IL-23 Receptor Expression and Effects of Signaling on T Cell Encephalitogenicity

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Alan Jay Smith

Graduate Program in Integrated Biomedical Science Program

The Ohio State University

2011

Dissertation Committee:

Amy Lovett-Racke PhD Advisor

Natarajan Muthusamy DVM/PhD

Abhay Satoskar MD/PhD

Larry Schlesinger MD
Abstract

Multiple Sclerosis (MS) is an immune mediated disease of the central nervous system characterized by the destruction of myelin sheaths surrounding nerve axons, and it is the leading cause of non-traumatic neurologic disability in young adults. The cause of MS remains unknown, but insights behind the mechanisms of this demyelinating disease are provided through use of animal models. Experimental autoimmune encephalomyelitis (EAE) is the most common mouse model used to study MS. The model is similar to MS in that it is also characterized by inflammation and demyelination within the central nervous system. Interleukin-23 (IL-23) has been implicated as a critical factor for the induction of EAE as mice deficient in IL-23 are resistant to EAE, and systemic administration of neutralizing anti-IL-23 antibodies significantly reduces the incidence and severity of EAE. There is limited knowledge of the functions of this cytokine beyond its ability to enhance IL-17 production in CD4+ T cells. The goal of this study is to determine the role IL-23 plays in enhancing the encephalitogenic capacity of myelin-specific T cells. To address this goal, the expression of IL-23 receptor (IL-23R) was characterized and was found to be positively regulated by IL-6 and IL-23 and negatively regulated by IL-4 and IFN-γ. This characterization of IL-23R expression was then translated to the EAE model. Using adoptive transfer of myelin-specific T cells, we found that signaling of IL-23 through the IL-23R significantly enhances T cell encephalitogenicity. Additionally, IL-23 caused an increase in inflammatory eye disease resembling optic neuritis, a condition commonly observed in MS patients. We also sought to determine if replacing the Mycobacterium tuberculosis found within the Complete Freund’s Adjuvant (CFA) with a bacterium that induces
an IL-23-dependent Th17 cell response during infection would induce EAE with a different phenotype. *Citrobacter rodentium*, a bacterium that requires IL-23 for protective immunity, was used as the adjuvant in EAE and compared to CFA. Mice immunized with *C. rodentium* adjuvant (CRA) developed classical signs of EAE, similar to CFA-immunized mice, but disease was less severe with a later onset and slower progression than CFA. The clinical course of CRA immunizations resembles that of MS patients better than CFA immunizations suggesting that the use of CRA immunizations may serve as a useful alternative model for studying EAE pathogenesis and potential therapeutics for MS. The effect of IL-23R signaling on T cell encephalitogenicity, the development of optic neuritis and the results of using an IL-23-dependent bacterium in the adjuvant suggest that IL-23 and IL-23R signaling are important factors in the pathogenesis of MS, and an understanding of these may give important insights into the development of demyelinating disease.
Acknowledgments

I would like to thank the following people for their contribution to my PhD training:

Amy Lovett-Racke PhD – I would like to thank you the most for my PhD training. You rescued me from a desperate situation. When I was changing labs, and I did not have any other lab that I was interested in, and you took me in. Since that time, you have been an excellent mentor giving me excellent feedback and advice. You have been patient with me in times of stress. Thank You very much for all you have done for me.

Michael Racke MD – I would like to thank you for your excellent support, for your feedback and comments. You have been an important part of my training helping me to keep my focus on the clinical disease. I have also appreciated your good humor that you bring to the lab.

Lovett-Racke and Racke lab members – Yuhong Yang PhD, Miereria Guerau PhD, Haiyan Peng, Dave Huss PhD, Yue Liu, Rachel Beers, Ryan Winger, and Jeff Weiner. Thank you all for your help, feedback and support.

Todd Shawler – I can honestly say that I owe you big time. You have made yourself available time and time again at the FACS machine. You have been patient with me when I run late, and you are always available to help troubleshoot problems. Thanks.
Committee members former and present – Raj Muthusamy PhD, Abhay Satoskar MD/PhD, Larry Schlesinger MD, and Brad Welling MD/PhD. Thank you for your feedback and support in my PhD. Thank you for taking time out of your busy schedules to help me become a scientist.

Former mentors – Virginia Sanders PhD and John Byrd MD. Although I did part from your mentorship, I am still indebted to you for my early training as a scientist. Thank you for your support and your training. I learned a great deal in those two years.

The Ohio State University – I would like to thank the university and all the people behind the scenes that made this opportunity available to me.

Reed and Lora Smith and family – You have also played an important part of my PhD training. Although I did not get my PhD training for you, you are the ones that gave me the foundation to actually earn this degree. Most of all, you taught me to work hard, and that is what got me through the PhD.

Erin Smith and kids – You are the other ones that I must thank the most. You supported me in getting through the PhD, and you provided me the perfect escape when I needed to get my mind on something else. I love science and it gives me a great deal of satisfaction and enjoyment. You, however, give me happiness and joy.
Vita

1996 .................................................. Clarkston High School

2002 .................................................. B.S. Zoology, Brigham Young University

2003 .................................................. Graduate Research Fellow, Medical Scientist

                                            Program, The Ohio State University

2005 to present .................................... Graduate Research Associate, Department of

                                            Molecular Virology Immunology and Medical

                                            Genetics, The Ohio State University

Publications

Lovett-Racke, A.E. T-bet is Essential for Encephalitogenicity of Both Th1 and Th17 Cells. J. Exp.
Med. 206(7):1549-64 (2009)

Th1 Bacteria Versus a Th17 Bacteria as Adjuvant in the Induction of Experimental Autoimmune

Fields of Study

Major Field: Integrated Biomedical Science Program

Area of Emphasis: Immunology
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1.1 Multiple Sclerosis (MS)

Multiple Sclerosis is the most common inflammatory disease of the central nervous system and a leading cause of disability. It is characterized by perivascular infiltrates within the central nervous system accompanied by areas of demyelination and axon loss. Patients who develop MS commonly present with symptoms that correlate with the damage within the central nervous system such as weakness, fatigue, numbness, or visual disturbances. MS is diagnosed by the presence of a variety of criteria. These criteria include the patient experiencing more than one neurological attack over time or the presence of lesions as observed by MRI. Cerebral spinal fluid is also measured for oligoclonal bands which are found in up to 98% MS patients (Rejdak et al., 2010).

Multiple Sclerosis has different disease courses each defined by the pattern of progression. About 85% of patients present with a relapsing-remitting MS characterized by neurological attacks followed by periods of complete or nearly-complete recovery. Permanent disability accumulates slowly in these patients; however, in time, most of these patients develop secondary-progressive MS in which there is steady progression of neurological disability without distinguishable relapses. About 10-15% of patients present with primary-progressive MS which is also described as a steady progression of neurological disability without relapses, but in these patients progression occurs from disease onset (Rejdak et al., 2010). The rate at which each type of MS typically advances is frequently correlates with the individual’s age, i.e. individuals who
are diagnosed at a later age commonly have a faster progressing disease than those who are
diagnosed at a young age (Vukusic and Confavreux, 2007). Individuals with primary-progressive
MS also have a faster progression of disease. There are limited factors that favor a better
prognosis though. They include an onset limited to one symptom, an onset with optic neuritis, a
complete recovery, a long time span between first and second relapse and fewer relapses within
first five years. Conventional MRI, biological markers, and clinical signs are currently unreliable
prognostic indicators for each individual (Vukusic and Confavreux, 2007).

The geographic distribution of people who develop MS follows a pattern of increasing
incidence with increasing latitude. The countries with the highest incidence of MS (>100 per
100,000) include the United States, Canada and Northern European countries. Countries that
are near the equator generally have a much lower incidence of MS (0-5 per 100,000) (Milo and
Kahana, 2010; Pugliatti et al., 2002). There are exceptions to this pattern however. Sardinia is a
Mediterranean island with a warm climate, yet the incidence of MS is relatively high. Despite
their living at very high latitude, the Inuit people of Northern Canada have a very low incidence
of MS. In addition to geographic propensity, women are about twice as likely to develop MS as
men (Milo and Kahana, 2010).

Although the cause of MS is unknown, there are several factors implicated in its
etiology. There has been great interest in finding a genetic link to the disease, but so far, this link
is only modest. The HLA DRB1 gene consistently shows the strongest association with MS. Other
genesis associated with MS include interleukin-2 receptor alpha, interleukin-7 receptor alpha,
CLEC16A, CD6, CD58, IRF8, RGS1 and TNFRSF1A genes (Oksenberg and Baranzini, 2010). Vitamin
D is believed to play a role in disease pathogenesis. Vitamin D is believed to inhibit the
production of inflammatory cytokines and enhance the production of anti-inflammatory
cytokines. This hypothesis is supported by the latitudinal gradient of disease incidence where individuals at the equator get more direct sunlight and therefore more vitamin D production than those at higher latitudes. Viral infections are believed to be a cause of MS under two different hypotheses. One hypothesis is based on timing of infection. Infection with a causative agent early in life is protective while an infection later on will result in disease. People at higher latitudes may be less exposed to the infection because of colder temperatures which would result in increased incidence of MS. Another hypothesis is that MS is caused by an infectious agent that is more common in regions of higher disease incidence (Marrie, 2004). Several infectious agents have been proposed to be either protective or causative of MS including Epstein-Barr virus, herpes simplex virus and Chlamydia pneumoniae (Milo and Kahana, 2010).

There are several approved first-line treatments for MS, and more potential treatments are currently in clinical trials. Interferon-β (IFNβ) therapy was developed under the rationale that MS may be caused by a virus and that viral infections were reported to increase the risk of relapses (Confavreux, 2002), and it is one of the most commonly used therapies in MS patients. There are three different formulations of IFN-β that differ in their dose, method of injection and recommended frequency of use. Avonex® (IFNβ-1a) is given weekly as an intramuscular injection. Rebif® (IFNβ-1a) and Betaseron® (IFNβ-1b) are given as subcutaneous injections three times a week and every other day respectively. The primary mechanism of action of IFNβ is uncertain. Proposed mechanisms of action include reduced antigen presentation, reduced expression of costimulatory molecules, inhibition of proliferation, reduction in cell migration, and shifting cytokine production from inflammatory to a more anti-inflammatory phenotype (Vosoughi and Freedman, 2010). Glatiramer acetate (Copaxone®) is also one of the most commonly used therapies for the treatment of MS. Glatiramer acetate is composed of a
polymerized sequence of four amino acids that, because of its effectiveness in the EAE model, was tested in MS and proven effective. The mechanism of action for this therapy is unclear. It is believed that glatiramer acetate increases MHC binding in the periphery possibly drawing myelin specific T cells away from the central nervous system (Vosoughi and Freedman, 2010). It may also induce regulatory T cells shifting T cells to a more anti-inflammatory phenotype.

Although IFNβ and glatiramer acetate are first-line agents for the treatment of MS, they have limited effectiveness in slowing the progression of disease. Clinical trials for each agent show about a 30% reduction in the number of relapses in comparison to placebo (1993; 1998; Johnson et al., 1995; Rudick et al., 1997). The effectiveness of treatments can be further reduced by neutralizing antibodies. Each patient has the potential to produce neutralizing antibodies to both IFNβ and glatiramer acetate (Vosoughi and Freedman, 2010). With respect to IFNβ, studies have shown a correlation between reduced effectiveness of the medication and the development neutralizing antibodies to IFNβ. It is unclear what effect neutralizing antibodies have on glatiramer acetate and IFNβ therapy; nevertheless, a need still exists to develop more effective first-line therapies for the treatment of MS.

There are several second-line agents some of which are more effective in treating MS, but they are not used frequently because they have more severe adverse side effects including death (Vosoughi and Freedman, 2010). Mitoxantrone was originally used as an anti-neoplastic agent, but has subsequently proven effective in treating MS. This drug intercalates with DNA causing damage to rapidly dividing cells. In the case of MS, it blocks activated immune cells causing cell death. Natalizumab is composed of monoclonal antibodies specific for VLA-4 (very late activating antigen-4). VLA-4 binds to cells adhesion molecules and allows immune cells to
traffic to sites of inflammation. Natalizumab blocks this binding, and thus inhibits cell migration into the central nervous system. In the most severe cases of MS autologous hematopoietic stem cell transplantation has been tested, and although effective, has a high risk of death. Fingolimod is the first oral treatment and was recently approved in 2010. This drug inhibits signaling through sphingosine-1-phosphate (S1P) receptors. This prevents T cells and B cells from leaving lymph nodes thus preventing cell migration.

1.2 Experimental Autoimmune Encephalomyelitis (EAE)

The discovery and use of several currently used MS treatments can be attributed to the successful application of animal models. The animal model most commonly used to study MS is experimental autoimmune encephalomyelitis (EAE). This model has also been referred to as experimental allergic encephalomyelitis. The origin of this model dates back over 100 years when Louis Pasteur immunized humans for rabies using dried spinal cord from rabies infected rabbits. Occasionally individuals would get an encephalomyelitis which revealed signs and pathology different from rabies. Key differences of the encephalomyelitis included demyelination within the CNS around blood vessels and the absence of hydrophobia whereas rabies is associated with hydrophobia and not associated with demyelination. This encephalomyelitis was further studied by the virologist Thomas M. Rivers in the 1930’s. Rivers wanted to determine if the encephalomyelitis was caused by the virus or by some other agent found within the vaccine (Rivers et al., 1933). He was plagued by difficulty inducing disease such that animals required numerous immunizations to develop encephalomyelitis. Then, in the 1940’s Jules Freund developed the use of Complete Freund’s Adjuvant (CFA) which is a mixture
of dried, killed *Mycobacterium tuberculosis* mixed in an oil emulsion. Shortly thereafter, his and several other groups found that encephalomyelitis could be rapidly induced by injecting brain material mixed with the adjuvant (Freund et al., 1947; Kabat et al., 1947; Morgan, 1947), and since that time the EAE model has become a well-established model for studying demyelinating disease.

There are several models of EAE that can utilize different animals, but due to cost, availability of reagents and transgenic/gene knockout strains, the most commonly used models are in mice (Baxter, 2007; Croxford et al., 2011). Most EAE experiments are done using a few inbred mouse strains including C57Bl/6, SJL, and B10.PL. The most common strain is C57Bl/6 mice because gene knockout and transgenic mice are most commonly available on this mouse strain. EAE can be induced in mice by a number of different methods (figure 1.1). In the immunization model, mice are immunized subcutaneously with myelin protein or an immunodominant peptide in emulsion with Complete Freund’s Adjuvant (CFA) (Stromnes and Goverman, 2006a). An i.p. co-injection of pertussis toxin is also given. Two days later, another i.p. injection of pertussis toxin is administered. After about 7-15 days the mice will subsequently develop EAE. Another method of EAE induction is through the passive transfer of myelin reactive T cells (Stromnes and Goverman, 2006b). In order to passively transfer EAE, myelin-specific T cells are isolated either from immunized mice or from T cell receptor (TCR) transgenic mice which have a high frequency of T cells specific for myelin peptides (Bettelli et al., 2003; Goverman et al., 1993; Lafaille et al., 1994; Pollinger et al., 2009; Waldner et al., 2000). The myelin-specific T cells are then activated *in vitro* with the appropriate myelin protein followed by a transfer of the cells into recipient mice. The recipient mice will subsequently develop EAE normally within 4-10 days. In addition to being useful for passive transfer of EAE, the TCR
transgenic mice have the potential to develop spontaneous EAE. This allows for the study of demyelinating disease in vivo without the use of immune-altering adjuvants. The availability of different methods to induce EAE makes it a useful model to study MS and the immune mechanisms associated with demyelinating disease.

1.3 MS and EAE

There are several similarities between EAE and MS that make EAE a useful model. Both MS and EAE are immune mediated diseases. This is shown by the fact that genes that are associated with MS are largely immunomodulatory genes (Oksenberg and Baranzini, 2010). Additionally, EAE can be induced by well-defined immunologic processes. Both show similar pathology of inflammatory infiltrates within the CNS along with loss of myelin surrounding axons. The most important similarity is the similar response shown in the EAE model to therapeutics that has later proven to be effective in the treatment of MS. These therapies include glatiramir acetate, mitoxantrone and natalizumab. The effectiveness of these therapies validates the usefulness of the EAE model system for the study of MS.

