INTERLEUKIN-11 IS A KEY MEDIATOR OF INTRAVENOUS IMMUNOGLOBULIN EFFECTS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine and Pathobiology
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Interleukin-11 is a key mediator of intravenous immunoglobulin therapy in experimental autoimmune encephalomyelitis

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2013

Abstract
Intravenous immunoglobulin (IVIg) has been used to treat a variety of autoimmune disorders including multiple sclerosis (MS); however, its mechanism of action remains elusive. Our results demonstrate a novel finding wherein IVIg treatment induces a dramatic surge (>1000-fold increase) in the levels of IL-11 in the circulation and that the liver is the organ of increased IL-11 transcription. Furthermore, we show that IL-11Rα knockout mice, although initially protected, become resistant to protection by IVIg during EAE and develop disease with a similar incidence and severity as control-treated IL-11Rα−/− mice. The inability of IVIg to prevent EAE in IL-11Rα−/− mice correlated with a failure of this agent to decrease IL-17 production by myelin-reactive T-cells in the draining lymph nodes. Finally, we show that IL-11 can directly inhibit IL-17 production by lymph node cells in culture. Together, these results implicate IL-11 as an important immune effector of IVIg in the amelioration of EAE.
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
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<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CNTF</td>
<td>Ciliary Neurotrophic Factor</td>
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<tr>
<td>DC</td>
<td>Dendritic Cells</td>
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<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
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<td>FcγR</td>
<td>Fc-gamma receptor</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FoxP3</td>
<td>Forkhead Box P3</td>
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<td>GBS</td>
<td>Guillain Barré Syndrome</td>
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<td>gp130</td>
<td>Glycoprotein 130</td>
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<td>HES</td>
<td>Hypereosinophilic Syndrome</td>
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<td>HSA</td>
<td>Human Serum Albumin</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-11Ra−/−</td>
<td>Interleukin-11 Receptor Knockout</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPOPI</td>
<td>International Patient Organization for Primary Immunodeficiencies</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ITP</td>
<td>Immune Thrombocytopenic Purpura</td>
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<td>IV</td>
<td>Intravenous</td>
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<tr>
<td>IVIg</td>
<td>Intravenous Immunoglobulin</td>
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<tr>
<td>JAK</td>
<td>Janus Kinase</td>
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<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
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<tr>
<td>LN</td>
<td>Lymph Node</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MOG</td>
<td>Myelin Oligodendrocyte Glycoprotein</td>
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<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
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<tr>
<td>Th1</td>
<td>T helper cell 1</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
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<td>Treg</td>
<td>T regulatory cell</td>
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<td>Wild-type</td>
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CHAPTER 1
INTRODUCTION

1.1 History of Intravenous Immunoglobulin

Immunoglobulin therapy can be dated back to the 1800s, where patients suffering from smallpox, rabies and diphtheria could be treated with animal sera. In the 1940s, a process to isolate proteins from human plasma was described (Cohn et al., 1946), which was developed into large scale purification of immunoglobulin G (IgG). In 1952, Dr. Ogden Bruton described a condition called agammaglobulinemia, which he explained was caused by low concentrations or absence of gamma globulin (Bruton, 1952). Gamma globulin refers to a class of plasma proteins, of which the most important component is immunoglobulins (IgG, IgM, IgA and IgD are the major classes of immunoglobulins) which comprise of antibodies that play a role in fighting infection and foreign antigens. To treat patients with agammaglobulinemia, Bruton relied on subcutaneous infusion of human serum immunoglobulin, which consisted mostly of IgG. For a number of years, infusions of these immunoglobulin preparations was carried out through intramuscular or subcutaneous administration, which was particularly painful with the large volumes that needed to be administered to the patient (Goddard, 2008). Additionally, the absorption of IgG into circulation from the site of injection was slow (Knezevic-Maramica and Kruskall et al., 2003). Furthermore, intravenous (IV) administration of the IgG preparation that was available at the time resulted in complications and adverse side effects to patients. As a result, further refinement and development in IgG preparation was required such that intravenous administration
would be easier (Hooper, 2008). It was not until the 1980s that FDA approved intravenous immunoglobulin (IVIg) was introduced as a therapeutic for the replacement of immunoglobulins in patients with primary immunodeficiencies. The IVIg offered patients a much less painful treatment option and allowed for larger volumes of the drug to be administered with fewer side effects (Garbett ND 1989).

1.2 Preparation of IVIg

IVIg is manufactured by isolating and preparing the immunoglobulin molecules from the plasma of healthy donors. IVIg production is carried out largely by using the Cohn alcohol fractionation method (Cohn et al. 1946). To ensure the high quality of the product, plasma from approximately 10,000 to 20,000 donors is pooled before the fractionation takes place (Goddard, 2008). There are a number of additional regulations which govern the quality of IVIg being produced. The IVIg needs to be contaminant free and contain no infectious agents. Furthermore, the product must contain at least 90% intact IgG and the IgG aggregate concentration needs to be as low as possible (Goddard, 2008, Hooper, 2008). Some of the popular IVIg manufacturers are Grifols, Baxter and CSL Behring. In this work, IVIg from both Grifols and CSL Behring was used.
1.3 Evidence for usage in autoimmune disorders

In 1981, Imbach et al. had administered IVIg to two patients suffering from agammaglobulinemia as well as immune thrombocytopenia (ITP; an autoimmune disorder where patients have lower than normal platelet counts). They made a remarkable observation, where those patients with ITP showed an increase in platelet counts following IVIg infusion (Imbach et al., 1981). This finding provided the first evidence that IVIg therapy could potentially be useful in autoimmune disorders. In the next few years, IVIg usage was increased remarkably for the treatment of various autoimmune disorders. IVIg is now widely used to treat patients suffering from diseases such as primary ITP, Kawasaki disease, Guillain-Barré syndrome, and chronic inflammatory demyelinating polyneuropathy (CIPD) (Schwab and Nimmerjahn, 2013; Imbach et al., 1981; Berkman et al., 1988; Schwartz, 1990; Chipps and Skinner, 1994; Gelfand, 2012). In addition to these approved therapeutic uses, IVIg is also efficacious in many “off-label” clinical applications, particularly in autoimmune disorders such as myasthenia gravis and multiple sclerosis (MS) (Ratko et al., 1995; Foster et al., 2010; Katz U et al., 2011). Figure 1.3 shows the breakdown of IVIg usage for different disorders in Australia for 2009.
Figure 1.3 IVIg usages for specific indications in Australia for 2009. Data is shown as a percentage of grams used per year. Source is the National Blood Authority, Australia.
1.3.1 Usage in Canada and North America

The International Patient Organisation for Primary Immunodeficiencies (IPOPI) has reported on the usage and demands of IVIg on a global scale, and highlights that IVIg usage per capita varies widely by country. Despite increasing costs of production, the demand for IVIg has continued to increase over the years exceeding rates of 10% per year, and is predicted to continue to increase beyond this rate (Canadian Blood Services, 2007). Historically, global IVIg demand has increased at an average rate of 12% per year between 1984 and 2008 with North America being a major contributor to the increase (Figure 1.3.1). Canada has been labelled as one of the highest users of IVIg per capita, with a staggering usage rate of approximately 140.1g/1000 population (2010) and an average cost of around $65-$75/g (Canadian Blood Services 2007, Canadian Blood Services 2011). IVIg is dosed between 400mg to 2g/kg of body weight depending on the age of the patient and the disease being treated (Knezevic-Maramica and Kruskall, 2003). It can cost between $7,000-$10,000 for a single treatment of IVIg over two days, and patients usually depend on multiple maintenance doses of IVIg to control disease symptoms (Goddard, 2008). In Canada, Canadian Blood Services is the sole provider of IVIg for clinical use outside of Quebec (where Hema-Quebec is the sole provider to that Province only). It has been estimated that the cost to CBS for the provision of IVIg, given free to hospitals, comprises 25% of their total operating budget. Taken together, it is easy to see why IVIg therapy can be a challenge if increased use and cost continue to rise at its current pace.
Figure 1.3.1 Increase in the demand for IVlg on a global scale shown here in metric tons, between the years of 1984 and 2008. Overall, the average rate of increase per year is around 12%. Data obtained from IPOPI.
1.3.2 Multiple Sclerosis and IVIg

Multiple Sclerosis (MS) is an autoimmune disorder which involves the degradation of the myelin insulation surrounding nerve fibers or axons, also known as demyelination. In Canada, the prevalence of MS is at an average of around 240 cases per 100,000 (Beck et al., 2005). There are different kinds of MS which are characterized by the pattern of symptoms, which include primary progressive MS (PPMS), secondary progressive MS (SPMS), relapse-remitting MS (RRMS) and progressive relapsing MS (PRMS). MS results from a combination of genetic and environmental factors, but there currently is no specific explanation as to the specific causes of the disease (Chataway et al., 1998). Studies have looked at the prevalence of MS in monozygotic twins, in an attempt to explain the heritability of MS. These studies have reported a broad range of 25-76% heritability (Hawkes and Macgregor, 2009), which continues to show the difficulty in understanding the underlying causes of the disease. Additionally, MS is about 2-3 times more common in women compared to men (Duquette et al., 1992).

