85%, 85.45%, 82.81%, 79.83% in the presence of 0.03 nM, 0.1 nM, 0.3 nM, 0.9 nM IL-17 respectively, when compared to 83.77% in the NT culture.
Figure 3

NT

PDGF+FGF

BrdU

BRDU

BRDU

Olig2

OLIG2

OLIG2

Dapi

DAPI

DAPI

Brdu/Dapi/Olig2

MERGE

MERGE
Figure 3. Immunohistochemistry of BrdU incorporation.
Figure 4

A. Experiment I

B. Experiment II

C. Experiment III

D. Composite
Figure 4. IL-17 promotes growth factor induced OPC proliferation in a dose-dependent manner.

A-C, Quantified percentage of BrdU+ OPCs (DAPI+) cultured in the absence of growth factors (N2B2 alone-NT) or in the presence of PDGF and FGF with IL-17 (1 nM stock) at 0 nM, 0.03 nM, 0.1 nM, 0.3 nM, 0.9 nM) or IL-17 alone (0.9 nM) followed by a three hour BrdU pulse. The cells were quantified for 25 fields using 2 coverslips per treatment. A, Experiment I, B, Experiment II, C, Experiment III, D, Composite for three experiments, (;p<0.0001*by One way ANOVA for the composite experiments).
Figure 5

MBP/DAPI
Figure 5. Immunohistochemistry of MBP expression. Representative images for treated OPCs, cultured in the presence or absence of IL-17 (1 nM stock) (0, 0.3 nM, 0.1 nM, 0.9 nM) and differentiated into mature OLs for 5 days; differentiation was assessed by the expression of MBP (Alexa546)/DAPI.
Figure 6
Figure 6. IL-17 does not have a significant effect on differentiated OLs.
A-C, Quantified percentage of MBP+/DAPI+ mature oligodendrocytes (OLs) differentiated for 5 days for 3 duplicate experiments, cultured in N2B2 +T3 in the absence or presence of IL-17(0.03 nM, 0.1 nM, 0.3 nM, 0.9 nM). The cells were treated every two days with Immunohistochemistry being performed on the 5th day. The cells were quantified from 25 fields using 3 coverslips per treatment. A, Experiment I, B Experiment II, C, Experiment III, D, Composite for three experiments, no significance (NS).
DISCUSSION

Multiple sclerosis is a demyelinating disease in which chronic inflammation targeting oligodendrocytes (myelin-producing) leads to their reduction in the central nervous system (Siffrin et al., 2010). Accordingly, the regulation of oligodendrocyte biology and lineage is crucial for the proper formation and aggregation of myelin along the axons. Several growth factors such as PDGF and FGF, chemokines such as GRO-α, IL-8, SDF-1α, and cytokine IFN-γ, and TNF-α have been shown to be key regulators in the oligodendrocyte lineage (McKinnon et al., 1990; Grinspan and Franceschini 1995; Eccleston and Silberberg 1984; Kadi et al., 2006; Saneto et al., 1986; Cannella et al., 2003). One particular cytokine, IL-17 has been implicated as a significant component in the formation of MS lesions (Tzartos et al., 2008) as well as in the progression of several inflammatory diseases such as rheumatoid arthritis (Hwang and Kim 2005), inflammatory bowel disease (Fujino et al., 2003), and psoriasis (Lowes et al., 2008). However, the pathogenesis of IL-17 and the role it plays in diseases is still relatively unknown.

IL-17 promoted growth factor induced proliferation of OPCs at low concentrations

In our present study, we investigated the effect of IL-17 on OPC proliferation as assessed by bromodeoxyuridine (BrdU) uptake in the presence or absence of PDGF+FGF. In the first and third set of experiments (Figure 4A, 4C) the NT cultures had little to no BrdU incorporation into the purified OPC cells, indicating that OPC proliferation was not initiated. In the presence of PDGF+FGF the number of BrdU
incorporating cells increased as expected. When cultures were co-treated with IL-17 we observed further increase in OPC proliferation. Interestingly, at higher doses, IL-17 appeared to have an inhibitory effect on growth factor induced OPC proliferation.

In the second set of experiments (Figure 4B) this trend deviates slightly. The NT cultures had little to no BrdU incorporation, in accordance with the first and third set of experiments. In the presence of PDGF+FGF the number of BrdU incorporating cells increased. However, in this set of experiments the cultures co-treated with IL-17 had a BrdU uptake similar to the cultures treated with growth factor alone. Interestingly, the cultures treated with IL-17 0.9 nM alone had increased proliferation relative to the control.