Despite its usefulness as a model to study MS, one must exercise some caution because EAE is not the same disease, and there are some distinct differences that make it unique. One important difference is the location of the lesions. In most models of EAE, the lesions are primarily found within the spinal cord, and the highest frequency is found within the lumbar region. MS patients are typically the opposite. Most lesions are found within the brain, and if a patient has lesions in the spinal cord, they are commonly in the cervical or thoracic region. Another important difference is some therapies that have proven effective in EAE have shown
no benefit in patients with MS. The differences between EAE and MS have unfortunately led to adverse outcomes in some clinical trials. One notable example was treatments using an altered peptide ligands (APL) to myelin basic protein (MBP\textsubscript{(83-99)}). The hypothesis behind APL therapy was that by altering amino acids in the immunogenic peptide, T cell responses can be reduced, blocked or altered to a more anti-inflammatory phenotype. Treatment of mice with APL resulted in significant improvements in clinical EAE scores, but a phase 2 clinical trial using the same approach resulted in mixed results, and some patients even experienced exacerbations resulting from the treatments (Bielekova et al., 2000; Brocke et al., 1996; Gaur et al., 1997; Karin et al., 1994). These outcomes highlight another important difference between MS and EAE. MS is a heterogeneous disease, and patients get lesions within different areas of the CNS resulting in different symptoms. Some will have a relapsing remitting disease course with little disease progression, whereas others will develop a very rapid primary progressive disease course. EAE, on the other hand, is a very homogeneous disease. The majority of experiments use only a limited number of inbred mouse strains that get a very stereotypical disease course with little variation in disease severity and clinical signs. It is by appreciating these differences that researchers can effectively use the EAE model to develop new therapies for MS.

1.4 Cell Types in MS and EAE

Each immune cell type plays different roles in MS and EAE development. MS and EAE lesions contain predominantly T cells macrophages, and B cells. Macrophages function as contributing antigen presenting cells within the CNS. Chemical depletion of macrophages with mannosylated liposome-encapsulated dichloromethylene diphosphonate is protective in both
the immunization and adoptive transfer model of EAE (Huitinga et al., 1990; Tran et al., 1998). Microglia, a resident cell population of the CNS that resemble macrophages, also contribute to EAE. Heppner et al. (Heppner et al., 2005) developed mice in which gancyclovir treatment would prevent microglial activation and release of cytokines. They found that immunization of gancyclovir treated mice resulted in reduced EAE severity demonstrating an important role for microglia in EAE pathogenesis. Studies show a role of macrophages and microglia in MS also. Immunohistochemistry of brain sections from MS patients reveal increased MHC class II expression on perivascular cells and microglia within lesion area but not in normal appearing white matter (Ulvestad et al., 1994). Culture of microglia with IFN-γ, a cytokine commonly expressed by infiltrating T cells, causes increased expression of MHC class II molecules. Other groups have also found increased expression of MHC class II and costimulatory molecules on infiltrating macrophages (Chastain et al., 2011). Expression of these molecules suggests macrophages and microglia contribute to pathogenesis by enhancing antigen presentation to T cells.

Dendritic cells are also critical antigen presenting cells that express high amounts of MHC class II and costimulatory molecules. In the EAE immunization model, dendritic cells play a role in antigen uptake and trafficking to the lymph node where they present antigen to T cells thus initiating disease (Dittel et al., 1999). In the CNS, however, dendritic cells are also present and they appear to play a role in antigen presentation reactivating T cells at the inflammatory site. Dendritic cells have been found within lesion areas in both MS and EAE. They appear to be juxtaposed to T cells and they express MHC class II and costimulatory molecules (de Vos et al., 2002; Serafini et al., 2006). In addition, dendritic cells may be critical for T cell activation within draining lymph nodes of the CNS. Myelin proteins have been found within cervical lymph nodes
of MS patients and EAE animals, and the cells that contain the myelin antigens express dendritic cell markers (de Vos et al., 2002; Fabriek et al., 2005). Even in the adoptive transfer model of EAE dendritic cells are believed to play a critical role. Greter et al. (Greter et al., 2005) showed that adoptive transfer of myelin reactive T cells in to *MAP3k14* null mice, which lack lymph nodes, peyers patches, and have disorganized spleen and thymus, was able induce EAE. Then using MHC class II-deficient mice, chimeric mice were generated where MHC class II expression was limited to CD11c+ peripheral immune cells or CNS immune cells. Adoptive transfer into these mice revealed that MHC class II expression limited to dendritic cells was sufficient to induce EAE also suggesting that T cell reactivation may be occurring within the CNS. Thus dendritic cells play an essential role as antigen presenting cells.

Astrocytes, like microglia, are also resident cells of the CNS, and due to their abundance in the CNS, their role deserves attention. Astrocytes are not efficient antigen presenting cells, so their role in T cell activation is likely a supportive role. Astrocytes have been shown to secrete inflammatory cytokines in response to activation (Constantinescu et al., 2005). They may also play a role in recruitment of immune cells to the CNS by secreting chemokines and increasing blood brain barrier permeability (Chastain et al., 2011). More work is needed to find the exact role astrocytes play in disease.

B cells have long been implicated in the pathogenesis of MS for several reasons. Cerebral spinal fluid from MS patients has revealed the presence of B cells and oligoclonal immunoglobulin (Hirotani et al., 2010). Compliment deposition has been found in MS brains, and more recently, follicle-like structures containing B cells have been found in the meninges of individuals with MS. Although B cells have been believed to influence MS and EAE, the impact of B cells was thought to be limited. Recently, however, B cells have received much more attention.
with the finding that a B cell-depleting therapy, Rituximab, caused a significant reduction in MS progression (Hauser et al., 2008). This was shown by a reduction in relapses as well as a reduction of lesions detected by MRI. The therapeutic benefit of Rituximab was also shown in the EAE model. Although there is conflicting data whether B cell depletion improves or worsens EAE if given before immunization, B cell depletion as a therapy improves EAE score (Matsushita et al., 2008; Monson et al., 2011). The mechanism by which B cell-depletion improves MS and EAE is not clear. Plasma cells are not a target of this therapy and antibody levels do not change significantly. Some proposed mechanisms included reduction cytokine secretion. Also, B cells may be more involved in antigen presentation than previously thought thus affecting T cell responses.

Although MS is believed to be a T cell mediated disease, CD8⁺ T cells have not received much attention. Much of this is because most genetic associations are not specific to CD8⁺ T cells. Histological sections of lesions within MS brains reveal a high frequency of CD8⁺ T cells suggesting the these cells do play a role in disease (Babbe et al., 2000). A correlation has been shown between the number of infiltrating CD8⁺ T cells and axonal damage (Bitsch et al., 2000). Both inflammatory and regulatory CD8⁺ T cells have been described, so it is unclear to what extent CD8⁺ T cells are protective or pathogenic in both MS and the EAE model (Friese and Fugger, 2009).

There are several lines of evidence that show CD4⁺ T cells as the primary mediators of MS and EAE. TCR-transgenic mice, in which the majority of myelin-specific T cells are CD4⁺, can develop spontaneous EAE without the need of immunization or passive transfer of myelin-specific T cells (Goverman et al., 1993; Lafaille et al., 1994). Adoptive transfer of purified CD4⁺ T cells into recipient mice can cause EAE, and depletion of the CD4⁺ T cell population prevents
EAE. (Trotter et al., 1985; Waldor et al., 1985). Also, gene knockout mice have been developed such that gene deletions are specific to CD4$^+$ T cells, and these mice produce different responses to immunization and in some cases, they are resistant to disease (Kleiter et al., 2010; Liu et al., 2008a). There are also several lines of evidence suggesting CD4$^+$ T cells are also the primary mediator of MS. This data includes the presence of CD4$^+$ T cells in MS lesions and data that most genetic linkages involve genes associated with CD4$^+$ T cell responses. Additionally, myelin-reactive T cells from MS patients have an effector or memory phenotype whereas myelin-reactive T cells from healthy individuals have a naïve phenotype (Lovett-Racke et al., 1998). One of the most convincing lines of evidence stems from the APL trial. The purpose of APL therapy was to block or alter T cell responses to a more suppressive phenotype; however, administration of altered peptides of myelin basic protein caused exacerbation of MS in three patients (Bielekova et al., 2000). These exacerbations included increased MS symptoms, MRI lesions and an expansion of the myelin-specific T cells. These results support the critical role of CD4$^+$ T cells in mediating MS and EAE pathogenesis.

1.5 T cell Differentiation

Whether or not T cells contribute to disease progression is dependent on the production of inflammatory cytokines, chemokines and other mediators. Naïve T cells can differentiate down different pathways that ultimately govern the immune response to antigen (figure 1.2). Th2 and regulatory T cell (iTreg) differentiation pathways are protective in MS and EAE whereas Th1 and Th17 pathways are known to be more inflammatory and contribute to demyelination. T cells activated in the presence of IL-4 differentiate into Th2 cells which are characterized by the
transcription factor GATA3 and the secretion of IL-4, IL-5 and IL-13. T cells activated in the presence of TGF-β will differentiate into regulatory T cells characterized by the transcription factor Foxp3 and the release of anti-inflammatory cytokines such as TGF-β and IL-10. Although not within the scope of this project, Th2 and iTreg cells are of interest in MS research because these cells have the potential to reduce inflammation and thus reduce disease severity.

As shown in figure 1.2, naïve T cells activated in the presence of IL-12 will differentiate into Th1 cells which are characterized by the transcription factor T-bet (aka T-box-expressed-in-T-cells, or Tbx21). The prototypical cytokine secreted by Th1 cells is IFN-γ. EAE has historically been considered a Th1-mediated disease. This has been largely due to the fact that EAE can be induced by Th1-mediated mechanisms. Induction of EAE by immunization relies heavily on the presence of Mycobacterium tuberculosis, the primary active ingredient in CFA. Passive transfer of myelin-specific T cells differentiated under Th1 conductions is also sufficient to induce EAE (Yang et al., 2009). Mice deficient in the p40 subunit of IL-12 are resistant to EAE (Gran et al., 2002; Segal et al., 1998). Mice deficient in the Th1 transcription factor T-bet are also resistant to EAE (Bettelli et al., 2004). Despite this, reports have been published suggesting that key components of Th1 differentiation do not contribute to EAE pathogenesis, and there may be other mechanisms at play in disease. IFN-γ-deficient and IFN-γ-receptor-deficient mice are susceptible to EAE as are mice deficient in the p35 subunit of IL-12 (Ferber et al., 1996; Gran et al., 2002; Willenborg et al., 1996). A potential reason for this discrepancy was later proposed when IL-23, an IL-12-related cytokine, was discovered and characterized (Oppmann et al., 2000). Cua et al. (Cua et al., 2003) subsequently developed mice deficient in p19, a subunit specific to IL-23, and he found that mice deficient in p19 and p40 were protected from EAE whereas mice deficient in p35 remained susceptible to disease. In addition to knocking out the gene,
treatment of mice with anti-p19 and anti-p40 antibodies also ameliorated disease (Chen et al., 2006). This led to the conclusion that EAE is not a Th1 disease but an IL-23-mediated disease.

IL-23 is a heterodimer that shares a common subunit with IL-12. It is composed of a p40 subunit and a p19 subunit, whereas IL-12 also has a p40 subunit but has a p35 subunit instead (figure 1.3). The receptor for IL-23 is also like IL-12 in that both receptors have a common subunit. The IL-23 receptor (IL-23R) is also a heterodimer composed of an IL-12Rβ1 subunit and an IL-23R subunit, and the IL-12 receptor (IL-12R) is composed of an IL-12Rβ1 and an IL-12Rβ2 subunit (figure 1.3). Both receptors signal through Jak/Stat signaling pathways. IL-12R signals primarily through a Stat4 signaling pathway (Watford et al., 2004), and IL-23R signals through Stat3 phosphorylation and to a lesser degree phosphorylation of Stat1, Stat4 and Stat5 (Cho et al., 2006; Parham et al., 2002). IL-23, like IL-12, is made by macrophages, dendritic cells and possibly by activated microglia (Li et al., 2003; Oppmann et al., 2000).

Shortly after the discovery of the importance of IL-23 in EAE pathogenesis, IL-23 was found to increase IL-17 production on CD4+ T cells, thus linking it to Th17 cells (Langrish et al., 2005). This led to a great amount of excitement in the field of MS and EAE research, and many labs shifted their research focus from studying Th1 mechanisms of disease to Th17 mechanisms. In MS, increases in IL-23 and IL-17 were identified in blood, cerebral spinal fluid and within lesions of MS patients (Krakauer et al., 2008; Lock et al., 2002; Masurevicius et al., 1999; Tzartos et al., 2008; Vaknin-Dembinsky et al., 2006). In the EAE model, several labs tried to replicate previous studies linking IL-23 to IL-17 production but with limited success. However, new methods were developed that could reliably generate T cells that would produce large amounts of IL-17 and virtually no IFN-γ, and this involved activating T cells in the presence of IL-6 and TGF-β (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). Therefore, over the
period of about two years, the focus of EAE research shifted from Th1 to Th17 mechanisms mediated through IL-6 and TGF-β. We were also interested in determining the role IL-17 played in EAE pathogenesis, and it did not take long for our lab and others to find that IL-17 production was not the primary mechanism by which IL-23 caused T cells to be pathogenic. We and others observed that T cells activated in the presence of IL-6 and TGF-β were not encephalitogenic (McGeachy et al., 2007; Yang et al., 2009). Later, IL-17-deficient mice were developed, and these mice produced a mildly less severe course of EAE after immunization; however, in sharp contrast to IL-23-deficient mice, the IL-17-deficient mice were all susceptible to disease (Haak et al., 2009; Lees et al., 2008; Park et al., 2005). Despite these findings, there is still great interest in Th17 cell differentiation and its role in demyelinating disease.

In mice, IL-23 was initially classified as the cytokine critical for generating Th17 cells (Langrish et al., 2005). Since that time, other cytokines have been proposed to contribute to Th17 differentiation including IL-6, TGF-β and to a lesser extent IL-1β (Bettelli et al., 2006; Ghoreschi et al., 2010; Mangan et al., 2006; Sutton et al., 2006; Veldhoen et al., 2006). All these cytokines contribute to the production of IL-17 by T cells; however, in the context of EAE, IL-6 and IL-23 appear to be critical for generating encephalitogenic Th17 cells (Ghoreschi et al., 2010; Yang et al., 2009). TGF-β appears to contribute to the production of IL-17 by inhibiting the production of IFN-γ, but activating T cells in the presence of TGF-β renders them nonpathogenic (McGeachy et al., 2007; Yang et al., 2009). RORγt was originally classified as the prototypical transcription factor for Th17 cells, but more recently T-bet has also been shown to be expressed on encephalitogenic Th17 cells (Ivanov et al., 2006; Yang et al., 2009). Although IL-17+ cells have been identified in MS lesions, it is uncertain to what degree IL-17 production contributes to MS pathogenesis (Tzartos et al., 2008). Several groups have tried to determine factors that are
critical for the differentiation of human Th17 cells, but there has been a lack of agreement with their findings. As in mice, RORγt induces expression of IL-17, but human Th17 cells are generated with different cytokine combinations including IL-23, IL-1β, and IL-21 (Chen et al., 2007; Manel et al., 2008; Wilson et al., 2007; Yang et al., 2008). It remains unclear if TGF-β contributes to IL-17 production.

As indicated by the above observations, there has been significant interest in characterizing Th17 cells and determining what factors produced by these cells contribute to disease; however, there has been limited research done on the mechanism of the original findings. These include the discovery that IL-23(p19)-deficient mice are resistant to EAE, and treatment of mice with neutralizing anti-IL-23(p19) antibodies ameliorates disease (Chen et al., 2006; Cua et al., 2003). Additionally, culturing T cells in the presence of IL-23 has the capacity to significantly enhance EAE severity, and this is correlated with an increase in the production of IL-17; however, the IL-23-mediated effect on EAE severity appears to be independent of IL-17 as IL-17 deficient mice are not resistant to EAE (Haak et al., 2009; Langrish et al., 2005). In order for IL-23 to produce a response, it must bind to its receptor; therefore, receptor signaling is the limiting factor. It is this rationale that led us to hypothesize that IL-23R signaling is critical for the development of pathogenic myelin-reactive T cells. We used the following three aims to address our hypothesis.

The first aim was to Characterize IL-23R expression on immune cells with our focus on the EAE model. IL-23R expression was characterized on each immune cell immune cell population typically involved in EAE comparing naïve mice with immunized mice. T cell receptor transgenic mice, that have T cells specific for myelin basic protein, were also included in this aim comparing cells from naïve mice to mice with spontaneous EAE. Additionally, in vitro cultures
were used to determine what cytokines regulate IL-23R expression. The goal of this aim was to determine how IL-23R expression is regulated. Then, by increasing IL-23R expression future data can be gathered to determine what signaling pathways, cytokines and chemokines result from IL-23R engagement thus providing an understanding of specific pathways involved in EAE and potentially MS pathogenesis.