IVIg has been used in an “off-label” fashion to treat MS exacerbations and was shown to reduce relapse rates and the number of brain lesions on MRI in patients with early relapse-remitting MS (Cohen et al., 2008). However, it currently remains a second line treatment for RRMS patients given that a number of clinical trials failed to show clinical benefit (Schwarz et al., 2009). IVIg is particularly used for patients who are refractory to steroid treatment or who are pregnant and need to avoid interferon-β therapy (Dudusek and Zetti, 2006). How IVIg exerts its clinical benefit in MS is not known, however many potential mechanisms have been proposed: 1) Circulating autoantibodies to myelin
proteins could be targeted by IVIg 2) IVIg can induce the expansion of regulatory T cells which can modulate the immune response in MS 3) IVIg can downregulate pro-inflammatory cytokines such as IL-2, IFN-γ 4) IVIg may prevent activated complement components from attaching to the surface of oligodendrocytes and myelin proteins (Durandy et al., 2009, Sorensen, 1994). In MS, one microarray study identified interleukin-11 (IL-11) as amongst several immune-related genes that were upregulated by IVIg in MS patient T-cells (Pigard et al., 2009).
1.4 Overview of Proposed Mechanisms of IVIg

The mechanism of IVIg has been and continues to be a controversial and widely debated topic (Branch, 2013). As it has been described, IVIg is capable of offering therapeutic benefit to patients that suffer from a wide variety of disorders, each of which has different immunological backgrounds. This brings about the question of whether the beneficial effects of IVIg are different in each disorder, or if they can be explained by a single unifying mechanism which then works to direct additional downstream effects. It has already been explained that IVIg is manufactured from plasma pools of over 20,000 or more donors and would therefore contain a vast repertoire of different immunomodulatory molecules. There has been support for the hypothesis that the mechanism of IVIg may entail a series of different actions involving many pathways depending on the type of autoimmune disorder (Dalakas, 2010). In the past few years, a number of different mechanisms have surfaced and suggested as being the primary modes of action of IVIg (Katz et al., 2011, Durandy et al., 2009). Some of these postulated mechanisms involve direct interaction of the IgG molecule with particular targets, while others suggest indirect pathways wherein IVIg may work through intermediate steps. The following sections summarize some of the proposed mechanisms of IVIg.
1.4.1 Neonatal Fc receptor (FcRn) and Fc gamma receptors (FcγRs).

The neonatal Fc receptor (FcRn) in humans plays an important role in the transfer of IgG from maternal to fetal circulation across the placenta (Roopenian and Akilesh, 2007). In adults, FcRn is expressed on the surface and in intracellular regions of macrophages, dendritic cells and monocytes (Zhu et al., 2001). It was proposed that upon IVIg administration, IgG binds to available FcRn thereby increasing its serum half-life (Roopenian and Akilesh, 2007). In a mouse model of ITP it was shown that the large quantity of IgG administered competitively saturates FcRn, which prevented pathogenic IgG antibodies from engaging the FcRn resulting in loss of protection and increased clearance of the pathogenic IgG antibodies, in this case anti-platelet antibodies (Hansen and Balthasar, 2002). However, one of the major challenges to this mechanism is the fact that anti-platelet antibodies are efficacious within a short period (1-4 hours), in contrast to the effects of IVIg wherein platelet counts are observed to increase 1-2 days after treatment (Baerenwaldt et al., 2010, Crow et al., 2011). The IgG molecule can contain a sugar moiety found on the asparagine 297 residue within the Fc constant region of the heavy chain. The ability of IVIg to interact with FcRn is independent of this sugar moiety, and therefore it can be expected that lack of this sugar side chain would not alter its efficacy (Baerenwaldt et al., 2010). Interestingly, it was shown that IVIg lacking this glycosylated side chain showed reduced ability to ameliorate disease thereby implicating that FcRn is perhaps not involved in the mechanism (Nimmerjahn and Ravetch, 2007). Furthermore, Crow et al., 2011 showed in an ITP mouse model that FcRn deficient mice are protected from thrombocytopenia with IVIg treatment, suggesting that further studies are required to understand the involvement of FcRn.
Another hypothesis on the mechanism of action of IVIg pertains specifically to the Fc portion of the IgG molecule. One of the original proposed mechanisms of IVIg action was blockade of the Fcγ receptors on phagocytes (Bussel, 2000). IgG saturates the activating Fc receptors on splenic macrophages, thereby blocking the interaction of these receptors with autoantibody covered targets (Pyne et al., 2002). However, studies by Leontyev et al., 2012 showed that IVIg was still capable of ameliorating ITP in splenectomised BALB/c mice, and a similar finding was reported in an ITP model using B6 (Schwab et al., 2012). Put together, these investigations challenged theories which suggested the involvement of splenic macrophages in the mechanism of IVIg. As the Fc portion binds to activating Fcγ receptors on hematopoietic cells, after which intracellular signalling can modulate inflammation (Kazatchkine and Kaveri, 2001, Reilly and McKenzie, 2002) the binding to activating Fcγ receptors remains a viable possible mechanism. The Lazarus group showed that the amelioration of experimental mouse ITP by IVIg involved signalling through activating Fc receptors on dendritic cells (Siragam et al., 2006). Additionally, it was claimed that IVIg upregulates the expression of the inhibitory Fc receptor FcγRIIB (Samuelsson et al., 2001), which could be clinically relevant to prevent phagocytosis or binding to activating Fcγ receptors which could also affect demyelination that occurs due to phagocytosis by macrophages. However, recent studies have indicated that the mechanism of IVIg is actually independent of FcγRIIB (Crow et al., 2003; Ichiyama et al., 2005; Leontyev et al., 2012; Othy et al., 2013), and the discrepancy in results may have been due to strain differences in mice (Leontyev et al., 2012).
1.4.2 Sialylation of IVIg.

It was reported in one study that the efficacy of IVIg is critically dependent upon the sialylation of the Fc portion of IgG, in an experimental model of inflammatory arthritis (Kaneko et al., 2006). Enhanced sialylation of IVIg is reported to be directly related to the anti-inflammatory effects of IVIg, and the same group linked high doses of IgG required for therapeutic efficacy to the 10% or less of sialylated IgG contained within the product. They showed that the sialylated IgG was involved in the induction of the inhibitory FcRγIIB (Anthony and Ravetch 2010). Furthermore a particular transmembrane protein called specific ICAM3 grabbing nonintegrin-related 1 (SIGNR1) was implicated in this pathway, which was shown to bind to sialic acid rich IgG Fc residues, and IVIg activity could be blocked by using a SIGNR1 specific antibody (Schwab et al., 2012). However, other studies have demonstrated that Fc and F(ab’)2 sialylation offer no additional benefits to the efficacy of IVIg (Guhr et al., 2011, bLeontyev et al., 2012). bLeontyev et al., 2012 have even shown that there is no induction of spleen FcRγIIB mRNA and conclude that IVIg works in an independent mechanism unrelated to terminal Fc sialic acid residues.
1.4.3 Anti-idiotypic and natural antibodies.

Due to the nature of the IVIg product, a wide range of idiotypic and anti-idiotypic antibodies can be found among the IgG molecules (Dalakas, 2010). It has therefore been hypothesized that the anti-idiotypic antibodies by interacting with specific autoantigens can inhibit the activity of pathogenic autoantibodies (Kaveri, 2012). As mentioned before, it has been theorized that IVIg induces the reduction of the half-life of autoantibodies, which can be mediated by intercepting their interaction with FcRn (Gelfand, 2012, Hansen and Balthasar, 2002). Particular attention has been placed on the F(\(\text{ab'}\))\(_2\) fragment, which has been shown to bind to and neutralize known autoantibodies (Kaveri et al., 1997, Malik et al., 1996). This mechanism is of clinical relevance particularly to antibody related neuromuscular disorders such as myasthenia gravis and GBS, wherein inhibition of autoantibody functionality results in better symptom management.

Normal human serum contains natural antibodies, which are produced in the body without the need for external immunization or subjection to foreign antigens (Kaveri, 2012). These natural antibodies are important in the immunoregulation in immune related disorders. IVIg has been shown to contain natural antibodies which can exert control over autoreactivity and induction of self tolerance (Vani et al., 2008). Some functions of these natural antibodies include defense against pathogen infection, clearance of senescent cells and tumours (Kazatchkie and Kaveri, 2001), and in particular the ability to induce remyelination which is of relevance to multiple sclerosis (Miller and Rodriguez, 1995). It has been demonstrated that IVIg preparations contain
antibodies to sialic acid-binding Ig-like lectin 8 and 9 (Siglec-8 and -9) (Gunten and Simon, 2008). Siglec-8 is primarily expressed on eosinophils and siglec-9 on neutrophils, and therefore anti-siglec-8 and anti-siglec-9 antibodies can be beneficial in immunoregulatory mechanisms. For example, patients suffering from hypereosinophilic syndrome (HES) or Churg–Strauss syndrome can be treated with IVIg, resulting in regulation of eosinophil numbers (Gunten and Simon, 2008, Tsurikisawa et al., 2004).

1.4.4 Suppression of inflammatory cytokines.
In most autoimmune disorders including neuromuscular disorders, disease is associated with the upregulation of pro-inflammatory cytokines (Santamaria, 2002). IVIg is known to contain antibodies against pro-inflammatory cytokines such as IFN-γ and TNF-α (Kaveri, 2012). Interestingly, studies have shown in in vitro and in vivo models that a suppression in the production of IFN-γ, TNF-α and IL-17 is observed following IVIg treatment (Amemiya et al., 2000, Abe et al., 1994, Maddur et al., 2013). It remains unclear however whether this effect is a result of an upstream regulation by IVIg, or if the effect of IVIg is directly related to the suppression of specific immune pathways. Additionally, IVIg may work to promote the production of regulatory cytokines such as IL-10 (Ephrem et al., 2008) and IL-11 (reported in thesis) which can then mediate anti-inflammatory effects on the immune system (Santamaria, 2002).
1.4.5 Modulation of T cells, B cells and dendritic cells

T cells are important components of the adaptive immune system. In autoimmune disorders, self-reactive T cells interact with local APCs which results in restimulation of the T cells in a positive feedback loop. Studies have demonstrated that IVIg attenuates the activation of T cells by interfering with activation factors (Padet and Bazin, 2013), and that antigen-dependent T cell activation requires signaling through FcγRs (Aubin et al., 2009).