The first and third sets of experiments were similar, while the second differed. In light of this a possible explanation could be that the cultures used for the second set of experiments were in-fact healthier than the cultures used for first and second proliferation assay. Thus, these cells may have been less susceptible to any harmful effects caused by the higher levels of the IL-17 cytokine.

The composite of the three experiments shown in Figure 4D suggest that IL-17, at low concentrations (0.1 nM, 0.3 nM), promotes growth factor induced proliferation. However, at high doses (0.9 nM) there was a significant inhibitory effect on OPC proliferation. Studies by Paintlia
et al have demonstrated this inhibitory effect produced by IL-17 (Paintlia et al., 2011). The study demonstrated that IL-17 (25 ng/ml; 0.72-0.75 nM) when synergized with TNF-α (10 ng/ml) induced a significant inhibition of (³H) thymidine incorporation (p<0.01), and the expression of cyclin D1 and cdk4 (p<0.001) in OPC-like B12 cell lines (Paintlia et al., 2011). Furthermore, IL-17 in combination with TNF-α accordingly promoted the expression of p21clip. Cyclin D1 and cdk4 are proteins important for cell cycle progression, while p21clip is an inhibitory cyclin kinase (Paintlia et al., 2011). These studies indicate that IL-17 synergized with TNF-α, induces cell cycle arrest in B-12 proliferating cells (Paintlia et al., 2011). Consistent with these inhibitory effects noted by Paintlia et al, findings from current histological studies have shown that brain lesions of patients with prolonged MS contain significant number of OPCs that remain in quiescence (Wolswijk, 1998). Further investigations by Lassmann et al identified lesions that maintain high levels of OLs with low levels of proteolipid mRNA and proteins associated with myelination, indicating that these oligodendrocyte cells may be dormant precursor or progenitor oligodendrocytes (Lassmann et al., 2002). These studies indicate that there may be elements within MS lesions inhibiting the cell cycle progression of OPCs. Accordingly, studies of MS lesions have shown elevated levels of several immunological factors, but their function is not yet known (Lucchinetti et al., 2000). In contrast, investigations by Paintlia et al found that IL-17 alone did not inhibit cell cycle progression or have any proliferative effects on OPC-like B-12 cells when compared with the control. However, due to the fact that the Paintlia et al study observed the effects of IL-17 on a cell line, it may be that primary OPCs are more susceptible to the inhibitory effects of IL-17, and IL-17 in high levels or in combination with other cytokines such as
TNF-α, may suppress primary OPC proliferation. Future studies regarding these parameters may provide insight into the progression of MS.

**IL-17 does not have a significant effect on the differentiation of OPCs**

To determine the role of IL-17 on OPC differentiation into mature OLs, the expression of MBP was assayed in OPC cultures stimulated with varying doses of IL-17 (0.03 nM, 0.1 nM, 0.3 nM, 0.9 nM) in mitogen deficient media (N2B2) supplemented with triiodothyronine (T3) every two days for five days.

In the differentiation experiments, the cultures without the cytokine treatment resulted in a considerable percentage of cells with MBP+ expression (Figure 5, 6D). The cultures treated with IL-17 had similar MBP+ cell expression, indicating that IL-17 did not significantly affect OL differentiation (Figure 6D). As corroborated by Paintlia et al, IL-17 did not affect the expression of MBP transcripts in maturing OPCs as compared to the control (Paintlia et al., 2011). Conversely, the maturing OPC cultures treated with IL-17 and TNF-α had a significant reduction in the expression of MBP transcripts (Paintlia et al., 2011). These findings, taken along with those found in the studies of Wolswijk, which show that there is lack of mature OLs in MS lesion (Wolswijk, 1998), suggest that IL-17 in combination with other cytokines may inhibit OL maturation within MS lesions.