The second aim was to determine how IL-23R signaling affects T cell differentiation and effector function. This aim was used in conjunction with the first aim to address specific questions. First, this aim was used to determine what conditions are required for IL-23 to enhance T cell encephalitogenicity and if these conditions correlate with enhanced IL-23R expression. This aim was also used to determine if the IL-23 effect on EAE was mediated through CD4+ T cells or antigen presenting cells. Additionally, preliminary studies were performed on an unexpected observation where IL-23 affected cell trafficking to optic nerves of mice with EAE and the subsequent development of eye disease resembling optic neuritis and possibly uveitis, symptoms commonly developed in patients with MS.

The third aim deviates back to the use of Complete Freund’s Adjuvant (CFA) in the EAE model. CFA has been used in the EAE model for over 50 years, and it is still classified as a Th1-inducing adjuvant. The goal of this aim was to determine if EAE course can be altered by replacing *M. Tuberculosis* with a Th17-inducing or more particularly an IL-23-dependent bacterium in the adjuvant. The bacterium chosen for this aim was *Citrobacter rodentium*, a bacterium that causes murine colon infections and is used to study enteropathogenic and enterohemorrhagic *E. coli* infections in humans. Immunizations using *C. rodentium* as an adjuvant were analyzed measuring cytokine production, cell proliferation as well as the development of EAE.
Figure 1.1: Methods of inducing EAE

- **Immunized Mouse**
- **Myelin Protein & Complete Freund’s Adjuvant (s.c.)**
- **Pertussis Toxin (i.p.)**
- 2 Days
- **Pertussis Toxin (i.p.)**
- 7-15 Days
- **EAE**

- **TCR Transgenic Mouse**
  - (Myelin Protein-Specific)
- **Adoptive Transfer**
- **in vitro CD4 T Cell Activation with Myelin Protein**
- **EAE**
- 4-10 Days
Figure 1.2: T cell differentiation pathways.
Figure 1.3: IL-12 and IL-23 – cytokines and receptors.
Chapter 2: Materials and Methods

2.1 Mice

C57Bl/6 mice were purchased from Jackson Laboratory. B10.PL Vα2.3 and Vβ8.2 TCR transgenic mice were obtained from J. Goverman (Goverman et al., 1993). Vα2.3/Vβ8.2 T cell receptor (TCR)-transgenic mice express a gene encoded a TCR Vβ8.2 chain and Vα2.3 chain that recognize MBP Ac1-11. IL-4-deficient mice were obtained from Jackson laboratory and backcrossed onto the B10.PL background. All mice were bred and kept in a pathogen-free facility at The Ohio State University (OSU).

2.2 Preparation of Citrobacter rodentium

C. rodentium (ATCC 51638) was grown to approximate late log phase growth in nutrient broth (Sigma-Aldrich), washed with PBS and heat killed at 65°C for one hour. C. rodentium was lyophilized, crushed and stored for later use. Heat killing of bacteria was confirmed by plating on nutrient agar plates.

2.3 Induction of EAE

B10.PL Vβ8.2 T cell receptor (TCR)-transgenic mice express a gene encoded a TCR Vβ8.2 chain that when paired with the appropriate TCR Vα chain, recognize MBP Ac1-11 (Goverman et al., 1993). The frequency of T cells that recognize MBP Ac1-11 in Vβ8.2 TCR transgenic mice is estimated to be 1:100-1:1000, making them highly susceptible to EAE. For immunizations, EAE was induced in C57Bl/6 or B10.PL Vβ8.2 T cell receptor (TCR)-transgenic mice by administering
subcutaneous injections over 4 sites at the flanks of mice with an emulsion of Complete Freund’s Adjuvant (CFA) (Difco) or Incomplete Freund’s Adjuvant supplemented with heat-killed C. Rodentium (CRA) or M. Tuberculosis H37Ra (CFA) (Difco) and 200µg MOG35-55 (C57Bl/6 mice) or MBP Ac1-11 (B10.PL mice). Pertussis toxin (Ptx) (List Biological Laboratories) was given i.p. at a dose of 200ng at the time of immunization and 2 days later.

For adoptive transfer experiments using C57Bl/6 mice, spleens and lymph nodes were removed from C57Bl/6 mice 18 days after immunization, and single cell suspensions were prepared. Cells were suspended in EAE media containing 1X RPMI 1640 supplemented with 1mM Sodium Pyruvate, 13mM HEPES, 1X MEM Nonessential Amino Acids, 2mM L-Glutamine, 100 I.U. Penicillin, 100 µg/ml Streptomycin (Cellgro), 10% FBS (Invitrogen), and 50µM 2-ME (Sigma-Aldrich) at 6X10^6 cells/ml. Cells were then cultured in 24-well plates at 2ml/well with the addition of MOG35-55(2µg/ml). After 3 days of culture, cells were washed with sterile PBS and 20X10^6 total cells were injected i.p. into recipient mice.

For experiments using B10.PL mice, spleens were removed from naïve Vα2.3/Vβ8.2 T cell receptor (TCR)-transgenic mice or TCR transgenic mice with spontaneous EAE (clinical EAE without immunization) and single cell suspensions were prepared. Feeder cells were also prepared by generating single cell suspensions from B10.PL mouse spleens and irradiating the cells at 3000rad. Alternatively, feeder cells were prepared from naïve and immunized B10.PL mouse spleens depleted of CD4^+ cells using CD4^+ T cell isolation kit and magnetic columns (Miltenyi Biotec). The cells were then mixed together at a ratio of 1 Vα2.3/Vβ8.2 TCR transgenic cell to 3-5 irradiated feeder cells and the mixed cells were suspended in EAE media at 3-4X10^6 cells/ml. Cells were then cultured in 24-well plates at 2ml/well with the addition of MBP Ac1-11(2µg/ml). For the transfer of memory/effector cells, cells were activated for 3 days followed
by resting for 4 days followed by reactivation with MBP Ac1-11(2µg/ml) for 3 days. For the transfer of anti-CD3/CD28 activated cells, cells were activated in 24-well plates coated with anti-CD3 (1µg/ml) and anti-CD28 (1µg/ml) antibodies (BD) at 2ml/well. After 3 days of culture cells were washed with sterile PBS and 2-10X10^6 total cells were injected i.p. into recipient mice.

For all adoptive transfer experiments, various cytokines (all from R&D Systems) were added to cell cultures at various concentrations and neutralizing anti-IL-23 antibody (eBioscience) was also added to some cultures. Mice were scored based on the following scale: 0-no clinical disease, 1-limp tail, 2-mild hind limb weakness, 3-severe hind limb weakness, 4-hind limb paralysis, 5-moribund, and 6-death from EAE.

2.4 Cell culture for IL-23R expression

For IL-23R analysis, spleens were removed from naïve Vα2.3/Vβ8.2 TCR-transgenic mice and single cell suspensions were prepared. Feeder cells were also prepared by generating single cell suspensions from B10.PL mouse spleens and irradiating the cells at 3000rad. The cells were then mixed together at a ratio of 1 Vα2.3/Vβ8.2 TCR transgenic cell to 3-5 irradiated feeder cells and the mixed cells were suspended in EAE media at 3-4X10^6 cells/ml. Cells were then cultured in 24-well plates at 2ml/well with the addition of MBP Ac1-11(2µg/ml) for 2 days.

For analysis of Vβ8.2 TCR-transgenic cells, spleens were removed from naïve Vβ8.2 TCR-transgenic mice and single cell suspensions were prepared, and cells were suspended in EAE media at 3-4X10^6 cells/ml. Cells were then cultured in 24-well plates at 2ml/well with the addition of MBP Ac1-11(2µg/ml) for 2 days.

For analysis of C57Bl/6 mouse cells, spleens were removed from naïve C57Bl/6 mice and single cell suspensions were prepared. Cells were suspended in EAE media at 2.5-3X10^6 cells/ml
and were then cultured in 24-well plates at 2ml/well with the addition of 2µg/ml MOG35-55 for 2 days.

For CD4 T cell-enriched cultures, single cell suspensions from C57Bl/6 mice, were enriched using CD4⁺ T cell enrichment kit and the EasySep® magnet (StemCell Technologies). Cells were suspended in EAE media at 0.75X10⁶ cells/ml and then cultured in 24-well plates at 2ml/well with the addition of 2µg/ml MOG35-55 for 2 days. Various cytokines and neutralizing antibodies (all from R&D Systems) were added to cell cultures at various concentrations.

2.5 FACS analysis

For cytokine analysis, draining lymph nodes were isolated and crushed between microscope slides to generate single cell suspensions. Cells were suspended in EAE media at 5-6X10⁶ cells/ml and cultured in 24-well plates at 2ml/well for three days with 2µg/ml MOG35-55. For cytokine analysis of adoptive transfer experiments, cells were cultured as indicated above. Six hours before cytokine staining, 50ng/ml PMA and 1µg/ml ionomycin were added, and one hour later 1µl/ml Golgi Plug (BD Biosciences) was added to the culture. CNS mononuclear cells were isolated by crushing brains and spinal cords between microscope slides. CNS tissue was washed with 37% Percoll (GE Healthcare) solution and subjected to a 15 minute 2118 x g centrifugation over a 30/70% Percoll gradient. The interphase layer was collected, washed and suspended in EAE media at 1-2.5x10⁶ cells/ml. Cells were cultured overnight in 24-well plates at 2ml/well with 2µg/ml MOG35-55, and Golgi Plug was added five hours before cytokine staining.

For flow cytometric staining, cells were washed and incubated with FC block (BD Biosciences) for 15 minutes. Cells were then incubated with surface staining antibodies for 30 minutes and washed. Cells were fixed in Cytofix/Cytoperm buffer (BD Biosciences) for 20
minutes and permeabilized in Perm/Wash buffer (BD Biosciences) for 30 minutes. Cells were suspended in Perm/Wash buffer and incubated with intracellular staining antibodies for 30 minutes. Cells were washed, suspended in stain buffer and 100,000 events were collected on a FACSCanto 2 (BD Biosciences). Data was analyzed using Flowjo software (Tree Star Inc.). 7-AAD stains used were from BD biosciences. Antibodies used included the following from BD Biosciences: PerCP-conjugated anti-mouse CD45 and anti-mouse CD4, V500-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD4 and IFN-γ, AlexaFluor 647-conjugated anti-mouse P-Stat3, PE-Cy7-conjugated anti-mouse IFN-γ, Pacific Blue-conjugated anti-mouse B220, and PE-conjugated anti-mouse IL-17 and anti-mouse CD4. The following antibodies were from eBioscience: APC-conjugated anti-mouse CD11b and eFluor® 450-conjugated anti-mouse CD44. Additional antibodies used include FITC-conjugated anti-mouse T-bet (Santa Cruz Biotech), APC-conjugated anti mouse IL-23R (R&D Systems), and anti-mouse IL-23R (R&D Systems) conjugated to FITC (Leinco Technologies Inc.).

2.6 Immunocytochemistry

Cells were washed and fixed in Cytofix/Cytoperm buffer (BD Biosciences) for 20 minutes and permeabilized in Perm/Wash buffer (BD Biosciences) for 30 minutes. Cells were then washed and blocked with 0.1% cold fish skin gelatin, 1% BSA, and 5% serum from the same source as the secondary antibody for 30 minutes. Cells were then washed and incubated in Perm/Wash buffer with primary antibodies for 90 minutes and washed. Cells were suspended in Perm/Wash buffer and incubated with secondary antibodies for 60 minutes. Cells were washed and mounted on slides using a Cytospin 4 (Thermo Scientific) and Vectashield mounting medium with DAPI (Vector Laboratories). Primary antibodies used include Rabbit anti mouse IL-23R (Imgenex), rat
anti-mouse CD4 and CD8 (Santa Cruz Biotech). Images were taken on an Olympus BX41 microscope.

2.7 Immunohistochemistry

Mice were perfused with 4% paraformaldehyde. Optic nerves were then removed and incubated in 4% paraformaldehyde for an additional 2 hours. Optic nerves were then transferred to 0.2M Phosphate buffer overnight followed by incubation within a 30% sucrose solution. Tissue was then cryopreserved at -20°C in OCT compound. Longitudinal sections 10µm thick were cut and sections were stained with hematoxylin and eosin. Images were taken using an Olympus BX41 microscope and lesion area was measured using ImageJ software (NIH).

2.8 Immunoprecipitation/Western Blot analysis

Cells were collected and lysed in 1% Triton X-100 lysis buffer containing 20mM Tris (pH7.5), 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1mM Sodium Orthovanadate, 1mM PMSF, and 1:100 protease inhibitor cocktail (Sigma-Aldrich).

For immunoprecipitation, cell lysates were divided according to immunoprecipitating antibody. For immunoprecipitations using rabbit polyclonal anti-mouse IL-23R antibody, 20µg antibody was added to 4-6ml cell lysate and incubated on a circular rotator overnight. A 40µl slurry of protein A beads (Thermo Scientific) were then added to the lysate and mixture was incubated on a circular rotator for 2 hours. For immunoprecipitations using goat polyclonal and rat monoclonal anti-mouse IL-23R antibodies, 50µg antibody was covalently conjugated to 50µl bead slurry using a coimmunoprecipitation kit (Thermo Scientific). The slurry was then added to 4-6ml cell lysate and incubated on a circular rotator overnight. All beads were then washed and
protein was eluted using heated (100°C for 5 minutes) Laemmli sample buffer (Bio-Rad) mixed with β-mercaptoethanol.

For Western Blot analysis, samples were run on a denaturing gel and transferred to Hybond-P PVDF membranes (GE Healthcare). Membranes were blocked with 5% dried milk mixed with a TBST solution containing 150mM NaCl, 20mM Tris (pH7.5), and 0.05% Tween 20 for 1 hour at room temperature. Membranes were then incubated with the primary antibody diluted in TBST and 5% dried milk overnight at 4°C. Membranes were washed and incubated with HRP-labeled secondary antibodies diluted in TBST and 5% dried milk for 1 hour at room temperature. The membranes were then washed and visualized on Hyblot CL autoradiography film (Denville Scientific). Spot densitometry was calculated using AlphaEase®FC software (Alpha Innotech).

2.9 ELISA

Supernatants were collected from in vitro cultures used for adoptive transfer and flow cytometric analysis. Immulon 2 HB 96-well plates (Thermo Scientific) were coated overnight with 2µg/ml anti-mouse primary antibody at 50µl/well diluted in 0.1M NaHCO₃ pH 8.2. Plates were washed twice with PBS/tween and blocked with PBS + 1% BSA for 2 hours. Plates were washed twice and incubated overnight with samples and standards at 100µl/well. Cytokine standards were prepared in EAE media by diluting each individual cytokine in serial dilutions from 2ng/ml – 0.03ng/ml. Plates were washed 4X with PBS/tween and incubated with 1µg/ml biotinylated anti-mouse secondary antibody at 100µl/well diluted in PBS + 1% BSA for one hour. Plates were washed 6X with PBS/tween and incubated with 2.5µg/ml avidin-peroxidase at 100µl/well diluted in PBS + 1% BSA for 30 minutes. Plates were washed 8X with PBS/tween
followed by the addition of 100µl ABTS substrate containing 0.03% H$_2$O$_2$ to each well. Following appropriate color development, plates were read at A405 on an Emax microplate reader (Molecular Devices).

2.10 Thymidine Incorporation assay

Spleens were isolated and crushed between microscope slides to generate single cell suspensions. Red blood cells were lysed in buffer containing 155mM NH$_4$Cl, 10mM KHCO$_3$, and 0.1mMEDTA for one minute at room temperature followed by two washes with PBS. Cells were cultured in 96-well round bottom plates at 2.5X10$^5$cells/well for 48-72 hours with MOG35-55 at 0.4, 2 or 10µg/ml or bovine MBP (bMBP) at 4, 20 or 100 µg/ml in replicates of four. Cells were pulsed with 0.5mCi/well [$^3$H]thymidine for 18 or 36 hours. Cells were then harvested onto Unifilter-96 GF/C plates (PerkinElmer), and plates were dried. Micriscint 20 (PerkinElmer) was added to each well, and [$^3$H]thymidine was measured using a TopCount NXT (PerkinElmer). The stimulation index was calculated determining the mean CPM for the no antigen and antigen replicates and determining the ratio of mean CPM of the antigen relative to the mean CPM of the no antigen. For the precursor frequency assays, cells were plated in 96-well plates at 2X10$^5$, 1X10$^5$ and 5X10$^4$ cells per well with no antigen, 2µg/ml MOG35-55 or 20µg/ml bMBP in replicates of 32. Cells were pulsed with 0.5mCi/well [$^3$H]thymidine after 72 hours and harvested as described above. The mean and standard deviation of the no antigen wells was determined for each sample. For the antigen stimulated wells, a positive well was defined has having a CPM greater than the mean of the no antigen wells plus three standard deviations. After determining the number of positive wells for each condition, a chi square minimization analysis was
performed to determine the estimated frequency of cells responding to a specific antigen for each sample.