Auto-antibodies produced by autoreactive B cells are thought to be the main effectors in auto-immune diseases. IVIg has been shown to downregulate the production of these autoantibodies (Bayry et al., 2007), and as mentioned earlier may contain specific anti-idiotypic antibodies to neutralize pathogenic antibodies (Kaveri, 2012). A specific factor called B-cell activating factor (BAFF) is important for the differentiation of B cells. It has been shown that IVIg can work to neutralize BAFF (Le Pottier et al., 2007), and that commercial preparations of IVIg contain BAFF antibodies which can prevent the further amplification of autoreactive B cells.

The effects of IVIg have also been linked to interactions with dendritic cells (DC) (Crow et al., 2009). DC are a set of APCs, whose primary function is to present antigens and stimulate a primary immune response in resting naïve T cells (Bayry et al., 2003). Studies by Bayry et al., 2003 showed that the differentiation and maturation of DC in the presence of IVIg was inhibited. Additionally, in an experimental model of ITP it was demonstrated that DCs were a direct target of IVIg. This group conducted experiments where the ameliorative effects of IVIg were observed in a mouse model of ITP, after an adoptive transfer of IVIg-primed DCs (Siragam et al., 2006).
1.4.6 Tregs

Regulatory T-cells (Tregs) are important players in immune tolerance and are distinguished by the characteristic markers CD4⁺CD25⁺, as well as the expression of the transcription factor forkhead box p3 (FoxP3) (Sakaguchi et al., 2006). Animal studies have shown that depletion of Tregs can result in spontaneous autoimmune disease development (Sakaguchi et al., 2009), and it has been reported that genetic defects which affect the normal development of Tregs in humans can be a cause for inflammatory and autoimmune disorders (Sakaguchi, 2004). Theories that have been postulated on the mechanism of IVIg have recently centred on the expansion of CD4⁺CD25⁺FoxP3⁺ Tregs (Ephrem et al., 2008). With respect to clinical studies, Tjon et al., 2013 have shown that patients treated with high dose IVIg show an expansion of Tregs following treatment. Furthermore a study on patients with Kawasaki disease showed that lack of CD4⁺CD25⁺FoxP3⁺ Tregs could be an indicator of IVIg resistance (Hirabayashi et al., 2013). Tregitopes are defined as a specific set of peptides that can activate CD4⁺CD25⁺FoxP3⁺ natural Tregs (nTregs) (Su et al., 2013). Studies by Cousens et al., 2013 have described that Tregitopes can be found in the Fc and Fab regions of IgG, and that this can be used to understand how Tregs are induced by IVIg. As described above, the numerous mechanisms of IVIg have created a lot of discussion and controversy, resulting in a lack of consensus as to how this therapeutic works. This uncertainty regarding the mechanism of action of IVIg stands as a major hindrance to establishing treatment alternatives to cope with the increasing demands and costs.
Figure 1.4  Summary of the mechanisms of IVIg highlighted in 1.4.  --→ indicates controversial theories wherein different groups have produced contradictory results.
1.5 Experimental Autoimmune Encephalomyelitis (EAE)

1.5.1 History of EAE

Experimental Autoimmune Encephalomyelitis or EAE is an animal model of inflammatory disease, which has been used to understand the mechanisms and pathways that occur in MS. The history of EAE dates back to Louis Pasteur's rabies vaccine which was first used in 1885. This vaccine consisted of spinal cord tissue which was collected from rabbits inoculated with the rabies virus. It was also observed that some patients who received the anti-rabies vaccine experienced ascending paralysis following vaccination (Stuart and Krikorian 1928). Dr. Thomas Rivers conducted studies in rhesus monkeys and showed that repeated injections of emulsified rabbit brain and spinal cord tissue resulted in ascending paralysis and demyelination in the CNS (Rivers et al., 1933, Rivers and Schwenteker, 1935). This combined with the studies by Freund lead to the development of an adjuvant which contained killed Mycobacterium tuberculosis to enhance the production of “brain-specific” antibodies. What resulted is the formation of an experimental model to study demyelinating disorders termed EAE, which could be used in many different species (Baxter 2007).
1.5.2 Mechanisms underlying EAE

With the introduction of EAE by Kabat et al., 1947 came the interest in studying the CD4/Th1 paradigm. Simply put CD4+ T-cells that show a polarization towards Th1, characterised by the secretion of IFN-γ (Mosmann et al., 1986), were thought to be the main effectors in EAE. However, studies have shown that it is actually IL-17 producing T-cells (Th17 cells) which are the main mediators of disease progression, which work alongside other effectors such as Th1 cells, CD8+ T-cells and B cells (Cua et al., 2003, Becher et al., 2002). Many variations of EAE have been used in experimental studies depending on the type of disease that is desired. In this work, EAE was induced in mice using myelin oligodendrocyte glycoprotein MOG_{35-55}. This type of EAE is characterized by an acute phase resulting in severe clinical scores, after which symptoms stabilize and animals begin to spontaneously remit.

1.5.3 Cellular background on EAE

It was initially proposed that CD4+ T helper type 1 cells or Th1 cells as being the main effectors in the pathogenesis of EAE (Batoulis et al., 2010). As mentioned before these cells produce IFN-γ and TNF-α, and are distinct from IL-4 producing Th2 cells, thought to have anti-inflammatory effects. Work by Leonard et al. showed the implications of IL-12 in EAE exacerbation, which is linked to the promotion of Th1 development and the production of IFN-γ. Interestingly, some studies have shown that mice that were deficient for IFN-γ actually showed a more exacerbated EAE, thereby challenging the classical Th1/Th2 model which had been proposed in the disease (Ferber et al., 1996). It was later in 2003 when Cua et al. showed that it was in fact IL-23 not IL-12 that was a
crucial cytokine in EAE. IL-23 was found to promote the secretion of IL-17 from CD4+ T-cells, thus giving rise to the discovery of a new lineage of T-cells called Th17. Th17 cells are now determined to be the main drivers of EAE pathogenesis.

1.5.4 Summary of EAE mechanism

The inflammation associated with EAE begins by T-cells priming in the peripheral immune system. Antigen presenting cells, mainly comprising of dendritic cells, produce cytokines in response to antigen presented by MHC class II complexes. These cytokines are responsible for the activation and expansion of naive CD4+ T-cells into either Th1 or Th17 cells. The production of IL-12 promotes the development of Th1 cells and TGF-β, IL-6 and IL-23 promote the differentiation and amplification of Th17 cells. These activated T-cells then traffic towards the CNS, and are able to cross the blood brain barrier (BBB) due to the ability to up-regulate integrins. The increased permeability of the BBB and the release of chemokines attracts other types of immune cells such as peripheral monocytes and macrophages. Infiltrating macrophages interact with the cognate myelin antigen through resident antigen presenting cells resulting in activation. The myelin sheath surrounding neurons is destroyed as a result of phagocytosis by macrophages. Upon entering the CNS, the activated T-cells interact with the CNS antigen presenting cells leading to an inflammatory cascade. This entails the release of pro-inflammatory cytokines from activated T-cells namely IFN-γ from Th1 cells and IL-17 from Th17 cells. This drives inflammation further such that more inflammatory cells cross into the CNS and cause additional damage to myelin. CD8+ T-cells may contribute additional damage by the further release of cytokines. The overall
consequence is the loss of myelin which is not capable of complete regeneration, coupled with axon loss due to the lack of the protective myelin sheath.

1.5.5 EAE and Multiple Sclerosis

After EAE was initially described it received critical acclaim for its ability to mimic a number of different pathologies associated with multiple sclerosis (Wolf et al., 1947, Baxter 2007). Some of these include, but are not limited to:

- Myelin sheath destruction surrounding the neurons in the CNS
- The formation of numerous lesions with a temporal and spatial distribution in the brain and spinal cord, and the perivascular localization of these lesions.
- The ability of these lesions to undergo further demyelination as a result of inflammation, and then show signs of remyelination at further stages in the disease.
- The detection of IgG within the CNS and the cerebrospinal fluid (Constantinescu et al., 2011).

Three well known examples of MS therapeutics which were developed after being studied in EAE are glatiramer acetate, mitoxantrone and natalizumab (Farooqi et al., 2010). The studies on IVIg in EAE are highlighted in 1.6.5.

One of the advantages of using the EAE model to study the pathogenesis of EAE is the fact that a lot of the variables are known. The concentration of adjuvant, the predicted outcome of the disease and the differentiation of induced T-cells can be directed (Batoulis et al., 2010). Many scientists continuously stress that EAE is not MS, in that EAE requires artificial induction of the disease be it through emulsion or adoptive
transfer. However, MS is a spontaneous disease and we still do not understand fully what brings about its onset. Furthermore, the disease is highly unpredictable and involves much more complexity and variables as compared to EAE.

EAE has also received criticism in that several different potential therapies which showed promising results in the experimental model failed to have any benefit to MS patients. Additionally, the adverse side effects and clinical complications that arise from these therapeutics could not have been predicted using animal models (Farooqi et al., 2010).
1.5.6 IVIg treatment of EAE

There have been numerous studies which have described the profound ability of IVIg to ameliorate EAE (Achiron et al., 1994, Jorgensen et al., 2005, Jorgensen and Sorensen, 2005, Ephrem et al., 2008). Different EAE models were used in these studies including induction by emulsions containing myelin basic protein or MOG emulsion and adoptive transfer experiments. The results are unanimous, wherein all have shown that animals which receive regular doses of IVIg have lower clinical scores, and the onset of EAE is delayed significantly. Jorgensen and Sorensen, 2005 showed in their work that administration of IVIg prior to EAE immunization was essential for it to have a beneficial effect, and that maintenance of the “amelioration” is only possible with daily doses of IVIg.