**IL-17 may be cytotoxic to OPC**

In the cultures exposed to the high dose of IL-17 (0.9 nM), there was a reduction in BrdU+ cells (Figure 4D). This reduction of OPC proliferation observed in our
experiments may be consistent with cytokine toxicity or apoptosis. Paintlia et al, using TUNEL assays, and an assay for caspase-8 activity, demonstrated that IL-17 potentiated the apoptotic activity of TNF-α in cultured OLs, and produced a significant increase in the release of lactate dehydrogenase (LDH) in OLs and the B-12 cell line (Paintlia et al. 2011). In addition, when the OLs were treated with a high dose (100ng/ml; 2.8-3nM) of IL-17 there was a significant increase (p<0.05) in the release of LDH (Paintlia et al. 2011). Moreover, IL-17 together with TNF-α induced greater oxidative stress to the B-12 cell line, when compared with IL-17 alone and the control (Panitlia et al., 2011). These studies indicate that IL-17 at higher concentrations produces a cytotoxic effect on OLs and when synergized with TNF-α, produces a cytotoxic as well as an apoptotic effect on OLs (Panitlia et al., 2011). Nevertheless, these studies use mature OLs and we have not tested the synergistic effect of IL-17 in addition with other cytokines on OPCS. It would be advantageous to perform cytotoxic and apoptotic assays on primary OPCs in conjunction with other cytokines, considering that studies have noted the presence of OPCs in MS lesions (Wolswijk, 1998)

**Future directions**

Studies done by Cannella and Raine have shown that a complex cytokine profile exist in MS lesions (Cannella and Raine, 1994). Paintlia et al and Matusoviccius et al have indicated the synergistic effects of the IL-17 cytokine with TNF-α (Paintlia et al., 2011; Matusoviccius et al., 1999) on mature OLs. It would be advantageous to look at the synergistic effects of IL-17 and other cytokine on primary OPCs and OLs.
Recent studies have revealed that IL-17R is expressed in OLs (Paintlia et al., 2011), indicating the possibility of IL-17 mediated regulation of OLs. In MS, it is unknown which stage in the oligodendrocyte lineage (OPC, immature OPCs, non-myelinating mature OLs, myelinating mature OLs; Figure 2B) responds to or is affected by the IL-17 cytokine. It will be important in future experiments to examine at which stage of OPC differentiation the IL-17 receptor is first expressed, as the expression of the IL-17 receptor could render OPCs susceptible to regulation by the IL-17 cytokine. Additionally, other cytokines could modulate the IL-17 receptor expression in each stage of OPC maturation, potentially making the cells more susceptible to the effects of IL-17. Hence, it will be beneficial to examine the effects of IL-17, as well as the receptor expression at the different stages in the OPC lineage in combination with other cytokines. These experiments can be performed using western blot analysis to assess the receptor levels.

Our initial study demonstrated that IL-17 had an effect on OPC proliferation in a dose-dependent manner, but had no effect on differentiation; however, we used a range of relatively low concentrations of the IL-17 cytokine (0.03 nM, 0.1 nM, 0.3nM, 0.9 nM). It will be prudent to examine a wide range of the IL-17 concentration on the different and intermediate stages of OPC development and repeat the proliferation and differentiation assays with these new concentrations.

It will also be crucial to determine the downstream pathway of IL-17, which may contribute to the inhibitory effect of IL-17. The studies of Panitlia et al demonstrated that in cultures using OLs, and OPC-like B-12 cell lines, IL-17 in combination with TNF-α inhibited the expression of cyclin D1 and cdk 4, and increased the expression of p21^{cip} (
Panitlia et al., 2011). Furthermore, they found that OLs treated with IL-17 and TNF-α, had elevated levels of phosphorylated ERK1/2, phosphorylated JNK1/2, Bid, Bax, TRAAD and TRAF6 (TNF receptor associating factor-6), all of which participate in the cell death pathway (Panitlia et al., 2011). However, in the cultures treated with IL-17 alone, only TRAF6 was increased (Panitlia et al., 2011). This increase in TRAF6 is consistent with the synergistic activities of IL-17 and TNF-α noted in this study (Paintlia et al., 2011). It would be beneficial to conduct a cell death assay, as well as examine the downstream pathways of IL-17, to determine if increasing the concentrations of the IL-17 cytokine eventually trigger apoptosis or programmed cell death in primary OPCs.

This study has revealed an effect of various doses of IL-17. Our results suggest that the dose of IL-17 plays an important role in determining the fate of OPCs. It will be interesting to test if this mechanism applies to other cytokines up-regulated in MS lesions. The studies proposed would provide further insight into the role of IL-17 on oligodendrocytes and the progression of MS, which will have important implications therapeutically.
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