2.11 Statistical analysis

All data were analyzed using GraphPad Prism software. Statistics on EAE clinical scores were performed using Mann-Whitney non-parametric analysis. Student t test was used for analysis of proliferation data. Significant differences represent analysis with a p<0.05.
Chapter 3: IL-23 Receptor Characterization

Introduction

Experimental autoimmune encephalomyelitis (EAE) has long been known to be a T cell mediated disease. This was shown in both the immunization and passive transfer models that the depletion of T cells was sufficient to abrogate EAE (Gonatas and Howard, 1974; Ortiz-Ortiz et al., 1976). Shortly thereafter, researchers found that not all T cells were pathogenic and some T cell populations even suppressed disease (Bernard, 1977). From that time, scientists have worked to characterize encephalitogenic T cells with the goal to find new therapies for the treatment of MS. This research led to the long standing belief that EAE was a Th1 disease mediated by T cells secreting key cytokines, such as IFN-γ. This paradigm was called into question when it was found that IFN-γ-deficient and IFN-γ receptor-deficient mice remained susceptible to EAE (Ferber et al., 1996; Willenborg et al., 1996); nevertheless, it still held support by data showing IL-12(p40)-deficient mice were resistant to EAE (Segal et al., 1998).

A mechanism for this discrepancy was proposed with the discovery of the IL-12 related cytokine IL-23 (Oppmann et al., 2000). Cua et al. (Cua et al., 2003) subsequently developed the necessary gene-deficient mice and determined if mice deficient in the individual subunits of L-12 and IL-23 could develop EAE. They found that mice deficient in the p40 subunit shared by IL-12 and IL-23 or the p19 subunit specific for IL-23 were protected from EAE, whereas mice deficient in the IL-12 specific p35 subunit were still susceptible to disease (Cua et al., 2003). Subsequent papers corroborated this finding demonstrating a critical role of IL-23 in the pathogenesis of EAE.
One proposed mechanism by which EAE mediates EAE pathogenesis is through the production of inflammatory Th17 cells (Langrish et al., 2005). There are likely more mechanisms by which IL-23 contributes to T cell encephalitogenicity, but these mechanisms are still unclear.

In order to characterize the IL-23 response, signaling must occur through the IL-23 receptor (IL-23R). Therefore, the first aim was to characterize IL-23R expression on immune cells in the context of EAE. The goal was to determine what conditions promote IL-23R expression in vivo and in vitro. IL-23R can be expressed on most immune cell types depending on what activation signals the cell receives (Parham et al., 2002). In EAE, IL-23 is believed to affect encephalitogenicity through CD4+ T cells. Initially, IL-23R was believed to be expressed only on memory T cells. Recently, IL-23R expression has been shown on a number of small T cell subsets including NKT cells, γδ T cells, lymphoid inducer-like cells and CD4 CD8- T cells (Awasthi et al., 2009; Rachitskaya et al., 2008; Riol-Blanco et al., 2010; Takatori et al., 2009). Most published data concerning IL-23R expression has been shown measuring mRNA expression and not IL-23R protein levels, and there was little agreement as to what factors regulated IL-23R expression. Knowing that mRNA is subject to downstream regulatory factors such as micro RNAs and may not always accurately indicate the level of protein expressed, the goal of this aim was to characterize IL-23R expression at a protein level. At the time this aim started there was a limited number of commercial antibodies specific for IL-23R, so different antibodies were tested to determine which antibody would be most useful in this characterization.
Results

3.1 IL-23 receptor (IL-23R) is upregulated in Th1, Th2 and Th17 conditions

The first antibody tested was a polyclonal rabbit anti-mouse IL-23R antibody. This antibody is indicated for Western Blot analysis and immunohistochemistry. IL-23R mRNA was shown to be regulated by several cytokines such as IL-6, IL-23, IL-1β and TGF-β (Harrington et al., 2005; Ivanov et al., 2006; Langrish et al., 2005; Manel et al., 2008; Mangan et al., 2006; McGeachy et al., 2007; Zhou et al., 2007), and IL-23R protein was shown to be regulated by the Th1 transcription factor T-bet (Gocke et al., 2007), therefore, a broad approach was initially employed to analyze IL-23R expression. Splenocytes from C57Bl/6 mice were polyclonally activated in the presence of Th1, Th2, and Th17 (IL-6 and TGF-β) cytokines, rested, and reactivated, and IL-23R expression was measured by Western Blot analysis. As expected, IL-23R expression increased several fold after primary and secondary activation but decreased in the resting phase (figure 3.1 A). Unexpectedly, IL-23R expression increased in a similar manner regardless of what cytokines were added to the culture and expression increased the most in Th2 cultures. This same antibody was also tested for compatibility with FACS analysis and immunocytochemistry, and was found to be ineffective (figure 3.1 B). Admittedly, the tests were not performed optimally because all stains were surface stains, and the antibody is specific for a cytoplasmic domain of the IL-23R. Therefore, at the time, this antibody was primarily used for Western Blot analysis.

3.2 Non-CD4 cell populations express IL-23R

Recent publications to this point had focused on IL-23R mRNA expression in purified CD4+ T cell populations, but when IL-23R was first characterized, it was shown to be expressed in
multiple cell types including macrophage, B cell and dendritic cell populations (Parham et al., 2002). To determine if IL-23R protein expression is upregulated in other cell populations in addition to CD4\(^+\) T cells, purified CD4\(^+\) T cells from C57Bl/6 mice were activated in the presence of IL-6, previously shown by Zhou et al. (Zhou et al., 2007) to upregulate IL-23R expression, and cell lysates were taken at different time points to measure IL-23R expression. A concurrent culture was also done with total splenocytes activated in the presence of IL-12 shown in figure 3.1 A to upregulate IL-23R expression. Surprisingly, purified CD4\(^+\) T cells cultures showed a decrease in IL-23R expression (figure 3.1 C), but total splenocyte cultures showed increased IL-23R expression (figure 3.1 D) with peak changes occurring at approximately two days. Although the difference in IL-23R expression may have been due to the different culture conditions, it suggested that non-CD4\(^+\) cell populations may be expressing IL-23R.

### 3.3 IL-23R expression is enhanced with IL-6, IL-1\(\beta\), and IL-23 and diminished with TGF-\(\beta\)

These findings led to a need for an IL-23R antibody that could be used for FACS analysis allowing the characterization of IL-23R on individual cell populations. A rat monoclonal anti-mouse IL-23R antibody was tested for use in FACS analysis. This antibody is indicated for use in Western Blot analysis and for neutralization of the IL-23R. Since the antibody was a monoclonal antibody indicated for neutralization, it was believed the antibody would likely bind to the protein in its native conformation possibly making it useful for FACS analysis. This antibody as well as the isotype control antibody was professionally conjugated and then tested for use. When compared to isotype control antibody staining, the rat monoclonal anti-mouse IL-23R antibody revealed positive staining (figure 3.2 A). Interestingly, although approximately 7-8 percent of total cells stained positive for IL-23R, very few of the positive staining cells were
CD4+. To determine which cytokines regulated IL-23R expression, B10.PL mice, which express
the Vβ8.2 T cell receptor (TCR) transgene specific for myelin basic protein (MBP) Ac1-11 were
used. These mice have an increased frequency of T cells specific for MBP allowing for antigen-
driven in vitro cell cultures; nevertheless, the MBP-specific T cell frequency is low enough to
remain physiologically relevant (Goverman et al., 1993; Ratts et al., 1999). Splenocytes from
Vβ8.2 TCR transgenic mice were activated with MBP Ac1-11 in the presence of different
combinations of IL-12, IL-6, TGF-β, IL-23 and IL-1β, as each of these cytokines has been shown to
regulate IL-23R expression either directly or indirectly (Gocke et al., 2007; Harrington et al.,
2005; Ivanov et al., 2006; Langrish et al., 2005; Manel et al., 2008; Mangan et al., 2006;
McGeachy et al., 2007; Zhou et al., 2007). Analysis of IL-23R expression revealed that although
IL-23R was commonly associated with Th17 cells and a Th17 response (Langrish et al., 2005; Park
et al., 2005), IL-23R expression correlated more with IFN-γ expression than with that of IL-17
(figure 3.2 B). IL-23R expression was enhanced by IL-6, IL-1β, and IL-23, but TGF-β diminished IL-
23R expression. Thus IL-23R receptor expression was enhanced by inflammatory cytokines and
inhibited by anti-inflammatory cytokines.

3.4 IL-23R expression is enhanced in EAE

In addition to in vitro culture, IL-23 responses were shown in an in vivo adoptive transfer
of myelin-specific T cells suggesting those cells express a functional IL-23R (Langrish et al., 2005;
McGeachy et al., 2007). Little is known about IL-23R expression in vivo in naïve mice as well as
mice with EAE. To address this question, healthy unimmunized mice were analyzed for IL-23R
expression using the rat monoclonal anti-mouse IL-23R antibody. First, splenocytes were taken
from a naïve B10.PL Vα2.3/Vβ8.2 TCR transgenic mouse, the complete transgenic mouse which
contains T cells, nearly all of which are specific for the peptide MBP Ac1-11. Additionally, splenocytes and lymph node cells were taken from naïve C57Bl/6 mice and directly stained for FACS analysis. Some IL-23R expression was detected, but expression was low ranging from 2-4 percent cells staining positive (figure 3.3 A-C). Again much of the positive staining was observed on non-CD4 cells. In particular approximately 80 percent of IL-23R⁺ splenocytes were B220⁺. Only lymph nodes showed an appreciable positive staining on CD4⁺ T cells. The high expression of IL-23R on B cells and low expression CD4⁺ T cells was unexpected as IL-23 has not been reported to affect B cell phenotypes.

IL-23R expression in naïve mice was then compared to the level of expression in mice under non-naïve conditions. C57Bl/6 mice were immunized and lymph node cells were isolated 7 days after immunization. Splenocytes and CNS infiltrating cells were also isolated 21 days after immunization and IL-23R expression was analyzed using the rat monoclonal anti-mouse IL-23R antibody. IL-23R expression was increased in lymph node cells after immunization (figure 3.4 A), whereas expression was only modestly changed in spleen cells (figure 3.4 B) in comparison to naïve mice (figure 3.3 B). Interestingly, very little IL-23R expression was observed in CNS isolates, and virtually none was observed in CD45⁺ infiltrating macrophage and lymphocyte populations (figure 3.4 C). IL-23R expression was also measured in a mouse with spontaneous EAE, and compared to immunized C57Bl/6 mice. Because they have a large number of myelin specific T cells, B10.PL Vα2.3/ββ8.2 TCR transgenic mice will occasionally develop spontaneous EAE without the need of immunization. IL-23R expression was not as high in draining lymph node cells from a mouse with spontaneous EAE (figure 3.5 A), and splenocytes from the mouse with spontaneous EAE showed almost no change in comparison to the healthy mouse (figure 3.5 B).
Again most of the positive staining was observed on B cell populations and only lymph nodes showed an appreciable positive staining on CD4$^+$ T cells.

### 3.5 Western Blot analysis identifies IL-23R protein not identified by FACS surface stains

The predominance of IL-23R expression on B220$^+$ cells and low expression on CD4$^+$ cells was unexpected, so an attempt was made to confirm this finding. To do this, splenocytes from C57Bl/6 mice were polyclonally activated in the presence of IL-12. FACS analysis was performed on cultured cells using the rat monoclonal anti-mouse IL-23R antibody. Cells from the same culture were also separated using magnetic beads into CD4$^+$ and non-CD4 populations, and Western Blot analysis was performed on the individual cell populations using the rabbit polyclonal anti-mouse IL-23R antibody. Interestingly, FACS staining on the cultures revealed very little IL-23R positive staining, and almost all positive staining was on B220$^+$ cells (figure 3.6 A); however, Western Blot analysis revealed easily detectable IL-23R expression, and there was almost no difference between the level of expression on both CD4$^+$ and non-CD4 cells (figure 3.6 B). This data suggests that the rat monoclonal antibody was not identifying all IL-23R that was expressed.

### 3.6 IL-23R is expressed within cytoplasm

This discrepancy between the two antibodies could be explained by a low expression of IL-23R on the plasma membrane. Up to this point, all FACS staining had been surface stains for IL-23R, but Western Blot analysis identifies the total protein whether it is located within the cytoplasm or on the plasma membrane. To determine if there is IL-23R expression within the cytoplasm after cell activation, splenocytes from wild-type B10.PL mice were polyclonally
activated in the presence of IL-12 and then stained for immunocytochemistry using the rabbit polyclonal anti-mouse IL-23R antibody. Intracellular staining revealed positive staining on CD4+ and non-CD4+ T cell populations (figure 3.7 A). Some CD8+ T cells also stained positive for IL-23R (arrows). Again, the rabbit polyclonal antibody was tested for use by FACS analysis, this time with intracellular staining, but there was no difference between staining of this antibody and a rabbit polyclonal isotype control antibody (figure 3.7 B). It is interesting to appreciate that positive intracellular staining reveals more a perinuclear or global cytoplasmic stain for the IL-23R and not staining more reminiscent of the plasma membrane, like that of CD4 and CD8, suggesting that IL-23R may be expressed predominantly within the cytoplasm, and there is limited surface expression.

Cytoplasmic staining for IL-23R was also tested using the rat monoclonal anti-mouse IL-23R antibody. Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were activated with MBP Ac1-11 in the presence of different cytokines and intracellular staining for FACS analysis was performed. Splenocytes stained positive for IL-23R to varying degrees (figure 3.8 A), but contrary to previous surface stains, a significant percentage of CD4+ T cells stained positive for IL-23R (figure 3.8 B). The relative expression of IL-23R expression on CD4+ and non-CD4 populations was similar with intracellular staining which parallels the Western Blot data analyzing the same two populations (figure 3.6 B). Thus, IL-23R is expressed in the cytoplasm of multiple cell populations, and functional receptor may be regulated as to its expression on the plasma membrane. Although this is interesting and useful data, cytoplasmic expression of IL-23R may not have a direct impact on T cell encephlitogenicity, so the focus still remained on how to characterize a functional IL-23R.
3.7 Different IL-23R antibodies reveal different patterns of expression

Previous attempts were made to test a goat polyclonal anti-mouse IL-23R antibody that had previously been used in publications by the lab (Gocke et al., 2007). Attempts to repeat previous experiments to show IL-23R expression using this antibody were unsuccessful due to difficulty isolating sufficient protein to be detected. Fortunately, Morishima et al. (Morishima et al., 2009) identified an in vitro culture technique to detect IL-23R protein using this antibody. To confirm this finding, splenocytes from C57Bl/6 mice were polyclonally activated either without the addition of any cytokines, with IL-12 or with IL-6, IL-23 and neutralizing antibodies to IL-4 and IFN-γ, as shown by Morishima et al. As previously observed, Western Blot analysis using the rabbit polyclonal anti-mouse IL-23R revealed similar upregulation of IL-23R in all three culture conditions (figure 3.9 A); however, use of the goat polyclonal anti-mouse IL-23R antibody revealed IL-23R expression only in the IL-6, IL-23 and neutralizing antibody condition. This experiment was repeated with the same three culture conditions. Again, cell activation in the presence of IL-6, IL-23 and neutralizing antibodies to IL-4 and IFN-γ caused a small but observable increase in IL-23R expression using the goat polyclonal anti-mouse IL-23R antibody (figure 3.9 B); however, intracellular staining of cells from the same culture using the rat monoclonal anti-mouse IL-23R antibody revealed similar positive staining in all three groups (figure 3.9 C). Thus, the rat monoclonal and the rabbit polyclonal antibodies revealed similar IL-23R expression patterns, but the goat polyclonal antibody revealed a unique expression pattern requiring specific culture conditions. Although it was promising that Western Blot analysis using the goat polyclonal anti-mouse IL-23R antibody showed that IL-23R expression may be regulated by specific signals previously shown by other groups, it was still uncertain whether this antibody was identifying a functional IL-23R.
3.8 Functional IL-23R is expressed on lymph node cells from immunized mice

It is very difficult to accurately determine if antibodies always bind a functional protein, but in the case of membrane receptors, an alternative strategy is to stain for downstream signaling intermediates. IL-23R, for example, signals primarily through the phosphorylation of Stat3, so to identify a functional IL-23R, the use of a phosphorylated-Stat3 (phospho-Stat3) stain was used. In brief, the protocol for this stain involves collecting cells and stimulating them, in this case with IL-23. After a short incubation period, before other signaling cascades can be activated, the cells are then fixed and prepared for FACS analysis. Langrish et al. (Langrish et al., 2005) showed that lymph node cells from immunized mice activated in the presence of IL-23 are more encephlitogenic, so functional IL-23R is likely expressed within lymph nodes of immunized mice. To test this hypothesis, lymph node cells were isolated from C57Bl/6 mice 7 days after immunization, and the cells were cultured with or without IL-23 for 15 minutes and then analyzed by FACS analysis for phospho-Stat3. Indeed, phospho-Stat3 was detected in lymph node cells from immunized mice (figure 3.10). Approximately 50 percent of the phospho-Stat3 positive cells were CD4+ and only about 25 percent of the phospho-Stat3 positive cells were B220+ which is in sharp contrast to previous stains for IL-23R.