Owing to the dramatic effects of IVIg, many theories have been postulated surrounding its mechanism in EAE. Histological analysis of the CNS revealed that animals receiving IVIg treatment exhibit a lower degree of inflammation in the CNS (Jorgensen and Sorensen, 2005). To give a better understanding of the T-cell repertoire in the immune compartments during the course of EAE, the production of cytokines in cells isolated from the lymph nodes and spleens is assessed. IVIg has been shown to down-regulate the production of pro-inflammatory cytokines IFN-γ and TNF-α (Ephrem et al., 2008, Chong et al., 2013, Achiron et al., 1994), which as mentioned earlier are primarily Th1 cytokines. IL-17, released by Th17 cells, has also been shown to be down-regulated with IVIg treatment (Chong et al. 2013). Furthermore, it has been documented that IVIg inhibits the differentiation and amplification of human Th17 cells in vitro (Maddur et al., 2011). Jorgensen et al., 2007 also highlighted that following permeabilization of the BBB
during EAE, IVIg may work by crossing into the CNS and localising towards inflammatory lesions.

Another important mechanism which has been implicated as a key effector in IVIG therapy is the induction of Tregs. This mechanism has been shown both in mouse EAE models (Ephrem et al., 2008, Maddur et al., 2010, Aslam et al., 2012) and in clinical studies (Hirabayashi et al., 2013, Tjon et al., 2013, Chi et al., 2007). In EAE, it was reported that IVIg induces a preferential expansion of these cells in the spleen (Ephrem et al., 2008). Chong et al., 2013 have recently described that the mechanism of this IVIg mediated Treg induction is dependent on natural killer cells (NK), and that adoptively transferred IgG treated NK cells are capable of suppressing EAE and the production of IL-17 and IFN-γ.
1.6 IL-11

Interleukin-11 is a 23kDa cytokine, which is in the IL-6 family of cytokines. IL-11, IL-6, leukemia inhibitory factor (LIF), Oncostatin M and ciliary neurotrophic factor (CNTF) utilize gp130 as a common chain for signal transduction on receptors (Leng and Elias, 1997). These receptors have additional chains that provide specificity to each of the IL-6 family of cytokines. IL-11 binds to its specific receptor, IL-11Rα, which leads to heterodimerization and activation of gp130. Downstream Janus kinases (JAK) are activated by the IL-11Rα-gp130 complex (Oleksowicz and Dutcher, 1994, Leng and Elias, 1997. Heinrich et al., 2003).

IL-11 is important for a number of different functions. It has been shown that IL-11 supports megakaryocyte formation and maturation (Bruno et al., 1991, Teramura et al., 1992) and also promotes neuronal development (Kobayashi et al., 1994). As mentioned earlier, interleukin-11 (IL-11) was identified amongst several immune-related genes that were upregulated by IVIg in MS patient T-cells (Pigard et al., 2009). Furthermore, there is evidence to suggest that IL-11 promotes oligodendrocyte maturation and survival (Zhang et al., 2011). A study by Gurfein et al., 2009 showed that IL-11 was important in regulating autoimmune demyelination in the CNS in a mouse model, and also reduced apoptosis of mouse oligodendrocyte progenitors. More support for the beneficial aspects of IL-11 for MS patients can be found in the study by Maheshwari et al., 2013. IL-11 was shown to ameliorate symptoms in the EAE mouse model (highlighted in 1.6), and promoted remyelination of damaged neurons. Interestingly, IL-11 has also been showed to inhibit the polarization of Th1 cells, and actually induces a Th2 polarization of CD4+ T-cells (Curti et al., 2001). Trepicchio et al., 1996 showed that human
recombinant IL-11 is capable of attenuating the inflammatory response, and that this is accomplished through the downregulation of pro-inflammatory cytokines such as IFN-γ and TNF-α. It was been suggested that this mechanism occurs as a result of direct interaction with macrophages. This same group later conducted an investigation in an animal model of psoriasis and were able to show that treatment with recombinant human IL-11 resulted in lowered expression of disease related genes such as IFN-γ and TNF-α (Trepicchio et al., 1999).

It was shown in a pilot study by Bussel et al., 2001 that recombinant IL-11 treatment for patients with refractory ITP appeared to offer no clinical benefits. However, this was a small and poorly constructed study with 7 subjects and used a very high dose of IL-11 that resulted in some significant toxicity. The conclusions by the investigators was that IL-11 cannot ameliorate ITP but that further dosing studies are warranted. Of note, all the patients that were used in this study were shown to be refractory to IVIg treatment. Therefore, this study does not challenge our hypothesis given that we propose a mechanism by which IL-11, produced by IVIg, acts as mediator where IVIg is efficacious.
1.7 Hypothesis

IVIg-mediated amelioration of EAE is by induction of IL-11 and IL-11 signaling.

1.8 Objectives and specific aims

The main objectives in this study were to investigate whether IVIg induces IL-11 and, if so, the role played by IVIg-induced IL-11 in the amelioration of EAE. This was accomplished by the following specific aims:

1. To determine whether IL-11 is produced following IVIg treatment.

2. To investigate the role of IL-11 in IVIg-mediated amelioration of EAE by using mice lacking a functional receptor for IL-11 (IL-11Rα−/− mice).

3. To examine the specific effects of IVIg on the inflammatory components of EAE, such as the production of pro-inflammatory cytokines and expansion and of myelin reactive T cells, and explore differences between wild-type and IL-11Rα−/− mice.
Chapter 2

MATERIALS AND METHODS

2.1 Mice

Female BALB/c wild-type mice were from Charles River Laboratories International, Inc. (Wilmington, MA). IL-11 receptor α-chain knockout (IL-11Rα−/−) or IL-11Rα+/+ (WT) littermate control females on the C57BL/6J background were from the Jackson laboratory (Bar Harbor, ME). Mice were housed and maintained according to guidelines of the Canadian Council on Animal Care under approved animal use protocols of the University Health Network. The genotypes of IL-11Rα−/− and WT mice were confirmed using PCR genotyping of tail DNA as described previously (Nandukar et al., 1997). Mice were housed at 22 ± 4º C under a natural light/dark cycle and were given a standard diet and water *ad libitum.*
2.2 Experimental Autoimmune Encephalomyelitis induction

2.2.1 Preparation of emulsion for injection

The emulsion for immunization was prepared by mixing myelin oligodendrocyte glycoprotein amino acids 35-55 (MOG<sub>35-55</sub>) (2 mg/ml) (Stanford University Pan Facility, Stanford, CA) with Complete Freund’s Adjuvant containing heat killed *Mycobacterium tuberculosis* *H37Ra* (4 mg/ml; Difco, Detroit, MI) (Dunn et al., 2010).

2.2.2 Immunization of mice

Mice were immunized by a subcutaneous injection of 100µl of the prepared emulsion in the ventral thorax, with an injection of 50µl in two sides in the chest (left and right side). At the time of immunization and 2 days after, mice were injected intraperitoneally with 75ng/ml pertussis toxin (List Biologicals, Campbell, CA).
2.3 IVIg

IVIg (Gammagard, Baxter Healthcare Corp., Toronto, ON; Privigen, CSL Behringer, Ottawa, ON, or Gammunex, Grifols, Los Angeles, CA) was administered daily (1 g/kg, i.p.) throughout all EAE experiments beginning on the day of immunization. Where necessary, IVIg was diluted with 1 x PBS. Control groups received either sterile 1 x PBS or an equivalent dose (1 g/kg) of human serum albumin (HSA; Canadian Blood Services, Toronto, ON).

2.4 EAE scoring

All mice were examined daily and were assessed for clinical scores of EAE as follows: 0=no symptoms; 1=tail paralysis; 2=hindlimb or foot weakness; 3=paralysis of one or both limbs; 4=hindlimb paralysis and weakness in one or both forelimbs; 5=moribund or dead.

2.5 Measurement of circulating IL-11

Mice were bled through the saphenous vein, up to a maximum volume of 100µl. The blood was then spun down at 1400 rpm for 10 minutes. The plasma was separated and spun once more to remove any debris. IL-11 levels were measured using an ELISA kit (R&D systems, Inc., Minneapolis, MN).
2.6 Measurement IL-11 mRNA expression.