3.9 Functional IL-23R is upregulated in the presence of IL-6

The phospho-Stat3 FACS stain was further employed to determine what cytokines play a role in the upregulation of a functional IL-23R. Splenocytes were taken from a naïve Vα2.3/Vβ8.2 TCR transgenic mouse and activated with MBP Ac1-11 in the presence of different cytokines implicated in the regulation of IL-23R expression and the development of encephalitogenic T cells, and then the cells were analyzed by FACS analysis. Functional IL-23R
expression was increased by the addition of IL-6 (figure 3.11). The addition of IL-23 did not have a significant effect on IL-23R expression, but this may be due to the absence of functional IL-23R. Neither IL-1β, IL-12 nor IFN-γ had any effect on the expression of functional IL-23R as shown by almost complete absence of positive phospho-Stat3 staining. Again, in contrast to previous cultures, the functional IL-23R response was found almost entirely on CD4+ T cells. This suggests that the rabbit polyclonal and the rat monoclonal anti-mouse IL-23R antibodies used previously do not identify functional IL-23R.

3.10 Surface IL-23R is upregulated by the presence of IL-6, IL-23 and absence of IL-4 and IFN-γ

Recently, the goat polyclonal anti-mouse IL-23R antibody was commercially conjugated for use with FACS analysis now allowing the characterization of IL-23R expression in a cell-specific manner. Expression of IL-23R was known to be affected by IL-6, IL-23 and neutralizing antibodies to IL-4 and IFN-γ, shown by Western Blot analysis using this same antibody; therefore, each of these factors were titrated for their individual effects on surface IL-23R expression. Splenocytes from C57Bl/6 mice were polyclonally activated with different concentrations of IL-6, IL-23 and neutralizing antibodies to IL-4 and IFN-γ, and FACS analysis was used to measure surface IL-23R expression using the goat polyclonal anti-mouse IL-23R antibody. IL-23R expression was primarily found on CD4+ T cells correlating closely with previous phospho-Stat3 stains (figure 3.12 A). Also, as predicted, surface expression of IL-23R on CD4+ T cells was enhanced by each of the titrated cytokines and antibodies (figure 3.12 B). Receptor expression appeared to be most influenced by the presence of IL-6, IL-23 and neutralization of IL-4. Interestingly, only small amounts of IL-6 is needed to enhance IL-23R expression as concentrations as low as 5ng/ml were still effective at upregulating surface expression of IL-23R.
3.11 Goat IL-23R antibody stain correlates with functional IL-23R expression

IL-6 is important for the upregulation of a functional IL-23R (figure 3.11); however, it was still uncertain whether the newly conjugated goat polyclonal anti-mouse IL-23R antibody bound to a functional IL-23R. To test this question, splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were polyclonally activated in the presence or absence of IL-6 or IL-23. As shown previously, surface IL-23R expression was enhanced by the addition of IL-6, and expression was further enhanced by the addition of both IL-6 and IL-23 (figure 3.13 A). As shown in the previous phospho-Stat3 stain (figure 3.11), IL-23 alone did not significantly increase IL-23R expression. Cultures in the presence of IL-6 were also tested for a functional IL-23R response. Nine percent of CD4+ T cells stained positive for phospho-Stat3 in response to IL-23 (figure 3.13 B) whereas 18 percent CD4+CD44+ cells (figure 3.13 A) and approximately 6.5 percent CD4+ cells (data not shown) were IL-23R+ as stained by the IL-23R antibody. Thus surface IL-23R staining with the goat polyclonal anti-mouse IL-23R antibody correlated with functional IL-23R expression as shown by a phospho-Stat3 stain.

3.12 Different IL-23R antibodies bind protein at different molecular weights

Because the rabbit polyclonal and the rat monoclonal anti-mouse IL-23R antibodies did not correlate with a functional IL-23 response, it was necessary to question whether these two antibodies are specific for and actually bind the IL-23R. Western Blots from figure 3.9 A were overlaid. Again, splenocytes from C57Bl/6 mice were polyclonally activated either without the addition of any cytokines or with the addition of either IL-12 or IL-6, IL-23 and neutralizing antibodies to IL-4 and IFN-γ. Western Blot analysis was then performed on cell lysates using the rabbit polyclonal and the goat polyclonal anti-mouse IL-23R antibodies. Again, each antibody
showed a different pattern of binding to IL-23R (figure 3.14 A). Additionally, each antibody identified bands at different molecular weights. The rabbit polyclonal antibody identified a band at approximately 75kDa whereas the goat polyclonal antibody identified a band at approximately 95kDa. This finding was unexpected as the predicted size for IL-23R is approximately 75kDa, yet the goat polyclonal antibody, which identifies a band at 95kDa, correlates with a functional IL-23R. This could suggest that there is more than one isoform of IL-23R. The smaller 75kDa isoform could be a nonfunctional isoform located within the cytoplasm whereas the 95kDa isoform could be a functional IL-23R protein.

The above data led to a number of questions concerning the different IL-23R antibodies. Previous Western Blots using the rabbit polyclonal antibody were done loading small amounts of total protein as the 75kDa band was easy to identify. On the other hand, Western Blots using the goat polyclonal antibody were a challenge because the 95kDa band was difficult to identify. It is possible that each of these antibodies have the capacity to bind IL-23R at both the 75kDa and the 95kDa molecular weights, but that has not previously been detected due to insufficient protein loaded into the gel. To address this question, total or CD4 enriched splenocytes from C57Bl/6 mice were polyclonally activated in the presence of IL-6 and IL-23 with or without the addition of neutralizing antibodies to IL-4 and IFN-γ. Each of these conditions was intended to produce high and low expression of IL-23R. Cell lysates were analyzed by Western Blot analysis this time loading additional protein. Western Blot using the goat polyclonal anti-mouse IL-23R antibody revealed bands at 95kDa as shown before (figure 3.14 B). As shown by FACS analysis, IL-23R was expressed primarily on CD4⁺ cells, and expression was enhanced by the addition of neutralizing antibodies to IL-4 and IFN-γ. Western Blot using the rabbit polyclonal anti-mouse IL-23R antibody did reveal an additional band at 95kDa; however, not only did the band appear
different, but there was not a significant difference between the individual treatment groups as observed on the Western Blot using the goat polyclonal antibody. Therefore, it is likely that the rabbit polyclonal antibody does not bind to the same protein isoform as the goat polyclonal antibody using Western Blot analysis.

3.13 Different IL-23R antibodies bind to the same protein

An additional method to determine if different antibodies bind to the same protein is through the use of immunoprecipitation. To further address this question of antibody specificity, splenocytes from C57Bl/6 mice were enriched for CD4+ cells and polyclonally activated in the presence of IL-6, IL-23 and neutralizing antibodies to IL-4 and IFN-γ. Lysates were then immunoprecipitated for IL-23R using the rabbit polyclonal, goat polyclonal, and rat monoclonal anti-mouse IL-23R antibodies. Eluents from the immunoprecipitations were then analyzed by Western Blot analysis for IL-23R using the non-immunoprecipitating antibody. Surprisingly, when IL-23R was immunoprecipitated with the rabbit polyclonal antibody, it was identified by Western Blot analysis using the goat polyclonal antibody (figure 3.15 A). Conversely, when IL-23R was immunoprecipitated using the goat polyclonal antibody, it was identified by Western Blot analysis using the rabbit polyclonal antibody (figure 3.15 B). Lastly, when IL-23R was immunoprecipitated using the rat monoclonal antibody, it was identified by Western Blot analysis using the rabbit polyclonal antibody (figure 3.15 B), but it was not observed in the Western Blot using the goat polyclonal antibody (figure 3.15 A). Thus, each of the anti-mouse IL-23R antibodies binds to the same protein.
Discussion

Functional IL-23R expression is thus positively regulated by IL-6 and possibly IL-23 and possibly negatively regulated by IL-4 and IFN-γ. Expression of functional IL-23R is primarily within CD4+ cells, but other cell populations appear to express IL-23R. Much of this expression is not on the cell surface, and the receptor is either non-functional or does not signal through the phosphorylation of Stat3. Regulation of functional IL-23R expression may provide insights into common mechanisms involved in EAE pathogenesis. Interestingly, mouse responses to gene deficiencies correlate well with the regulation of IL-23R expression. Mice deficient in IL-23(p19), IL-23R, IL-6 as well as common downstream signaling proteins (i.e. Stat3) are all resistant to EAE (Awasthi et al., 2009; Cua et al., 2003; Liu et al., 2008a; McGeachy et al., 2009; Mendel et al., 1998; Okuda et al., 1998; Samoilova et al., 1998; Thakker et al., 2007). Conversely, mice deficient in IL-4 and IFN-γ are more susceptible to EAE (Falcone et al., 1998; Ferber et al., 1996; Willenborg et al., 1996). Our lab has previously shown that the transcription factor T-bet regulates IL-23R expression, and T-bet-deficient mice are resistant to EAE as well (Bettelli et al., 2004; Gocke et al., 2007; Lovett-Racke et al., 2004). Although this correlation may be coincidental, it is possible signaling through the IL-23R may be a common link between each of these genetic deficiencies.

The accurate binding of individual antibodies is dependent on controls set by the individual manufactures. The use of antibodies in research relies on appropriate testing done by each individual company to confirm that each antibody binds its predicted target. In the case of the IL-23R antibodies, immunoprecipitation confirms that each anti-IL-23R antibody binds to the same protein; however, it is still not certain if the three antibodies are actually binding to IL-23R. To prove this, future experiments will need to be performed to confirm each antibody binds to
IL-23R. Attempts have been made to confirm this using mass spectrometry, but these attempts have not yet been successful due to difficulty in isolating pure protein without contamination. Although this type of analysis may be continued in the future, another method may likely be used due to its comparative effectiveness and increase in simplicity. This method is through using each individual antibody measuring IL-23R expression in cells known to be IL-23R-deficient comparing them to known IL-23R⁺ cells. This method would allow the use of cell lines which are easier to grow and manipulate genetically. A clarification of the specificity of each antibody may be necessary given the different staining patterns observed.

A notable discrepancy in the above data is why immunoprecipitates using the goat polyclonal and rabbit polyclonal antibodies were detected by Western Blot analysis using the other antibody (figure 3.15), yet Western Blot analysis on total cell lysates indicate that neither antibody appears to bind to both potential isoforms of the IL-23R (figure 3.14). A possible explanation for this observation is that immunoprecipitations are performed on total cell lysates that have not yet been subjected to treatments that alter its native structure. Western blots, however, employ the use of SDS and reducing agents such as beta-mercaptoethanol. Whatever protein the antibody binds is reduced and linearized, so much of the specificity for native protein may be lost. Although not the focus of this project, it is interesting to note that immunoprecipitation with the rabbit polyclonal anti-mouse IL-23R antibody appeared to also pull down a protein that appears at approximately 52kDa (figure 3.15 A). This appears to be the same protein that was on the Western Blot from figure 3.14 B. This may indicate that there are actually several different isoforms of IL-23R the function of which are unknown.

The predicted size of IL-23R is approximately 75kDa, and there is no data predicting IL-23R at anything larger. There are several possible mechanisms that could yield a protein beyond
its predicted size including phosphorylation, ubiquitination, and glycosylation. Human IL-23R has been shown to be glycosylated at multiple sites accounting for a change in molecular weight from 15-30kDa (Suen et al., 2010; Zhao et al., 2010). Like with human IL-23R, it is possible that the 95kDa protein is larger because of glycosylation. If this is true, deglycosylation of the protein followed by Western Blot analysis would reveal the loss of the 95kDa band.

Intracellular staining for immunocytochemistry yielded promising results showing that multiple cell types appear to express IL-23R. Closer examination of the staining patterns found in figure 3.7 revealed an unexpected staining pattern for IL-23R. Immunofluorescent staining for proteins that are found on the plasma membrane appear more ring-like as shown by the CD4 and CD8 stains; however, IL-23R staining was found to be diffuse within the cytoplasm and in some instances, the staining appeared to be perinuclear. This staining resembles that of endoplasmic reticulum stains. This might suggest that IL-23R is regulated with respect to surface expression as opposed to total protein expression. When compared with other proteins that signal through Stat signaling pathways, the prominent cytoplasmic staining is not a unique phenomenon. Other cell surface receptors, such as the leptin receptor, growth hormone receptor, IL-6 receptor and prolactin receptor, are also identified within the cytoplasm of cells (Barrenetxe et al., 2002; Buteau et al., 1998; De Matteis and Cinti, 1998; Lara-Ramirez et al., 2008; Lincoln et al., 1998). In fact, Bani et al. (Bani et al., 2001) demonstrated that different antibodies bind to IL-2 receptor subunit gamma at different molecular weights. The antibody that binds both the higher lower molecular weight stains protein localized to the cytoplasm of the cell whereas the antibody that binds only to the higher molecular weight stains protein localized to the plasma membrane. This may therefore be a phenomenon common to several
cell surface receptors or it may be unique to many receptors that signal through Stat signaling pathways.

A potential concern could account for the reduced plasma membrane binding observed in some protein stains. IL-23R is a heterodimer composed of an IL-12Rβ1 and an IL-23R subunit, and it is possible that the association with the two subunits interferes with the ability of IL-23R antibodies to bind to their targeted site. Although this possibility cannot currently be ignored, there is evidence suggesting that this may not be an issue. Canda-Sánchez et al. (Canda-Sanchez et al., 2009) investigated the association of the IL-12Rβ1 with the IL-12Rβ2 subunit using human cells. They found that the IL-12Rβ2 subunit is located within lipid rafts and the IL-12Rβ1 subunit is located outside lipid rafts. With the addition of IL-12, the IL-12Rβ2 subunit leaves the lipid raft to cross-link with the IL-12Rβ1 subunit. Since the IL-23R subunit is closely related to the IL-12Rβ2 subunit in that both subunits associate with the IL-12Rβ1 subunit, it is therefore likely that the IL-23R subunit will show a similar pattern of cellular localization. Nevertheless, cellular localization of the IL-23R is an important question to address.

Signaling through the IL-23R is critical for the development of EAE and possibly MS. Characterization of IL-23R has revealed a complex pattern of expression regulated by IL-6, IL-23, IL-4 and IFN-γ. Further work still needs to be done on how the expression of functional IL-23R is regulated; nevertheless the characterization of the IL-23R given in this project provides a useful foundation to take into the EAE model. A better understanding of IL-23R signaling gives not only an increased understanding of immune mechanisms of T cell differentiation but also the role of this important cytokine in demyelinating disease.
Figures

**Figure 3.1:** IL-23R expression increases after cell activation. All experiments used polyclonal rabbit anti-mouse IL-23R antibody (A) Splenocytes from C57Bl/6 mice were activated using plate bound anti-CD3/CD28 adding recombinant IL-12 (0.5ng/ml), IL-4 (10ng/ml), or IL-6 (25ng/ml) + TGF-β (1ng/ml). After 3 days, cells were rested in culture media for 3 days. Cells were then reactivated with plate bound anti-CD3. Cells were collected at indicated time points and cell lysates were analyzed by Western blot analysis. (B) Splenocytes from B10.PL mouse were activated using plate bound anti-CD3/CD28 adding recombinant IL-12 (0.5ng/ml). After 2 days, cells were stained for FACS analysis using rabbit polyclonal anti-mouse IL-23R antibody or rabbit IgG isotype control antibody. (C) CD4+ T cells from C57Bl/6 mice were activated using plate bound recombinant IL-6 (25ng/ml). (D) Splenocytes from C57Bl/6 mice were activated using plate bound anti-CD3/CD28 adding recombinant IL-12 (0.5ng/ml). (C-D) Cells were collected at indicated time points and cell lysates analyzed by Western blot analysis.
Figure 3.2: IL-23R is expressed primarily on B lymphocytes. Splenocytes from Vβ8.2 TCR transgenic mice were activated with MBP Ac1-11 (2µg/ml) and different combinations of IL-12 (0.5ng/ml), IL-6 (25ng/ml), TGF-β (1ng/ml), IL-23 (10ng/ml) and IL-1β (10ng/ml). After 2 days, cells were stained for FACS analysis using monoclonal rat anti-mouse IL-23R antibody. All gated cells are 7-AAD negative. (A) Cells were activated with MBP Ac1-11, IL-6 and IL-1β. Gated splenocytes were analyzed for IL-23R expression. (B) Represents percent B220⁺ cells express IL-23R (top). IFN-γ and IL-17 production was measured from supernatants by ELISA (bottom) (mean ± SD). Data are representative of 2 independent experiments.
Figure 3.3: IL-23R is minimally expressed in naïve mice. Splenocytes from a healthy Vα2.3/Vβ8.2 TCR transgenic mouse (A) or C57Bl/6 mouse (B) were isolated and stained for FACS analysis. (C) Cells were isolated from brachial, axillary and inguinal lymph nodes from a healthy C57Bl/6 mouse and stained for FACS analysis using monoclonal rat anti-mouse IL-23R antibody. Gated IL-23R⁺ cells were further analyzed for expression of B220, CD11b and CD4 shown in row 3 and 4.
Figure 3.4: IL-23R expression is enhanced in secondary lymphoid organs after immunization. C57Bl/6 mice were immunized with MOG35-55. (A) 7 days after immunization, cells were isolated from brachial, axillary and inguinal lymph nodes and stained for FACS analysis using monoclonal rat anti-mouse IL-23R antibody. (B-C) 21 days after immunization, cells were isolated from spleen (B) and CNS (C) and stained for FACS analysis. (A-B) Gated IL-23R⁺ cells were further analyzed for expression of B220, CD11b and CD4 shown in row 3 and 4. continued
(C) Gated IL-23R cells were further analyzed for expression of CD45 and CD11b shown in row 3. Gated CNS are shown to represent populations of microglia (CD45 CD11b+), macrophage (CD45+CD11b+) and lymphocytes (CD45+CD11b-). Row 4 shows a representative stain of each individual population from total CNS isolates. Lymph node data (A) are representative of 2 independent experiments.
Figure 3.5: IL-23R expression is enhanced in cervical lymph nodes of mouse with spontaneous EAE. (A-B) Cells were isolated from cervical lymph nodes (A) and spleens (B) of a Vα2.3/Vβ8.2 TCR transgenic mouse with spontaneous EAE (clinical score 3) and stained for FACS analysis using monoclonal rat anti-mouse IL-23R antibody. Gated IL-23R⁺ cells were further analyzed for expression of B220, CD11b and CD4 shown in row 3 and 4. Data are representative of 2 independent experiments.
Figure 3.6: Western blot for IL-23R does not correlate with FACS analysis. Splenocytes from C57Bl/6 mice were activated using plate bound anti-CD3/CD28 adding recombinant IL-12 (0.5ng/ml) for 2 days. (A) Cells were collected and stained for FACS analysis using monoclonal rat anti-mouse IL-23R antibody. Gated IL-23R+ cells were further analyzed for expression of B220 and CD4 shown in row 3. (B) Remaining cells were separated using magnetic beads into CD4+ and CD4- fractions and cell lysates from both fractions were analyzed by Western blot analysis using polyclonal rabbit anti-mouse IL-23R antibody.
Figure 3.7: Cell cytoplasm stains positive for IL-23R. Splenocytes from B10.PL mice were activated using plate bound anti-CD3/CD28 adding recombinant IL-12 (0.5ng/ml) for 2 days. (A-B) Cells were collected and stained for immunocytochemistry (A) and for FACS analysis (B) using polyclonal rabbit anti-mouse IL-23R antibody. (A) Immunocytochemistry includes CD4 (row 2) and CD8 (row 3) costaining. Data are representative of 3 independent experiments.
Figure 3.8: FACS analysis reveals positive intracellular staining for IL-23R. Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were activated with MBP Ac1-11 (2µg/ml) and different combinations of IL-12 (0.5ng/ml), IL-6 (25ng/ml), TGF-β (1ng/ml), IL-23 (10ng/ml), IL-1β (10ng/ml) and neutralizing antibodies for IL-12, IL-4, IL-6, IFN-γ and TGF-β. After 2.5 days, cells were stained for FACS analysis using monoclonal rat anti-mouse IL-23R antibody. Data are representative of 5 independent experiments.
Figure 3.9: Different antibodies for IL-23R reveal different expression patterns. Splenocytes from C57Bl/6 mice were activated using plate bound anti-CD3/CD28 adding recombinant IL-12 (0.5ng/ml) or IL-6 (25ng/ml) and IL-23 (10ng/ml) + neutralizing antibodies for IL-4 and IFN-γ for 2 days. (A-B) Cells were collected and analyzed by Western blot analysis using polyclonal rabbit anti-mouse IL-23R antibody (A) or polyclonal goat anti-mouse IL-23R antibody (A-B). Data are representative of 2 independent experiments. (C) Same cells as used in (B) were collected and stained for FACS analysis using monoclonal rat anti-mouse IL-23R antibody. MFI represents total cell mean fluorescence intensity of IL-23R stain – isotype control stain.
Figure 3.10: Functional IL-23R is present on LN cells after immunization. C57Bl/6 mice were immunized with MOG35-55. 7 days after immunization, cells were isolated from brachial, axillary and inguinal lymph nodes and cultured for 15 minutes with and without IL-23 (50ng/ml). Cells were then stained for FACS analysis using monoclonal mouse anti-mouse phospho-Stat3 antibody. Data are representative of 4 independent experiments.
Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse mice were activated with MBP Ac1-11 (2µg/ml) with or without the addition of IL-6 (25ng/ml), IL-23 (10ng/ml), IL-1β (10ng/ml), IL-12 (0.5ng/ml) or IFN-γ (10ng/ml). After 3 days, cells were washed and cultured for 15 minutes with and without IL-23 (50ng/ml). Cells were then stained for FACS analysis using monoclonal mouse anti-mouse phospho-Stat3 antibody. Positive phospho-Stat3 stain for IL-6 and IL-23 cultures was further analyzed for expression of B220 and CD4 (right).

Figure 3.11: Functional IL-23R is upregulated after T cell activation in the presence of IL-6.
Figure 3.12: IL-23R expression is positively regulated by IL-6 and IL-23 and negatively regulated by IL-4 and IFN-γ. (A) Splenocytes from C57Bl/6 mice were activated using plate bound anti-CD3/CD28 adding recombinant IL-6 (25ng/ml) and/or IL-23 (10ng/ml) with or without neutralizing antibodies for IL-4 (2μg/ml) and IFN-γ (2μg/ml) for 2 days. (B) Splenocytes from C57Bl/6 mice were activated using plate bound anti-CD3/CD28 adding different concentration of recombinant IL-6, IL-23, neutralizing antibodies for IL-4 and IFN-γ for 2 days. (A-B) Cells were collected and analyzed by FACS analysis using polyclonal goat anti-mouse IL-23R antibody.
Figure 3.13: Surface IL-23R expression correlates with a functional response. Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were activated using plate bound anti-CD3/CD28 adding recombinant IL-6 (20ng/ml) and/or IL-23 (5ng/ml). (A) After 2 days, CD4⁺CD44⁺ cells were collected and stained for FACS analysis using polyclonal goat anti-IL-23R antibody. (B) Cells activated with plate bound anti-CD3/CD28 adding recombinant IL-6 (25ng/ml) from (A) were washed and cultured for 15 minutes with and without IL-23 (50ng/ml). CD4⁺ cells were then stained for FACS analysis using monoclonal mouse anti-mouse phospho-Stat3 antibody.
Figure 3.14: Different antibodies for IL-23R identify bands of different molecular weights. (A) Splenocytes from C57Bl/6 mice were activated using plate bound anti-CD3/CD28 adding recombinant IL-12 (0.5ng/ml) or IL-6 (25ng/ml) and IL-23 (10ng/ml) + neutralizing antibodies for IL-4 and IFN-γ for 2 days. Cells were collected and cell lysates were analyzed by Western blot analysis using polyclonal rabbit anti-mouse IL-23R antibody (left) or polyclonal goat anti-mouse IL-23R antibody (middle). (Right) Western blots were aligned according to molecular weight and overlaid. (B) Total splenocytes or CD4+ T cells from C57Bl/6 mice were activated using plate bound anti-CD3/CD28 adding recombinant IL-6 (25ng/ml) and IL-23 (10ng/ml) with or without neutralizing antibodies for IL-4 and IFN-γ for 2 days. Cells were collected and cell lysates were analyzed by Western blot analysis using polyclonal rabbit anti-mouse IL-23R antibody (left) or polyclonal goat anti-mouse IL-23R antibody (right).
Figure 3.15: Different IL-23R antibodies bind to the same protein. Splenocytes from C57Bl/6 mice were enriched for CD4+ T cells. (A-B) Cells were then activated using plate bound anti-CD3/CD28 adding recombinant IL-12 (0.5ng/ml) or IL-6 (25ng/ml) and IL-23 (10ng/ml) + neutralizing antibodies for IL-4 and IFN-γ for 2 days. Cell lysates were collected and IL-23R was immunoprecipitated using polyclonal rabbit anti-mouse IL-23R antibody, polyclonal goat anti-mouse IL-23R antibody or monoclonal rat anti-mouse IL-23R antibody. Eluted protein was then analyzed by Western blot analyses using polyclonal goat anti-mouse IL-23R antibody (A) or polyclonal rabbit anti-mouse IL-23R antibody (B).
Chapter 4: IL-23 Receptor Signaling and T Cell Encephalitogenicity

Introduction

Experimental autoimmune encephalomyelitis (EAE) can be induced through multiple methods. Mice can be immunized with a myelin peptide or protein mixed with Complete Freund’s Adjuvant and Pertussis toxin. EAE can also be induced with passive transfer of myelin-reactive cells. Almost 40 years ago, T cells were shown to be critical mediators in both the immunization and passive transfer models of EAE (Gonatas and Howard, 1974; Ortiz-Ortiz et al., 1976). Since that time, researchers found that certain T cell populations were pathogenic and others were nonpathogenic while some T cell populations even suppressed disease (Ando et al., 1989; Bernard, 1977). Initially, EAE was believed to be mediated by Th1 cells, but Cua et al. (Cua et al., 2003) more recently showed EAE may actually be mediated by the IL-12 related cytokine IL-23. Subsequent papers corroborated this finding demonstrating a critical role of IL-23 in the pathogenesis of EAE (Awasthi et al., 2009; Chen et al., 2006; Thakker et al., 2007).

In addition to the inability of IL-23-deficient mice to develop EAE, the addition of IL-23 to myelin-specific T cell cultures has the potential to significantly enhance T cell encephalitogenicity (Langrish et al., 2005). However, the ability of IL-23 affect EAE severity depends on the presence of functional IL-23R. Nevertheless, passive transfer of myelin-specific T cells without the addition of IL-23 is still sufficient to induce EAE. Additionally, activation of myelin-specific T cells in the presence of IL-12 can effectively generate highly encephalitogenic T cells (Yang et al., 2009). It is unknown if IL-23 is required for the passive transfer of EAE in Th-
neutral or Th1 differentiating conditions. It is also unknown if the IL-23-mediated effect on EAE was dependent on CD4⁺ T cells or if other cell populations are involved in the IL-23 response. Thus the goal of the second aim was to determine how IL-23R signaling affects T cell differentiation and effector function.

Results

4.1 IL-23 enhances encephalitogenicity of myelin-specific T cells from an immunized mouse

Langrish et al. (Langrish et al., 2005) had shown that adoptive transfer of myelin-specific T cells reactivated in the presence of IL-23 significantly enhances encephalitogenicity. To begin, this finding was confirmed by isolating lymph node cells and splenocytes from immunized C57Bl/6 mice 18 days after immunization and reactivating the cells in the presence or absence of IL-23. The cells were then transferred into recipient mice, and the recipient mice were monitored for the development of EAE. As expected, the adoptive transfer of myelin-specific T cells reactivated in the presence of IL-23 significantly enhanced EAE severity (figure 4.1 A). Adoptive transfer of reactivated myelin-specific T cells in the absence of IL-23 is normally sufficient to induce EAE in recipient mice, but in this experiment EAE was not induced because a suboptimal number of cells were transferred into recipient mice. If more cells were transferred, any enhancement of encephalitogenicity caused by the addition of IL-23 may not have been as readily observed. Cytokine expression was measured by both FACS analysis and ELISA. There was increased production of IL-17 in IL-23-treated cultures as measured by ELISA, but there was not any pronounced difference in any other of the parameters measured (figure 4.1 B-C). Thus, IL-23 can significantly enhance encephalitogenicity of myelin specific T cells.
4.2 IL-23 does not affect Encephalitogenicity of Th1 cells

Adoptive transfer of naïve myelin-specific T cells activated in Th1-driving conditions can efficiently transfer EAE; however, it is unknown if encephalitogenicity is mediated through IL-23 or if other mechanisms are involved. To address this question, splenocytes from Vα2.3/Vβ8.2 TCR transgenic mice were activated in Th1 conditions either adding IL-23 or a neutralizing anti-IL-23 antibody and then transferred into wild-type recipient mice. Unexpectedly, there was no difference in EAE severity between recipient mice from all three treatment groups (figure 4.2 A). Cytokine expression analysis revealed about a two-fold increase in IL-17 expression in the IL-23 treated group as measured by ELISA (figure 4.2 B). There was also a small decrease in IL-10 production in both the IL-23 treated group and the neutralizing antibody treated group, but this did not correlate with EAE severity. Analysis of other cytokines did not reveal any difference between treatment groups (figure 4.2 B-C). T-bet expression, which has also been shown to be critical for generating encephalitogenic T cells (Yang et al., 2009), was also analyzed, but there was no difference between any of the treatment groups (figure 4.2 D).

IL-23R expression appears to be upregulated with the activation and reactivation of Th1 cells as shown in figure 3.1 A. Although the addition of IL-23 to Th1 differentiating cultures did not affect EAE severity, it is possible adding IL-23 at the memory/effector stage of T cell development might produce different results. To determine if the encephalitogenic potential of reactivated Th1 cells is affected by IL-23, splenocytes from Vα2.3/Vβ8.2 TCR transgenic mice were activated in Th1 conditions and then rested for 3 and 4 days respectively. The cells were then reactivated in the presence or absence of IL-23 and then transferred into wild-type recipient mice. Again, there was no difference in EAE severity between recipient mice from both treatment groups (figure 4.3 A). Cytokine expression analysis revealed about a two-fold increase
in IL-17 expression as measured by ELISA (figure 4.3 B). Analysis of other cytokines did not reveal any difference between treatment groups (figure 4.3 C). Thus, Th1 differentiation of encephalitogenic T cells in both the differentiation and effector phase is independent of IL-23.

**4.3 IL-23 enhances encephalitogenicity of MBP-specific T cells from a mouse with spontaneous EAE**

IL-23R was classically defined as a receptor found on memory T cells and absent on naïve T cells. Adoptive transfer of myelin-specific T cells from immunized mice reactivated in the presence of IL-23 significantly enhances encephalitogenicity and the resulting clinical EAE score (figure 4.1 A). Although IL-23 can affect encephalitogenicity of myelin-specific T cells from immunized mice, it was uncertain if IL-23 affects encephalitogenicity of myelin-specific T cells from mice with spontaneous EAE. Because they have such a large number of myelin specific T cells, B10.PL Vα2.3/Vβ8.2 TCR transgenic mice will occasionally develop spontaneous EAE without the need of immunization. To answer this question, splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse with spontaneous EAE were activated with MBP Ac1-11 either adding IL-23 or a neutralizing anti-IL-23 antibody, and then the cells were transferred into wild-type recipient mice. As expected, recipient mice that received cells activated in the presence of IL-23 developed a more severe disease course (figure 4.4 A). Mice that received cells activated in the presence of neutralizing anti-IL-23 antibodies developed a modestly less severe disease course than untreated cells, but the difference was not significant. As seen in previous experiment, splenocytes activated in the presence of IL-23 produced increased IL-17 as measured by ELISA, but there was not a significant difference in other cytokines produced and T-bet expression.
Therefore, activation of myelin-specific T cells from a mouse with spontaneous EAE in the presence of IL-23 enhances EAE severity.

4.4 IL-23 enhances encephalitogenicity primarily through CD4+ T cells

As previous analysis revealed IL-23R expression on antigen presenting cells in addition to CD4+ T cells, it was necessary to determine if IL-23 was mediating its encephalitogenic effects through antigen presenting cells. The previous adoptive transfer revealed that the addition of IL-23 in cultures from immunized mice significantly enhanced encephalitogenicity (figure 4.1 A). If IL-23 mediated its effects through antigen presenting cells, then antigen presenting cells from immunized mice would be more likely to respond to IL-23 than those from naïve mice. This was evidenced by increased IL-23R expression on spleen and lymph node cells from immunized mice (figure 3.3-3.4). Numerous attempts were made to address this question first testing individual cell populations. These attempts were performed by isolating individual antigen presenting cell populations such as B220+ cells or adherent cells (macrophage and dendritic cells) from both immunized and unimmunized mice. The cells were then divided and pulsed with antigen with or without IL-23 followed by washing and culturing cultured with myelin specific T cells. In each case, the myelin-specific T cells were insufficiently activated to induce EAE.