Total RNA was prepared from BALB/C mice tissue (lymph node, bone marrow, liver and spleen) using the TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription reactions of RNA (1 µg) were performed using the First-Strand cDNA Synthesis Kit (GE Healthcare/Amersham Pharmacia Biotech Inc. Baie d'Urfe, QC). Primers for PCR reactions were based on GenBank-published sequences and are as follows: Mouse interleukin -11 (IL-11, NM_008350) forward primer (5’-TGGTGGTGCTGAGCCTCTGG-3’), reverse primer (5’-CAGGAAGCTGCAAAGATCCCA-3’). Mouse hypoxanthine guanine phosphoribosyl transferase (HPRT, NM_013556) forward primer (5’-TCAGTCAACGGGGGACATAAA-3’), reverse primer (5’-GGGGCTGTACTGCTTAACCAG-3’). PCR amplification was conducted using 1 µl of cDNA TaqDNA polymerase (Sigma, Oakville, ON). Cycling conditions included an initial denaturation step (94°C for 3 min), followed by 35 denaturation cycles (94°C for 30 s), annealing (66°C for 30 s), and extension (70°C for 30 s) followed by a final extension (72°C for 7 min).
2.7 Assessment of MOG p35-55 Reactive T-cell Responses

2.7.1 Spleens

Spleens were harvested from mice, and were transferred to a cell sieve (70μm) placed in a sterile petri dish filled with 1X PBS. The spleens were subjected to mechanical disaggregation, and the resulting cell suspension was transferred to a Falcon tube. The suspension was topped up with 1X PBS and spun at 1400rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended by mechanical agitation. 1 ml of ACK Lysis buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA, pH 7.2-7.4, filtered through 0.22µm) per spleen was added to the resuspended pellet which was incubated for 1 minute and 15 seconds exactly. The solutions were topped up with 1X PBS and spun again at 1400 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in complete media (10% FCS (Omega Scientific Cat FB-01), 1X 2-mercaptoethanol, 1% L-glutamine (Gibco), 1% non-essential amino acids (Gibco), 1X sodium pyruvate, 1X penicillin-streptomycin (Gibco). The volume for resuspension was around 5 ml per pellet of spleen cells or lymph node cells. The cells were counted using a hemacytometer and were plated in triplicates in 96-well plates at a concentration of 1 x 10⁶ cells/well. The cells were stimulated with different concentrations of MOG₃₅-₅₅ (2µg/ml, 5µg/ml and 10µg/ml) and incubated at 37°C/ 5% CO₂. Plates were collected at 48h, 72h and 96h time intervals for ELISA analysis.
2.7.2 Lymph Nodes

Draining lymph nodes (LN) were collected: brachial, axillary and brachial from the mice and were subjected to mechanical disaggregation as mentioned before. The suspension was topped up with 1X PBS and spun at 1400rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended by mechanical agitation. The volume for resuspension was around 5 ml per pellet of spleen cells or lymph node cells. The cells were counted using a hemacytometer and were plated in triplicates in a 96-well plates at a concentration of 1 x 10^6 cells/well. The cells were stimulated with different concentrations of MOG\textsubscript{35-55} (2µg/ml, 5µg/ml and 10µg/ml) and incubated at 37ºC/ 5% CO\textsubscript{2}. Plates were collected at 48h, 72h and 96h time intervals for ELISA analysis.

2.7.3 ELISA

The productions of cytokines in culture supernatants were measured using Ready-SET-Go ELISA kits (eBioscience, San Diego, CA) at 48h (IL-2) or 72h (TNF-α, IFN-γ and IL-17) of culture.

2.7.4 Proliferation

To measure the proliferation of MOG-reactive cells, cultures were pulsed at 72 h of culture with [H3]-thymidine (Perkin Elmer, Canada) and 18 h later, cells were harvested onto filter paper and incorporated [H3]-thymidine was read using a beta counter.
2.8 T regulatory cell assessment

Flow cytometry staining of Tregs was done using a kit from eBioscience. 1 X 10⁶ cells were stained per sample. Cells were washed in cold FACS buffer a minimum of three times (pellet spun down 1400 rpm for 5 minutes). Cells were then Fc blocked for 15 minutes at 4°C, using a minimum of 0.5μg per test. Cells were then washed again to remove excess Fc block before the first staining step. The pellet was resuspended with the appropriate surface staining markers (CD4, CD25). Incubation was then carried out at 4°C in the dark for a minimum of 30 minutes. Following the surface antibody staining, the cells were fixed and permeabilized by addition of a pre-mixed “Fix/Perm buffer” for a further 30 minutes in the dark. The final staining step is the intracellular antibody stain FoxP3, which is added and incubated for 30 minutes in the dark at 4°C. The final wash is done in a permeabilization buffer, before being resuspended in FACS buffer for analysis by FACS. Data were acquired using the LSRII analyzer (BD Biosciences, Mississauga, ON) and were analyzed with FlowJo software (Treestar, Ashland, OR). Tregs were identified in the live (negative for Aqua live/dead stain, Invitrogen, Burlington, ON) CD4⁺ gate as being double-positive for CD25 and FoxP3. Gates were set using fluorescence minus one controls.
2.9 Histology

2.9.1 Spinal cord inflammation

Brains and spinal cords were harvested from EAE mice at the end-point of disease, were fixed in formalin and embedded in paraffin. Brain (6 sections) and spinal cord transverse sections (10 sections/cord) were cut (4 µm thick) and were stained with Haematoxylin and Eosin and Luxol Fast Blue (Pathology Core, Toronto Center for Phenogenomics, Toronto, CA). Inflammation was largely in the spinal cord and the severity of this inflammation was scored as follows. Spinal cord sections (10/mouse) were divided into four quadrants: the ventral funiculus, the dorsal funiculus and each lateral funiculus. An observation of meningitis or perivascular cuffing in any of the quadrants was scored as 1. Observations were then summed across all sections and the overall pathological score was expressed as the percentage of affected quadrants/total number of quadrants.

2.9.2 Demyelination

To score demyelination in the spinal cord, the area of myelin pallor in each section of spinal cord was quantified using an image analysis program and attached microscope (Leica Application Suite, Leica Microsystems) and was expressed as a percentage of total white matter area.
2.10 Treatment of lymph node cells with mouse recombinant IL-11.

Lymph nodes were harvested from C57BL6/J mice, were pooled, and were dissociated into a single cell suspension. Lymph node cells were resuspended in complete-10%FCS-RPMI containing no added cytokines (Th0 conditions) or under Th17-skewing conditions (with added 30 ng/ml IL-6, 3 ng/ml TGF-β, and 10 µg/ml anti-IFN-γ, all reagents from eBioscience) in the presence or absence of mouse recombinant IL-11 (R&D systems, Minneapolis, MN). Cells were then plated (0.5 x 10⁶/well) in 96-well flat-bottomed plates that were pre-coated with 0.5 µg/ml anti-CD3 (145-2C11) and 0.5 µg/ml anti-CD28 (37.51) (both from eBioscience). The proliferation and cytokine productions of cells were measured as described above.
3.1 Effects of IVIg on EAE

3.1.1 IVIg ameliorates EAE symptoms

Before embarking on our investigation of the role of IL-11 as an effector of IVIg, we first aimed to characterize the immune mechanisms of IVIg treatment in our EAE model. EAE was induced in female C57BL/6J mice via immunization with MOG p35-55 and CFA and mice were administered daily injections of high-dose IVIg (1 g/kg) or 1 x PBS as a control, starting at day of disease induction. Similar to previous studies (Ephrem et al., 2008; Othy et al., 2013), we observed that IVIg virtually prevented the development of EAE (Figure 3.1.1) with 100% of mice developing EAE in the PBS group and only 10% of mice developing EAE in the IVIg group.
Figure 3.1.1 EAE scores of wild-type mice with and without IVIg.

**Figure 3.1.1** EAE was induced in C57BL/6J mice by immunization with MOG p35-55 and CFA plus pertussis toxin and mice were administered daily i.p. injections of PBS or IVIg (1 g/kg) beginning at day of disease induction. Mice (N=10/group) were followed for clinical signs. Shows the mean + SEM severity of clinical signs of mice in each group over the period of observation. ***p<0.001 by Mann-Whitney U test.
3.1.2 IVlg down regulates pro-inflammatory cytokines

To address the underlying immune effects of IVlg treatment, we harvested the spleens of mice and examined the recall proliferation and cytokine responses of splenocyte mononuclear cells to MOG p35-55 in culture (Figure 3.1.2). We observed that IVlg profoundly reduced the production of the T helper 1 (Th1) cytokines IFN-γ and TNF-α and moderately reduced the production of IL-2 and the Th17-associated cytokine IL-17 by MOG p35-55-reactive T-cells.

3.1.3 IVlg reduces T-cell proliferation

In addition, IVlg reduced the proliferation of splenocytes to MOG p35-55 by almost half (Figure 3.1.3). Thus, IVlg has an effect in dampening both Th1 and Th17 inflammatory pathways that are pathogenic in EAE and MS.
Figure 3.1.2 Shows the levels of IL-2 (at 48 h), IFN-γ, TNF-α and IL-17 (at 72 h) in splenocyte cultures as measured by ELISA. Values are means ±SEM of triplicate cultures in one representative experiment of 2 independent experiments. *p<0.05, **p<0.01

Figure 3.1.3. The proliferation of cells in triplicate splenocyte cultures in response to MOG p35-55 (5 µg/ml) was measured in counts per minute (cpm) and was expressed relative to the background cpm. This ratio is the stimulation index. Data is representative of 3 independent experiments. *p<0.05
3.1.4 No effect observed on Treg population after IVIg treatment

Theories that have been postulated on the mechanism of IVIg have recently centred on the induction of CD4^+CD25^+FoxP3^+ regulatory T-cells (Treg) by this agent (Ephrem et al., 2008). To address whether IVIg induced an expansion of Treg during EAE, we measured the frequency and number of these cells in the lymph nodes and spleens of IVIg or PBS-treated mice at various time-points post-immunization. Although, the frequency of Treg tended to be elevated in the spleen with IVIg at 6 days post-immunization, this was not observed at other time points examined (Figure 3.1.4 A, C). Moreover, the numbers of Treg also were not different between PBS- and IVIg-treated groups (Figure 3.1.4 B), nor were the levels of the T-reg-associated cytokine IL-10 (not shown as it was not detected above background). Together, these results suggest that the dramatic effect of IVIg in preventing EAE in our hands was due to the inhibition of the expansion, Th1 and Th17 cytokine production, and the CNS trafficking of MOG p35-55 reactive T-cells.
Figure 3.1.4 Effect of IVIg on Tregs