Finally, a successful protocol was developed. Splenocytes were isolated from immunized and unimmunized wild-type mice, and they were depleted of CD4+ cells. T cells from a Vα2.3/Vβ8.2 TCR transgenic mouse were mixed with the CD4-depleted cells from immunized and unimmunized mice and then activated with MBP Ac1-11 with or without the addition of IL-23. The cells were then transferred into wild-type recipient mice under the hypothesis that if IL-23 mediated its effect through antigen presenting cells, then the IL-23 would enhance EAE.
severity more in the immunized group than in the unimmunized group. Although it appears that the addition of IL-23 enhanced encephalitogenicity when the myelin-specific T cells were cultured with antigen presenting cells from unimmunized mice, the difference in mean clinical score is due to reduced incidence of EAE in the culture without IL-23 (figure 4.5 A). Therefore, it cannot be confidently concluded that IL-23 enhances the encephalitogenicity of myelin-specific T cells cultured in Th-neutral conditions. However, myelin-specific T cells cultured with antigen presenting cells from immunized mice were clearly unaffected by the addition of IL-23. Cytokine analysis revealed an increase in IL-17 production as measured by ELISA in cultures that included IL-23 (figure 4.5 B). Contrary to all previous adoptive transfers, there was a decrease in the percentage of cells that produced IFN-γ in cultures that included IL-23 (figure 4.5 C), but there was no difference in the total amount of IFN-γ produced (figure 4.5 B). Therefore, in the EAE model, IL-23 appears to mediate its effects primarily through CD4⁺ T cell populations. This conclusion will be further confirmed with additional experiments using more mice and the addition of cultures that include CD4⁺ cells from immunized mice as positive controls.

4.5 Signaling through IL-23R significantly enhances T cell encephalitogenicity

Functional IL-23R expression is upregulated on CD4⁺ T cells when they are activated in the presence of IL-6 and IL-23 (figure 3.11-3.13). To determine if an increase IL-23R signaling affects T cell encephalitogenicity, splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were polyclonally activated with plate bound anti-CD3/CD28 in the presence of different combinations of IL-6 and IL-23. Adoptive transfer into recipient mice revealed that contrary to activation with antigen, myelin-specific T cells activated with plate bound anti-CD3/CD28 were unable to transfer EAE; however, when both IL-6 and IL-23 were added to the culture, EAE was
efficiently transferred (figure 4.6 A). There was no change in the amount of IFN-γ produced in the cultures (figure 4.6 B), but there was an increase in the amount of IL-17 produced by the addition of both IL-6 and IL-23 (figure 4.6 C). Thus, signaling through the IL-23R has a potent effect in enhancing encephalitogenicity of myelin-specific T cells.

4.6 IL-23 causes an increase of eye inflammation

There was an unexpected observation found in the adoptive transfer experiments employing IL-23. Recipient mice that received T cells cultured in the presence of IL-23 had an increased tendency to develop inflammation within and around the eye. This inflammation appeared as mild as a small amount of mucus buildup around the eye, and could get severe to the point of physical damage and blindness (figure 4.7 A). The inflammatory eye disease was mostly unilateral and more mild cases would commonly last only 2-5 days. Not all mice developed inflammatory eye disease in all experiments (figure 4.7 B), but this was not unexpected because adoptive transfers do not always cause 100 percent incidence of EAE either. Although it is uncertain if the increase of EAE severity contributes to the incidence of inflammatory eye disease, it is not likely the sole cause as the mean clinical score of mice, at the time they develop the inflammatory eye disease, was not different (figure 4.7 C). The development of the inflammatory eye disease is significant because it is a potential indicator of optic neuritis and/or uveitis. Although, both of these conditions occur with increased frequency in patients with MS, Optic neuritis is one of the most common symptoms experienced by patients. Thus, IL-23 may be a critical cytokine involved in the development of optic neuritis.
4.7 Eye inflammation is a positive indicator of inflammation within the optic nerve

The effect of IL-23 on inflammatory eye disease was further investigated using an adoptive transfer of cells cultured under Th1 conditions with or without the addition of IL-23. Vα2.3/Vβ8.2 TCR transgenic mouse cells were activated with MBP Ac1-11 and IL-12 with or without the addition of IL-23 and then transferred into wild-type recipient mice. As shown previously (figure 4.2 A), there was no difference in the clinical EAE score of mice receiving cells activated in the presence or absence of IL-23 (figure 4.8 A). Optic nerves were isolated from mice at the time they developed any signs of inflammatory eye disease and at the end of the experiment if no inflammatory eye disease developed. The optic nerves were then analyzed for the presence of lesions and inflammatory infiltrates (figure 4.8 B). The lesion area was measured within the optic nerves, and there was no difference observed between the two treatment groups; however, the mice that had developed outward signs of eye inflammation had the largest lesion area within their optic nerves (figure 4.8 C-D). It is important to note that the presence of eye inflammation may be an accurate indicator of general inflammation within the optic nerves. Nevertheless, although the mice that had the largest area of inflammation within their optic nerves showed outward signs of eye inflammation, the eyes that had eye inflammation did not all have large lesion areas on the ipsilateral optic nerves (figure 4.8 C). Thus the presence of inflammatory eye inflammation is a positive indicator of optic neuritis.

Discussion

The adoptive transfer of myelin-specific T cells activated in the presence of IL-23 has the potential to significantly enhance the severity of EAE. This is shown in adoptive transfers of cells
from immunized mice such that when IL-23 is added to the culture, there is a significant enhancement in encephalitogenicity. When IL-23 was added to Th1 cultures however, there was no difference in the resulting EAE. These results occurred at both the differentiation phase and effector phase. There are some possible explanations that can account for these results. First, the addition of IL-12 into cultures to differentiate T cells down a Th1 pathway may inhibit the upregulation of IL-23R thus preventing any IL-23 response. This may not be true though, as IL-23R expression was shown to be regulated by T-bet, an essential Th1 transcription factor (Gocke et al., 2007). One likely explanation is that addition of IL-12 to activating myelin specific T cells can, like IL-23, significantly enhances T cell encephalitogenicity. It is possible that IL-12 may enhance T cell encephalitogenicity using similar downstream mechanism as IL-23, and the addition of IL-12 may maximize the response such that no additional effect is observed with the addition of IL-23. This possibility is supported by the fact that although T cell encephalitogenicity was not enhanced, the addition of IL-23 did cause an increase in the amount of IL-17 produced suggesting there was signaling through the receptor. Another possibility at least at the differentiation phase is that IL-12 and IL-23 receptors are heterodimers that share a common IL-12Rβ1 subunit. The IL-12 found within a Th1 culture may effectively compete for the IL-12Rβ1 subunit found on the cell surface thus inhibiting IL-23R signaling. Each of these possibilities could account for inability of IL-23 to enhance encephalitogenicity of Th1 differentiated cultures.

When IL-23 was added to T cells activated with MBP Ac1-11 alone, there was an enhancement of encephalitogenicity. This occurred in cultures derived from Vα2.3/Vβ8.2 TCR transgenic mice with spontaneous EAE and possibly naïve transgenic mice also. As the mouse with spontaneous EAE would contain more T cells already activated against myelin proteins, one might expect the IL-23-induced enhancement of encephalitogenicity to resemble more the
adoptive transfer from immunized mice where IL-23 had a very pronounced enhancement of encephalitogenicity (figure 4.1 A). Many of these differences in clinical EAE are likely due to differences in IL-23R expression. This is supported by the difference in clinical EAE score shown in adoptive transfer of immunized mice (figure 4.1 A) and the increase incidence and disease severity in the adoptive transfer of Vα2.3/Vβ8.2 TCR transgenic mouse cells activated with plate bound anti-CD3/CD28 (figure 4.6 A). In agreement with the adoptive transfer data, there was much higher expression of functional IL-23R in cultures polyclonally activated in the presence of IL-6 than there was in draining lymph nodes of immunized mice. More work still needs to be done to determine if any difference in the expression of functional IL-23R receptor consistently correlates with IL-23 responses in adoptive transfers.

The development of inflammatory eye disease was an exciting observation because of its similarity optic neuritis and possibly uveitis, conditions commonly seen in MS patients. Optic neuritis is an inflammatory condition of the optic nerve. It is the first reported symptom in 15–20 percent of patients with MS, and about 50 percent of patients with MS will develop at least one episode of optic neuritis during the course of the disease (Balcer, 2006; Shams and Plant, 2009). The clinical manifestations of optic neuritis usually involve only one eye and include vision loss, color blindness, and pain around the eye. Vision recovers to near normal in many patients, but there is a high risk of recurrence, and some patients suffer permanent vision loss. Uveitis is a less common condition that consists of inflammation within the eye itself. Symptoms include tearing, blurred vision, pain, and photophobia. MS patients are about 17 times more likely to develop uveitis than the general population (Le Scanff et al., 2008; Zein et al., 2004). There are no models of optic neuritis currently used in MS research, but the IL-23-induced inflammatory eye disease may serve as an effective model of optic neuritis providing insights about what
causes optic neuritis to develop in MS patients. Possible similarities to uveitis may also be confirmed in the future by analyzing histological sections of the eyes. It is interesting to note that IL-23 may not only affect the encephalitogenicity of myelin-specific T cells, but it may also affect where cells traffic within the CNS, as illustrated by the enhanced inflammation of the optic nerves and eyes.

Although the development of inflammatory eye disease is dependent on the presence of IL-23, there are likely other factors involved in the process. The addition of IL-23 to Th1 differentiating cultures did not have a significant effect on whether or not recipient mice developed inflammatory eye disease. When Vα2.3/Vβ8.2 TCR transgenic mouse splenocytes were mixed with irradiated antigen presenting cells, approximately 20-40 percent of recipient mice would develop inflammatory eye disease with the addition of IL-23. However, in one experiment, when IL-23 was added to cultures containing Vα2.3/Vβ8.2 TCR transgenic mouse splenocytes mixed with non-irradiated antigen presenting cells depleted of CD4+ cells (figure 4.5A), 100 percent of the recipient mice developed inflammatory eye disease whereas none of the mice developed eye disease without the addition of IL-23. In addition to the culture conditions required, the development of inflammatory eye disease may also be dependent on the strain of mouse as eye disease only developed in adoptive transfers using B10.PL mice and was not observed in C57Bl/6 mice. Further work will need to be done to determine what other factors may be contributing to the inflammatory eye disease.

IL-23 is an important cytokine that can significantly affect the results of the EAE model. Activating T cells in the presence of IL-23 has the potential to significantly enhance T cell encephalitogenicity. Nevertheless, the degree to which IL-23 affects T cell encephalitogenicity is, in a large part, dependent on adequate expression of the IL-23R. Activation of T cells in the
presence of IL-23 also has the potential to modify cell trafficking to specific sites within the CNS resulting in increased inflammation of eyes and optic nerves. Thus IL-23R signaling is a critical factor in the development of demyelinating disease.
Figures

Figure 4.1: IL-23 affects encephalitogenicity of T cells from immunized mice. C57Bl/6 mice were immunized with MOG35-55. 18 days after immunization, lymph node cells and splenocytes were reactivated with MOG35-55(2µg/ml) with or without IL-23(10ng/ml) for 3 days. (A) Cells were collected, washed and 20x10^6 total cells were then transferred into C57Bl/6 mice, and mice were monitored for clinical signs of EAE (mean ± SEM) (P<0.0001). (B) Supernatants were collected and cytokines were measured by ELISA (mean ± SD) (for IL-17, P=0.0005). (C) Cells were collected and stained for FACS analysis measuring expression of IFN-γ and IL-17 on CD4^+CD44^{hi} cells. Data are representative of 2 independent experiments.
Figure 4.2: IL-23 does not affect encephalitogenicity of Th1 differentiated cells. Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were activated with MBP Ac1-11 (2µg/ml) and IL-12 (0.5ng/ml) with or without the addition of IL-23 (10ng/ml) or neutralizing anti-IL-23 antibody (5µg/ml) for 3 days. (A) Cells were collected, washed and 4x10^6 blast cells were then transferred into wild-type B10.PL mice, and mice were monitored for clinical signs of EAE (mean ± SEM) (P>0.05). (B) Supernatants were collected and cytokines were measured by ELISA (mean ± SEM) (for IL-10, P<0.01 in all comparisons of all treatment groups) (for IL-17, P<0.0001 in comparison of Th1+IL-23 group to other groups). (C-D) Cells were collected and stained for FACS analysis measuring expression of IFN-γ, IL-17 (C) and T-bet (D) on CD4^+ cells. Data are representative of 2 independent experiments.
Figure 4.3: IL-23 does not affect encephalitogenicity of reactivated Th1 cells. Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were activated with MBP Ac1-11 (2µg/ml) and IL-12 (0.5ng/ml) for 3 days. Cells were then cultured in media alone for 4 days followed by reactivation with MBP Ac1-11 (2µg/ml) with or without the addition of IL-23 (10ng/ml) for 3 days. (A) Cells were collected, washed and 2x10⁶ total cells were then transferred into wild-type B10.PL mice, and mice were monitored for clinical signs of EAE (mean ± SEM) (P>0.05). (B) Supernatants were collected and cytokines were measured by ELISA (mean ± SEM) (for IL-17, P<0.0001). (C) Cells were collected and stained for FACS analysis measuring expression of IFN-γ and IL-17 on CD4⁺ cells.
Figure 4.4: IL-23 affects encephalitogenicity of T cells from mice with spontaneous EAE. Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse with spontaneous EAE (clinical score 3) were activated with MBP Ac1-11 (2µg/ml) with or without the addition of IL-23 (10ng/ml) or neutralizing anti-IL-23 antibody (5μg/ml) for 3 days. (A) Cells were collected, washed and 4x10^6 blasting cells were then transferred into wild-type B10.PL mice, and mice were monitored for clinical signs of EAE (mean ± SEM) (P≤0.0104 in comparison of MBP Ac1-11+IL-23 group to other groups) (P>0.05 in comparison of MBP Ac1-11+αIL-23 group to MBP Ac1-11 group). (B) Supernatants were collected and cytokines were measured by ELISA (mean ± SEM) (for IL-17, P<0.0001 in comparison of Ag+IL-23 group to other groups). (C-D) Cells were collected and stained for FACS analysis measuring expression of IFN-γ, IL-17 (C) and T-bet (D) on CD4^+ cells. For cytokine and T-bet analysis, data are representative of 2 independent experiments.
Figure 4.5: IL-23 affects encephalitogenicity of T cells from healthy mice, and IL-23 response is not enhanced by antigen presenting cells from immunized mice. Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were activated with MBP Ac1-11 (2µg/ml) with or without the addition of IL-23 (10ng/ml) for 3 days. (A) Cells were collected, washed and 10x10^6 total cells were then transferred into wild-type B10.PL mice, and mice were monitored for clinical signs of EAE (mean ± SEM) (P=0.0104 in comparison of MBP Ac1-11+IL-23 group to MBP Ac1-11 in the unimmunized group) (P>0.05 in comparison of MBP Ac1-11+IL-23 group to MBP Ac1-11 in the immunized group). (B) Supernatants were collected and cytokines were measured by ELISA (mean ± SD) (P≤0.0022 in comparison of Ag+IL-23 group to Ag Only in both unimmunized and immunized groups). (C) Cells were collected and stained for FACS analysis measuring expression of IFN-γ, IL-17 on CD4+ cells.
Figure 4.6: IL-6 and IL-23 are able to restore encephalitogenicity to anti-CD3/28 activated T cells. Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were activated using plate bound anti-CD3/CD28 adding recombinant IL-6 (20ng/ml) and/or IL-23 (5ng/ml) for 3 days. (A) Cells were collected, washed and 5x10^6 cells were then transferred into B10.PL IL-4-deficient mice, and mice were monitored for clinical signs of EAE (mean ± SEM) (P≤0.0005 in comparison of αCD3/28+IL-6+IL-23 group to all other groups). (B-C) Supernatants were collected at 3 days and IFN-γ (B) and IL-17 (C) were measured by ELISA (mean ± SD for IFN-γ) (mean ± SEM for IL-17) (P value comparisons are listed in table below).