A) % Tregs

B) Treg numbers

C) CD25 vs FoxP3 for HSA and IVIg
Figure 3.1.4 C57BL6/J mice were immunized with MOG p35-55/CFA (without pertussis toxin) and were treated with PBS or IVIg daily. After 4, 6, 8, or 10 days, spleens were harvested, were processed into a single cell suspension and CD4^+CD25^+FoxP3^+ Treg were stained and were enumerated by flow cytometry. A & B show the frequency and number of these cells at these different time points after immunization in the spleen. C shows representative staining of FoxP3 and CD25 in the live, CD4^+ gate. Data in A-C are means + SEM of 4-5 mice per group at each time-point. **p<0.01, *p<0.05 as determined using a t-test or Mann-Whitney U test.
3.2 IVIg upregulates interleukin-11

3.2.1 IL-11 was upregulated in both C57BL/6J and BALB/c after IVIg injection

Previous microarray studies of MS patient T-cells indicated that IVIg upregulated the mRNA expression of six immune related molecules that included IL-11 (xcl2, kir2ds1, map4k2, ptger4, casp2, il11) (Pigard et al., 2009). To investigate whether IL-11 is produced at higher levels in mice with IVIg, we collected plasma from mice prior to and at various times post injection with IVIg (1 g/kg) and measured IL-11 levels by ELISA. Only low levels (50-100 pg/ml) of IL-11 were detectable in the circulation prior to IVIg administration. However, after IVIg injection, we observed a dramatic ~1000 fold increase in the levels of IL-11 that peaked at 6 hours post-injection. This response was observed in both C57BL6/J and BALB/c mice (Figure 3.2.1).
Figure 3.2.1 Induction of IL-11 after IVIg injection in BALB/c and C57BL/6 mice

A. BALB/c

B. C57BL/6

IL-11 pg/ml

Hours post-IVIg injection
**Figure 3.2.1** BALB/c (A) or C57BL/6J (B) mice were administered IVIg (1 g/kg) and serum was collected for IL-11 measurement using ELISA at time 0, 3, 6, 12, 18 and 24 hours post-injection. Values are means + SEM of individual mice. Stars indicate a statistical difference (**p<0.01, ***p<0.001) from time zero as determined using a one-way ANOVA and a Tukey post-hoc test.
3.2.2 Heat map showing the induction of IL-11

It was of interest to investigate other cytokines and the expression levels following IVIg treatment. Therefore, we tested the serum in order to compare the IL-11 response against the production of other cytokines and chemokines in the circulation. We found that IL-11 was the predominant cytokine induced by IVIg as seen in Figure 3.2.2.
**Figure 3.2.2** Shows a heat map of serum cytokine/chemokine levels taken at 6 h post IVIg or PBS injection with N=8 mice/group. Each data box in the heat map represents a reading from an individual mouse.
3.2.3 IL-11 induced by IVlg was upregulated in the liver, and consistently upregulated at later time points.

IL-11 is reported to be widely-expressed by many cell types (Leng and Elias, 1997). To investigate the potential source of the IL-11, we conducted RT-PCR analysis of IL-11 mRNA abundance in various tissues including the bone marrow, liver, spleen and lymph nodes of the IVlg-treated or PBS-treated mice. We observed that IL-11 mRNAs were up-regulated only in the liver (Figure 3.2.3 A), suggesting that this is the source of IL-11 production with IVlg. We investigated the induction of IL-11 post-IVlg injection and found it to be induced even at late stages in the disease (Figure 3.2.3 B).
Figure 3.2.3  A Lymph nodes, bone marrow, liver and spleens of BALB/c mice were harvested without IVIg treatment (-) or 6 hours after IVIg treatment (2 g/kg)(+), mRNA was extracted and RT-PCR analysis performed to detect a qualitative difference in IL-11 mRNA abundance. Hypoxanthine guanine phosphoribosyl transferase (HPRT) serves as a quality and loading control. M=molecular weight marker. This image represents data obtained from one mouse per group. B EAE was induced in C57BL/6J mice with MOG p35-55/CFA and pertussis toxin. Mice were injected with IVIg or HSA daily (1g/kg, i.p.). Shown are the IL-11 levels (pg/ml) in the sera of mice at 6 h post-injection on day 45 post-immunization. Values are means ± SEM of readings from individual mice.
3.3 IVIg effects on EAE in IL-11Rα⁻/⁻ mice

3.3.1 IL-11Rα⁻/⁻ mice do not show complete amelioration with EAE with IVIg.

To address whether IVIg utilizes an IL-11 signaling mechanism to ameliorate EAE, we compared the efficacy of IVIg in IL-11R⁺/⁺ (WT) and IL-11Rα⁻/⁻ mice. In these studies we used human serum albumin (HSA) as a control. As in our preliminary EAE studies, WT mice that received HSA treatment developed EAE with moderate severity and with high incidence while WT mice treated with IVIg had a greatly attenuated disease course (Figure 3.3.1, Table 1). On the other hand, in IL-11Rα⁻/⁻ mice, EAE not only presented differently than in WT, but also responded differently to IVIg treatment. First, IL-11Rα⁻/⁻ mice displayed a milder form of disease than WT mice as evidenced by lower mean clinical scores (Figure 3.3.1, Table 1). The IL-11Rα⁻/⁻ mice also tended to show a lowered incidence of disease than WT mice (Table 1). Secondly, we observed that IL-11Rα⁻/⁻ mice were resistant to the protective effects of IVIg. Though IVIg had an equivalent effect in delaying the onset of EAE in IL-11Rα⁺/⁺ and IL-11Rα⁻/⁻ mice, IL-11Rα⁻/⁻ mice, unlike WT counterparts progressed to develop moderate clinical symptoms (Figure 3.3.1, Table 1). The severity of these symptoms or the incidence of EAE did not differ between HSA- and IVIg-treated IL-11Rα⁻/⁻ mice (Table 1). These findings indicate that a portion of the effects of IVIg in the attenuation of EAE are mediated through IL-11R signaling.
Figure 3.3.1 EAE was induced in C57BL/6J IL-11R<sup>+/+</sup> (WT) or IL-11R<sup>−/−</sup> mice that were administered daily treatment of IVIg or HSA treatment (1g/kg) beginning on the day of EAE induction. Mice were followed for clinical signs and a histological analysis of inflammation and demyelination was conducted at the end-point of the experiment. Shows the mean ± SEM clinical scores of mice over a time-course of EAE. The graph shows combined data from three consecutive EAE studies that each contained (N=5-10 mice/group).
Table 1 Clinical Features of EAE in IL-11Rα⁺/⁺ and IL-11Rα⁻/⁻ mice with IVlg or HSA treatment.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Peak Score</th>
<th>Day of Onset (EAE cases)</th>
<th>Cumulative Score</th>
<th>% Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-11R⁺/⁺ HSA</td>
<td>19</td>
<td>2.2 ± 0.3</td>
<td>13.3 ± 0.8</td>
<td>27.4 ± 4.2</td>
<td>84.2</td>
</tr>
<tr>
<td>IL-11R⁺/⁺ IVlg</td>
<td>16</td>
<td>0.4 ± 0.2*</td>
<td>16.8 ± 0.9*</td>
<td>3.8 ± 1.7*</td>
<td>26.7*</td>
</tr>
<tr>
<td>IL-11R⁻/⁻ HSA</td>
<td>18</td>
<td>1.2 ± 0.3#</td>
<td>14.8 ± 1.0</td>
<td>16.0 ± 3.9</td>
<td>66.7</td>
</tr>
<tr>
<td>IL-11R⁻/⁻ IVlg</td>
<td>21</td>
<td>1.0 ± 0.2</td>
<td>19.6 ± 1.3*</td>
<td>9.8 ± 3.0</td>
<td>47.6</td>
</tr>
</tbody>
</table>

Legend. Values are means ± SEM. Between-group peak score, day of onset and cumulative score features were analyzed by one-way ANOVA and Tukey post-hoc test. A chi-square test was used to analyze whether disease incidence differed between IVlg and HSA counterparts. *indicates a significant difference (p<0.05) from the HSA counterpart. # indicates a significant (p<0.05) difference from WT counterpart. Sample sizes (N) are as indicated.
3.3.2 Analysis of histology of wild-type and IL-11Rα−/− mice spinal cords

To gain further insights into the differential effects of IVIg in the WT and IL-11Rα−/− mice, we also conducted a histopathological analysis of inflammation and demyelination in the spinal cords of mice at the end-point of the EAE experiment. We observed that WT mice treated with HSA exhibited the highest inflammation and demyelination scores, while IVIg treated WT mice showed considerably less inflammation and demyelination in the spinal cord (Figure 3.3.2 A-C). Consistent with our finding of equivalent EAE severity in IL-11Rα−/− mice treated with IVIg or the HSA, we observed no difference between these groups in the degree of inflammation or demyelination in the spinal cord. Taken together, these results suggest that optimal amelioration of EAE by IVIg requires an intact IL-11 receptor and IL-11 signaling.
Figure 3.3.2 Histology results showing assessment of inflammation and demyelination.

A

Wild-type

IL-11Rα−/−

HSA

IVIg

HSA

IVIg

B

WT, HSA

WT, IVIg

IL-11Rα−/−, HSA

IL-11Rα−/−, IVIg

C

% Demyelination

0 5 10 15 20 25

Quadrants affected %

0 20 40 60 80

Meningitis

Perivascular Cuffing

*
**Figure 3.3.2** A shows representative spinal cord sections from control- or IVIg-treated IL-11Rα/− or WT mice stained with haematoxylin and eosin and luxol fast blue. B shows the percent quadrants of spinal cords (N=10 sections/mouse) that were positive for meningitis or perivascular cuffing in EAE mice. C shows the percent demyelination in these spinal cord sections. Values in B and C are means ± SEM of N=4-6 mice per group for one representative experiment. *represents a significant difference (*p<0.05) from the HSA-treated, genotype-matched counterpart as determined by one-way ANOVA and a Tukey post-hoc test.
3.4 Cytokine and proliferation analysis in IL-11Rα<sup>−/−</sup> mice

3.4.1 IVIg downregulates pro-inflammatory cytokine production in both wild-type and IL-11Rα<sup>−/−</sup> mice, with the exception of IL-17 in the lymph node.

To gain further insights into the disparate effects of IVIg in IL-11Rα<sup>+/+</sup> and IL-11Rα<sup>−/−</sup> mice during EAE, we examined recall proliferation and cytokine responses to MOG<sub>p35-55</sub> in the spleen and draining lymph nodes of mice. The first thing we noted was that IL-11Rα<sup>−/−</sup> mice develop a more Th17 biased disease than WT counterparts HSA- IL-11Rα<sup>−/−</sup> mice exhibiting a higher MOG p35-55-induced IL-17 production and a lower MOG p35-55-induced IFN-γ production in the spleen as compared with IVIg- IL-11Rα<sup>−/−</sup> counterparts (Figure 3.4.1).
Figure 3.4.1 Effect of IVIg on the production of specific pro-inflammatory cytokines.
**Figure 3.4.1** IL-11Rα\(^{+/+}\) (WT) and IL-11Rα\(^{-/-}\) mice were immunized with MOG p35-55/CFA (no pertussis toxin), and were injected with either HSA or IVIg (1g/kg, i.p.) daily. On day 12 post-immunization, spleens and draining lymph nodes were collected, were processed into a single cell suspension, and were cultured with MOG p35-55 (5 µg/ml). The productions of IFN-\(\gamma\), TNF-\(\alpha\), IL-2 and IL-17 were measured in supernatants of lymph node A and splenocyte B cultures of IVIg- or HSA-treated IL-11Rα\(^{+/+}\) (WT) and IL-11Rα\(^{-/-}\) mice. In A-B, values are means + SEM of triplicate cultures. These data are representative of three individual experiments.
3.4.2 IVIg down regulates proliferation in both WT and IL-11Rα/− mice, but IL-11Rα−/− mice show higher circulating IL-17.

When contrasting the effects of IVIg in these mice, it was evident that IVIg was only effective at reducing the MOG p35-55 elicited IL-17 production in the lymph nodes of WT, but not IL-11Rα/− mice (Figure 3.4.1). On the other hand, IVIg induced an equivalent reduction in the proliferation and IL-2, IFN-γ, and TNF-α production by MOG p35-55-reactive immune cells in these immune compartments in IL-11Rα+/+ and IL-11Rα−/− mice (Figure 3.4.2). Higher levels of IL-17 were also detected in the circulation of IL-11Rα−/− versus IL-11R+/+ mice (Figure 3.4.2 B). Taken together, these data suggest that IVIg has dual effects in inhibition of EAE, an effect in inhibiting the expansion or cytokine production by Th17 cells that is dependent on IL-11 signaling and an effect in inhibiting the proliferation or expansion of Th1 cells that is independent of IL-11. The defect in the IL-11 inhibition of IL-17 is what was associated with progression to EAE development in the IL-11Rα−/− mice.
Figure 3.4.2  

A The proliferation in counts per minute of spleen cells was measured using a [H\(^3\)]-thymidine incorporation assay. Shown is the stimulation index, which is the mean + SEM counts per minute (cpm) in the MOG p35-55-stimulated wells divided by the cpm in the media control wells. Values are representative of means + SEM of individual mice (N=4-5/group) in one experiment. *p<0.05 indicates a difference of the IVIg group from the HSA-treated, genotype-matched counterpart.  

B, Sera was taken from mice at 12 days post-immunization and the levels of IL-17 were measured using FlowCytomix assays. Values are means + S.E.M. of individual mice (N=4-5/group).
3.5 IL-11 has direct effects in inhibiting IL-17 production by lymph node cells.

Our results suggested that a resistance of IL-11Rα−/− mice to protection by IVIg could relate to defective IL-11 inhibition of IL-17 production. To further test this possibility, we stimulated lymph node cells in vitro with anti-CD3 and anti-CD28 in the presence of mouse recombinant IL-11 and measured the proliferation, IFN-γ, and IL-17 production by these cells. We observed that under Th0 conditions (no added cytokines), the addition of IL-11 to cultures had no significant effect on the proliferation, IFN-γ, or IL-17 production by these cells (Figure 5). However, under Th17-promoting conditions (with IL-6, TGF-beta and anti-IFN-γ), IL-11 reduced the proliferation and IL-17 production by stimulated lymph node cells. Taken together, these data provide further evidence that Th17 cells may be an immune population that is affected by IL-11 in the attenuation of EAE by IVIg.
Figure 3.5 Direct effects of IL-11 on IL-17 production and proliferation.

A

Th0

Th17

Proliferation

Concentration of IL-11 (ng/ml)

CPM

Concentration of IL-11 (ng/ml)

Stim

No stim

Concentration of IL-11 (pg/ml)

B

IL-17A

Concentration of IL-11 (ng/ml)

pp/ml

Concentration of IL-11 (pg/ml)

C

IFN-γ

Concentration of IL-11 (ng/ml)

pg/ml

*
Figure 3.5 IL-11 inhibits the proliferation and IL-17 production by lymph node cells under Th17-skewing conditions. Lymph node cells were harvested from un-immunized C57BL/6J mice (N=3/group). They were dissociated into a single-cell suspension and were stimulated with plate-bound anti-CD3 and anti-CD28 (0.5 µg/ml) in the absence of added cytokines (Th0) or in the presence of IL-6 (30 ng/ml), TGF-beta (3 ng/ml) and anti-IFNγ neutralizing antibody (10 µg/ml). The proliferation (A), IL-17 (B), and IFNγ (C) cytokine production by lymph node cells was measured using H³-thymidine incorporation and ELISA assays, respectively. Values represent mean ± S.E.M. pg/ml or cpm in triplicate cultures. * indicates a significant (p<0.05) difference from the 0 ng/ml concentration group as determined using a one-way ANOVA and Tukey post-hoc test. Note that IFNγ was not detected under Th17 skewing conditions and is not shown.
IVIg was shown in several clinical trials to reduce relapse rates and the number of brain lesions on MRI in patients with early relapsing-remitting MS (Cohen et al., 2008). This agent is currently used in an “off-label” fashion to treat MS exacerbations, particularly in patients who are refractory to steroid treatment or who are pregnant and need safer treatment alternatives (Dudusek and Zetti, 2006). How IVIg exerts its clinical benefit in MS is not known, however many potential mechanisms have been proposed (Durandy et al., 2009) and have been discussed in the Introduction of my Thesis. The major objective of my Thesis was to test the novel hypothesis that IL-11 could be an immune effector of IVIg in the treatment of CNS autoimmunity in an animal model of MS. We show that IL-11 is the main cytokine upregulated in the serum of mice post-IVIg treatment and that IL-11 has an effect in attenuating Th17 cytokine production in vitro. Furthermore, mice that are deficient in the receptor for IL-11 are more resistant to IVIg-amelioration of this disease, correlating with an ineffectiveness of IVIg to inhibit IL-17 by MOG p35-55-reactive T-cells. Taken together, these results suggest that the amelioration of EAE by IVIg occurs in part through IL-11 signaling following induction of IL-11 due to administration of IVIg.
4.1 Amelioration of EAE by IVIg and Role of Tregs.

The finding that high-dose IVIg virtually prevented EAE development is in line with previous results in mouse and rat EAE models (Pashov et al., 1997, Humle Jorgensen et al., 2005, Jorgensen et al., 2005, Ephrem et al., 2008). Similar to previous studies, we also observed a profound effect of IVIg in the inhibition of the expansion and Th1 and Th17 cytokine production by myelin-reactive T-cells (Othy et al., 2013). Treg have been implicated as key effectors in IVIg therapy, both in mouse EAE models (Ephrem et al., 2008, Maddur et al., 2010, Aslam et al., 2012) and in clinical studies (Hirabayashi et al., 2013, Tjon et al., 2013, Chi et al., 2007). In EAE, it was reported that IVIg induces a preferential expansion of these cells in the spleen (Ephrem et al., 2008). However, we did not detect a remarkable increase in Treg frequency in the spleen after IVIg treatment at the multiple time points that we examined. The reason for the discrepancy between this study and previous reports is not clear. However it can be speculated that given the anti-proliferative properties of IVIg, that the higher dose used in this study (1.0 g/kg) may have limited the expansion of Tregs that is reported to occur at the lower dose of 0.8 g/kg (Ephrem et al., 2009; Othy et al., 2011). These results underscore the importance of dose consideration in the design of future clinical trials that aim to induce T-cell tolerance with IVIg.
4.2 Induction of IL-11 post-IVIg administration

The finding that IL-11 was induced at very high levels in the serum of mice post-IVIg treatment is a novel observation, but is consistent with one report of the induction of IL-11 mRNA in IVIg-treated MS patient T-cells (Pigard et al., 2009). In this report, the investigators confirmed the increased IL-11 mRNA by using real-time RT-PCR but did not investigate IL-11 protein production. Examination of the IL-11 mRNA levels following IVIg treatment across various tissues identified the liver as opposed to immune tissues as the organ of origin of the up regulation of IL-11 transcription. In this work, only liver, lymph nodes, bone marrow and spleen were examined. Purified T-cells were not tested. However, it has been reported that IL-11 is induced in hepatocytes in response to tissue injury and oxidative stress and that IL-11 induces the compensatory proliferation of hepatocytes to mediate liver repair post-injury (Masubuchi et al., 2003; Nishina et al., 2012). IL-11 has also been shown to protect against the elevation of liver enzymes or TNF production in various models of acute liver injury (Bozza et al., 1999; Trepicchio et al., 2001; Maeshima et al., 2004; Nishina et al., 2012). Given that a transient elevation in liver enzymes can occur in patients post-IVIg infusion (Antonelli et al., 1992; Oomes et al., 1996), it is possible that a surge in IL-11 evoked by IVIg treatment is reflective of a stress response in the liver to this treatment. Other than the single report of IL-11 mRNA increased following IVIg treatment, there have been no peer-reviewed studies of IL-11 protein production in humans following IVIg therapy; although preliminary results of IL-11 production in humans following IVIg therapy has been documented (Dr. D. Branch, unpublished).
4.3 EAE studies in IL-11Rα−/− mice and IL-11 in vitro studies.

Our studies in the IL-11Rα−/− mice revealed an important role for IL-11 as an anti-inflammatory mediator of IVIg in the treatment of EAE. We found that IL-11Rα−/−-mice treated with IVIg developed EAE with a similar severity and extent of CNS inflammation as HSA-treated counterparts. The major immune correlate of this resistance to IVIg protection was that this therapy failed to inhibit the IL-17 production by MOG p35-55 reactive T-cells in the draining lymph nodes of IL-11Rα−/− mice. Further establishing the link between IL-11 and inhibition of IL-17, we showed that IL-11 inhibited the proliferation and IL-17 production by lymph node cells when cultured with IL-6 or TGF-beta. These findings suggest that the IVIg induction of IL-11 may somehow interfere with the IL-6 receptor-STAT3 or TGF-β receptor-SMAD signaling pathways that lead to Th17 cell development. Indeed, there is a precedent in gastric cancer that IL-11 can inhibit TGF-β signaling through the induction of the inhibitory Smad protein, Smad7 (Ernst et al., 2008). Although, IL-6 and IL-11 both transduce signals through gp130 and activate STAT3 and MAPK signaling, it is also documented that these cytokines can have opposing effects (i.e., IL-6 is pro-inflammatory and IL-11 is anti-inflammatory) in cardiovascular and allergic lung disease (Garbers and Scheller, 2013). Future studies will explore whether IL-11 and IL-6 indeed have opposing effects on Th17 inflammation.

The finding that IVIg was just as effective at attenuating the expansion of Th1 cells and delaying the onset of EAE in IL-11R+/+ and IL-11Rα−/− mice indicates that IVIg has protective effects in EAE that are independent of IL-11. In this respect, various mechanisms have been proposed to explain the immune modulatory mechanisms of IVIg including modulation of the expression or function of FcyRs, induction of inhibitory
cytokines, cytokine neutralization, scavenging of complement fragments, and the induction of T regulatory cells (Durandy et al., 2009). Our finding that the anti-proliferative effect of IVIg was still observed independently of alterations in Treg frequency, suggests that Treg were not involved in this protection. Interestingly, Othy et al. (2013) recently reported that the inhibition of the expansion of myelin reactive Th1 cells during EAE still occurred in mice treated with only the F(ab')2 portion of IVIg, suggesting that these effects of IVIg are also independent of FcγR signalling. Certainly, more work remains to be done to fully understand the anti-inflammatory effects of IVIg in CNS autoimmune disease.

One finding that IL-11Rα−/− mice developed a milder form of EAE than WT mice was one observation that did not fit our IL-11 model of IVIg-mediated protection of EAE. It has been shown previously that the development of severe paralysis in the acute phase of EAE requires the production of CCL2 in the CNS and the subsequent recruitment of CCR2+ inflammatory monocytes (Huang et al., 2001; Ajami et al., 2011). The Th1 cytokine IFN-γ is absolutely required for the induction of CCL2 by microglia (Tran et al., 2002), but also for the entry of pathogenic Th17 cells into the CNS during EAE (O’Connor et al., 2008). Taken in this light, our finding of a less robust Th1 response in IL-11Rα−/− mice may explain the propensity of these mice to develop milder EAE. Of note, the observation of milder EAE in IL-11Rα−/− mice does conflict with one previous report of more severe EAE in these mice (Gurfein et al., 2009). One difference in this study compared to ours was the induction of EAE using 10-fold higher amounts of the Th1/Th17 adjuvant and blood-brain-barrier activator, pertussis toxin (Fedele et al., 2011). We speculate that the higher levels of pertussis toxin may have compensated for
the less robust Th1 response in these mice and thereby permitted more Th17 cells to enter the brain and evoke a more severe CNS inflammatory response.

It is previously reported that IL-11 is expressed in the MS lesion by reactive astrocytes and that IL-11 can increase oligodendrocyte progenitor cell numbers, leading to an increase in the number of mature oligodendrocytes (Zhang et al., 2006). More recently, it was shown that overexpression of IL-11 in the brain reduces the extent of demyelination and enhances remyelination in the cuprizone-induced demyelinating disease model (Maheshwari et al., 2013). We did not observe histological evidence of more severe demyelination in IL-11Rα−/− mice during EAE. However this is not unexpected given that the acute inflammatory response that triggers this demyelination in this model was less severe in IL-11Rα−/− versus WT mice. Our studies therefore do not discount a role for IL-11 in myelin protection during EAE.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Our studies have suggested a novel mechanism of IVIg, involving immunomodulation by an induced cytokine, IL-11. Also, we have explored how lack of normal IL-11 signalling can influence the type of immune response in EAE, and this in turn affects the efficacy of IVIg. We show that IL-11 may be linked towards the regulation of IL-17 in IVIg treatment, however further in vivo studies are necessary to understand the implications of this finding. Firstly, the specific capability of IVIg to inhibit both a Th1 and Th17 type EAE disease can be tested by adoptively transferring MOG\textsubscript{p35-55} specific Th1 or Th17 cells into wild type mice and treating each group with IVIg. This would allow us to examine specific disease patterns with respect to Th1 and Th17 cells, and compare this to our existing data with IL-11R\alpha\textsuperscript{-/-} mice. If in fact IVIg can regulate EAE inflammation caused by Th1 and Th17 cells, then we would expect to see amelioration in both these branches of disease. In addition, as we observed that IL-11R\alpha\textsuperscript{-/-} mice show a Th17 biased type EAE disease, it would be interesting to investigate whether IVIg is efficacious in ameliorating a Th17 biased EAE in IL-11R\alpha\textsuperscript{-/-} mice. MOG\textsubscript{p35-55} specific Th17 polarized cells can be adoptively transferred into IL-11R\alpha\textsuperscript{-/-} mice, and IVIg can be administered daily. In this instance, we would hypothesize that IVIg would not be efficacious in the amelioration of a Th17 biased EAE in the absence of IL-11 signalling. This would support the idea that IVIg induces IL-11 which modulates Th17 cells.

We have also described certain aspects of IVIg-mediated amelioration of EAE that are not IL-11 dependent, for example down regulation of IFN-\gamma, IL-2, TNF-\alpha and T
cell proliferation. Also, there were no differences found for Tregs

All of our studies showing the effects of induced IL-11 in EAE were carried out in mice that lack a functioning receptor. To demonstrate the direct effects of this induced IL-11, a model can be designed utilising recombinant IL-11 and/or neutralizing antibody to IL-11 (which would be administered in conjunction with IVlg). Previous investigators have shown that overexpression of IL-11 in the mouse brain was highly efficacious in ameliorating EAE (Maheshwari et al., 2013), supporting our hypothesis. By administering a dose of IL-11 that matches induced levels post-IVlg injection, a degree of EAE amelioration should be observed based on previous work (Maheshwari et al., 2013). To explore this further, the efficacy of recombinant IL-11 versus natural IL-11 can be compared. Natural IL-11 may be more potent than recombinant IL-11 as it will be properly glycosylated and folded and it can be isolated by affinity chromatography from serum by taking advantage of the IVlg mediated induction of high levels at 6 hours post IVlg administration. This natural, purified IL-11 can then be used in EAE and other disease models where IVlg has efficacy. Also, effects of recombinant or natural IL-11 can be assessed on Th1 and Th17 cytokine profiles.

Experiments with neutralizing anti-IL-11 would also be of value and would directly address the role of production of IL-11 following IVlg administration. With respect to EAE, one would still expect to see a mild amelioration of disease taking into account the IL-11 independent mechanisms, but it can be hypothesized that the EAE curve of symptoms would show a Th17 pattern. As mentioned before, IL-11 belongs to the IL-6 family of cytokines. Therefore, one can speculate that in IL-11Rα<sup>−/−</sup> mice may exhibit redundancy in signalling that may involve these other IL-6 family members. It would
therefore be interesting to carry out further neutralization experiments to perhaps rule out any compensatory mechanisms that may contribute towards IVIg effects.

Whether our findings of IVIg-induced IL-11 as playing a role in the mechanism of action of IVIg extends to other disease models where IVIg shows efficacy remains to be examined. Experimental models of ITP, inflammatory arthritis and Alzheimer’s exist and it would be of great interest to examine if IL-11 plays any role in the efficacy of IVIg in these models. The quickest way to ask this question in ITP and inflammatory arthritis is to use the IL-11Rα⁻/⁻ mice. In the transgenic Alzheimer’s model for IVIg efficacy, use of recombinant or natural IL-11 may be useful but neutralizing anti-IL-11 would be the best first choice. It is exciting to speculate that the mechanism of IVIg may involve a common, unifying mechanism that involves production of IL-11 as an effector of the pleiotropic nature of IVIg. If it can be shown that the IL-11 induction that is described in this thesis is linked to amelioration of other disease models, then it is possible that we can provide a rationale to explore IL-11R agonists as alternative therapy to IVIg.
CHAPTER 6
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