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Figure 4.7: IL-23 enhances inflammatory eye disease in recipient mice. (A) Image of same mouse showing eye inflammation on right eye while left eye appears unaffected. (B) Number of mice where inflammatory eye disease was observed after adoptive transfer of Vα2.3/Vβ8.2 TCR transgenic cells in the presence or absence of IL-23. (C) Clinical score of mice was measured at the time inflammatory eye disease was first observed. Data are gathered from 3 independent experiments.
Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were activated with MBP Ac1-11 (2µg/ml) and IL-12 (0.5ng/ml) with or without the addition of IL-23 (10ng/ml) for 3 days. Cells were collected, washed and 3x10^6 blasting cells were then transferred into wild-type B10.PL mice. (A) Mice were monitored for clinical signs of EAE (mean ± SEM) (P>0.05). (B) H&E stain of optic nerve containing inflammatory lesions (arrows). (C-D) Mice were monitored for inflammatory eye disease. When eye disease observed, mice were fixed and optic nerves were dissected for histology. (C) Lesion area as well as location and severity of eye inflammation were analyzed (mean ± SEM) (P>0.05). (D) Lesion area on optic nerves was compared with the presence or absence of eye inflammation (mean ± SEM) (P<0.0001).
Introduction

Experimental autoimmune encephalomyelitis (EAE) has a long history as a model of Multiple Sclerosis (MS), and it has expanded our understanding of inflammatory processes that occur in demyelinating disease. EAE originated from observations over one hundred years ago when individuals immunized with Pasteur’s rabies vaccine developed an encephalomyelitis caused by a reaction to the CNS tissue contaminating the inactivated virus. When scientists attempted to replicate the observed encephalomyelitis by injecting animals with homogenized CNS tissue, they had only limited success inducing disease (Baxter, 2007; Mackay and Anderson, 2010). It was not until Jules Freund introduced an adjuvant containing dried, killed Mycobacterium tuberculosis that disease could be induced consistently in animals, thus establishing the EAE model (Freund et al., 1947; Kabat et al., 1947; Morgan, 1947). Today EAE induced by immunization with myelin protein emulsified in Complete Freund’s Adjuvant (CFA) is one of the most studied immune-mediated diseases and has enhanced our understanding of autoimmunity, as well as fundamental immunologic mechanisms.

Partially spurred by the use of M. tuberculosis in the adjuvant, EAE has long been considered a Th1 disease mediated by T cells secreting key cytokines, such as IFN-γ. This paradigm was called into question with observations that IFN-γ-deficient and IFN-γ receptor-deficient mice remained susceptible to EAE (Ferber et al., 1996; Willenborg et al., 1996). Since IL-12 is a critical cytokine for the differentiation of Th1 cells, Cua et al. (Cua et al., 2003)
developed mice deficient for the subunits of IL-12 and a related cytokine, IL-23, and determined if these mice could develop EAE. Mice deficient in the p40 subunit shared by IL-12/IL-23 or the p19 subunit specific for IL-23 were protected from EAE, whereas mice deficient in the IL-12 specific p35 subunit were still susceptible to disease (Cua et al., 2003). Subsequent papers corroborated this finding demonstrating a critical role of IL-23 in the pathogenesis of EAE (Awasthi et al., 2009; Chen et al., 2006; Thakker et al., 2007). The mechanism by which IL-23 mediates EAE pathogenesis remains unclear. One mechanism that has gained the most attention has been the observation that IL-23 promotes the production of inflammatory Th17 cells (Langrish et al., 2005).

Although *M. tuberculosis* infections generate small amounts of Th17 cytokines, control of infection is dependent on a Th1 response (Cooper et al., 1993; Cooper et al., 2002; Khader et al., 2005). Immunizations with CFA also generate high levels of IFN-γ and other Th1 cytokines (Cua et al., 2003; Langrish et al., 2005). IL-23 plays a contributory role, but it is not required to limit *M. tuberculosis* growth (Khader et al., 2005). On the other hand, infections with other organisms are associated with dominant Th17 responses and are dependent on IL-23 for clearance. With the observed dependence of IL-23 in EAE pathogenesis, we hypothesized that the clinical course of EAE could be altered by replacing *M. tuberculosis* in the adjuvant with a Th17-inducing or more particularly an IL-23-dependent bacterium in the adjuvant. *Citrobacter rodentium* is a gram-negative bacterium that causes a distal colitis in mice that is typically cleared within 2-3 weeks. *C. rodentium* is commonly used as a model to study enteropathogenic and enterohemorrhagic *E. coli* infections. Infections in mice produce high levels of IL-17-producing lymphocytes in the lamina propria, and mice deficient in the IL-23-specific p19 subunit develop severe infections resulting in death by day 14 (Mangan et al., 2006; Zheng et al.,
2008), indicating that IL-23 plays a critical role in protective immunity to *C. rodentium*. In the current aim, EAE induction in mice using the standard CFA adjuvant is compared to EAE induced with *C. rodentium* emulsified in Incomplete Freund’s Adjuvant to determine if bacterium that induce different T cell-mediated immune responses during infection generate different phenotypes of EAE.

**Results**

5.1 *Citrobacter rodentium adjuvant (CRA) induces late onset EAE*

*C. rodentium* was heat-inactivated, lyophilized, and mixed with Incomplete Freund’s Adjuvant (IFA) to generate *C. rodentium* adjuvant (CRA). To make the CRA preparation as similar to CFA as possible, heat-inactivated *M. tuberculosis* was also emulsified in IFA at the same concentration. B10.PL mice, which express the Vβ8.2 T cell receptor (TCR) transgene specific for myelin basic protein (MBP) Ac1-11 and thus have an increased frequency of T cells specific for MBP, were used in the initial experiment to determine if CRA had the capacity to induce EAE in a highly susceptible strain of mice (Goverman et al., 1993; Ratts et al., 1999). Vβ8.2 TCR transgenic mice were immunized with MBP Ac1-11 in emulsion with CFA, CRA and IFA, with and without co-administration of pertussis toxin (Ptx). Mice that received CFA developed severe EAE with an early onset, whereas mice that received CRA developed less severe EAE with a later onset (figure 5.1 A). As expected, mice that received IFA did not develop EAE. Ptx caused an earlier onset of disease in CRA-immunized mice, but it did not have any effect with any other adjuvant. To determine if *C. rodentium* could induce EAE in a more physiological-relevant model, C57Bl/6 mice were immunized with MOG35-55 in emulsion with CFA or CRA, and Ptx was also given in
this and all subsequent experiments. Mice that received the CFA developed a typical rapid-onset, chronic disease course followed by only a mild diminution of disease (figure 5.1 B). Mice that received the CRA developed a disease course that resembled that of CFA, but there was more variation in disease onset. Most mice did not get EAE until after day 30, which accounts for the late rise in mean clinical score. In both strains of mice, the clinical signs of EAE were that of classical EAE, and they were similar between the CFA and CRA-immunized mice.

To determine if the differences in disease onset and severity in the CFA and CRA immunizations was due to the dose of bacterium, both *C. rodentium* and *M. tuberculosis* were titrated, and C57Bl/6 mice were immunized with decreasing doses of each ranging from 2 mg/ml to 0.5 mg/ml. The concentration of *M. tuberculosis* and *C. rodentium* that generated the greatest severity and incidence of EAE was 2 mg/ml, and as the concentration of bacterium decreased there was a decrease in incidence and/or severity of disease (figure 5.2 A,C). Again, CRA-immunized mice continued to develop a later onset and less severe disease course than CFA-immunized mice as disease onset was observed as late as 62 days after immunization, whereas the latest all mice in the CFA group developed disease was day 27. *C. rodentium* was also titrated using increasing doses, and the 2 mg/ml dose still generated the highest disease incidence and severity (figure 5.2 B), indicating the bacterium concentration was not responsible for the difference in disease incidence or severity observed relative to CFA.

5.2 CRA-immunization generates cytokine responses similar to CFA

*C. rodentium* infections generate an immune response that is dominated by high IL-17 and low IFN-γ production in lamina propria lymphocytes (Mangan et al., 2006), so we speculated that EAE induced with *C. rodentium* would generate myelin-specific Th17 cells. CD4 T cells from
draining lymph nodes of C57Bl/6 mice immunized with MOG35-55 in emulsion with CFA or CRA were evaluated for IFN-γ and IL-17 production. Unexpectedly, the CRA-immunized mice did not produce the same pattern of cytokine expression as shown in the infection model. The pattern of IFN-γ and IL-17 expression was similar to CFA-immunized mice with a higher number of CD4+ T cells expressing IFN-γ than IL-17 in both CFA and CRA groups (figure 5.3 A), and there was a modest increase in the amount of both cytokines being produced in the CRA-immunized mice compared to the CFA mice (figure 5.3 B). The production of IL-4 and IL-10 was also measured, but there was no difference detected (data not shown). Additionally, proliferation was measured in response to reactivation with antigen. Again, there was no difference between CFA and CRA-immunized mice (figure 5.4). This data suggests that C. rodentium induces a similar immune response as M. tuberculosis when used as an adjuvant for subcutaneous immunization. In addition, neither the number of Th1 and Th17 cells, nor the amount of IFN-γ or IL-17 in the periphery appears to determine the incidence or severity of EAE.

The phenotype of infiltrating immune cells within the CNS provides a more accurate picture of the inflammatory response directly responsible for EAE. CNS mononuclear cells were isolated from CFA and CRA-immunized mice with clinical signs of EAE. Cells were isolated at early (day 16) and late (day 42) time points, corresponding with the time when CFA and CRA-immunized mice reach peak disease activity respectively. At both early and late time points, CFA-immunized mice had increased numbers of IFN-γ and IL-17-producing T cells relative to EAE-affected CRA mice (figure 5.5 A,B). When comparing the number of CNS infiltrating cells that have an activated T cell phenotype (CD45+CD4+CD44hi), the CFA-immunized mice had more activated T cells infiltrating the CNS at day 16. However, at day 42, although there was a decrease in the number of CNS infiltrating cells in both groups, there were more activated T cells
within the CNS of CRA-immunized mice compared to CFA mice (figure 5.5 C), correlating with
the differences in time of disease onset between CFA and CRA-immunized mice.

5.3 Epitope spreading correlates with development of EAE

Epitope spreading is believed to play a role in the development of relapses and/or the
chronic phase of EAE, and possibly with the relapsing-remitting nature of MS. It is used to
determine if additional myelin-specific T cells had been activated during the course of EAE in
immunized mice that may contribute to disease. Although the pattern of epitope spread is well
characterized in some models of EAE, such is not the case for MOG35-55 immunization in
C57Bl/6 mice, now the most commonly used model of EAE. To determine if epitope spreading
occurred in MOG35-55 induced EAE in C57Bl/6 mice, whole bovine myelin basic protein (bMBP),
as opposed to specific peptides, was used as the antigen in lymphocyte proliferation assays.
Splenocytes from CFA-immunized mice showed robust proliferation in response to MOG35-55,
whereas the stimulation index from CRA-immunized mice was reduced by approximately 50%.
Interestingly, the proliferative response to bMBP was equal between both CFA and CRA-
immunized mice thus showing both adjuvants are equally capable of inducing epitope spreading
(figure 5.6).

Since all the mice used in the lymphocyte proliferation assay had EAE regardless of
whether the mice were immunized with CFA or CRA and both developed similar T cell responses
to bMBP, we wanted to determine if epitope spreading also occurred in CRA-immunized mice
that failed to develop EAE. To this end, precursor frequency analysis of MOG35-55 and bMBP-
specific T cells in the spleen was determined in CRA mice that developed EAE and CRA
immunized mice that remained free of disease. There was a correlation between mice that
developed EAE and an increase frequency of bMBP-specific T cells, suggesting that epitope spreading may be a critical factor to develop signs of EAE signs when \textit{C. rodentium} is used as the adjuvant (table 5.1). The same correlation was not observed with the frequency of MOG35-55-specific T cells in the CRA-immunized mice. The frequency of both MOG35-55 and bMBP-specific T cells in CFA-immunized mice was similar to that of CRA-immunized mice that developed EAE, suggesting that epitope spreading may be important in reaching a threshold of pathogenic T cells to generate signs of EAE, and CFA may do this more efficiently than CRA resulting in differences in disease incidence and severity.

\textbf{Discussion}

In light of the many recent studies in both EAE and MS that myelin-specific Th1 and Th17 cells both contribute to disease, but may yield distinct clinical phenotypes (Kroenke et al., 2010; Stromnes et al., 2008), we sought to determine if the bacterium in the adjuvant used in the induction of EAE influenced the phenotype of myelin-specific T cells and the clinical signs of disease. Since it is well established that \textit{M. tuberculosis}, the bacteria in CFA, induces a strong Th1 cell response and IL-12 p35-deficiency results in an impaired capacity to control infection (Cooper et al., 2002; Khader et al., 2005), we performed a comparison study of EAE using a bacteria that in known to induce Th17 cells and is dependent on IL-23 p19 to establish protective immunity (Mangan et al., 2006). This study shows bacterium other than \textit{M. tuberculosis} can serve as an effective adjuvant. The disease course of CRA immunizations is less severe and has a later onset in comparison to CFA immunization. When C57Bl/6 mice were immunized with CRA, 54% of the mice developed EAE within 25 days of immunization; however,
29% of the mice developed EAE at a later time point. The decreased incidence and severity of EAE in C57Bl/6 may make this a valuable model for studying certain aspects of MS. C57Bl/6 mice are readily available, and are the dominant genetic background for gene-deletion and gene-insertion studies; however, the clinical course of EAE in C57Bl/6 mice is not a good representation of that observed in MS patients. MS is most frequently a relapsing-remitting disease with slow progression. In the CRA mice, there was more heterogeneity in the time of disease onset and slower progression of disease, more reminiscent of MS. These features are beneficial for studies that investigate novel therapies, because there is an increased window of time for the drug to mediate its effect before there is severe neurological damage that would be resistant to most therapeutic intervention. This model would also be beneficial for studying molecules that contribute to disease severity. Since C57Bl/6 mice develop rapid-onset, severe EAE, it can be difficult to assess enhanced disease severity in these mice, which could be overcome by using CRA. Thus, C57Bl/6 mice immunized with CRA may serve as an alternative model of EAE in which the onset of disease is often delayed and severity reduced, yet most mice will develop EAE with the same peak clinical score as CFA induced EAE.

*C. rodentium* infections induce a strong IL-17 response within the lamina propria, so we hypothesized that we would see a similar response after immunization; nevertheless, we were surprised to observe a CD4 T cell immune response similar to immunization with CFA (Mangan et al., 2006). Lymph node cells showed similar ratios of IFN-γ and IL-17 expressing CD4 T cells in the CRA and CFA-immunized mice. There was a slight increase in the amount of IFN-γ and IL-17 observed in the CRA mice, but this did not seem to be biologically relevant, since the mice in both groups developed typical EAE characterized by ascending paralysis and not atypical EAE characterized by ataxia with minimal limb weakness often observed in Th17-mediated EAE.
The observation that the peripheral CD4 T cell response to the immunogen was similar in both the CFA and CRA groups suggests that other factors were contributing to the time of disease onset and altered severity in the CRA immunized mice.

Three explanations can account for the difference between the cytokine profile seen in *C. rodentium* infection and the profile seen after CRA immunization. First, the nature of the bacteria may have changed in the preparation. The bacteria were heat-inactivated, and the high temperature may have broken down proteins and ligands critical for activating immune receptors. Immunizations with heat-killed bacteria or protein subunits commonly produced immune responses different from infection (Roberts et al., 1981; Zhan et al., 1993). *M. tuberculosis* is not an exception as infections are not dependent on IL-23 for protection (Khader et al., 2005), but EAE, which uses *M. tuberculosis* as an essential component of the adjuvant is dependent on IL-23 (Cua et al., 2003). The change in cytokine profile may also be due in part to the instability of the IL-17-producing phenotype as described by several groups (Hirota et al., 2011; Lee et al., 2009; Yang et al., 2009). The phenotype of the immune response may be dependent on the location. *C. rodentium* infects the colon of mice, and only in extreme infections are bacteria seen within other tissues (Simmons et al., 2003). Pepper et al. (Pepper et al., 2010) compared antigen-specific IFN-γ and IL-17 production in response to intravenous and intranasal infection with *Listeria monocytogenes*, and they found that intravenous infection resulted in a long-lasting memory population of IFN-γ-producing cells, whereas intranasal infection resulted in a short-lasting memory population of IL-17-producing cells. Therefore, use of *C. rodentium* as a mucosal adjuvant may produce a cytokine response more reminiscent of natural infection with enhanced production of IL-17 and reduced production of IFN-γ. Nonetheless, it appears that subcutaneous immunization of myelin peptides with heat-killed *C.
rodentium induces an immune response dominated by Th1 cells, in contrast to natural infection dominated by Th17 cells, resulting in EAE with classical ascending paralysis.

Toll like receptors (TLRs) play a critical role in driving the immune response necessary to develop EAE (Marta et al., 2008; Prinz et al., 2006). Different TLRs may affect the ratio of inflammatory and anti-inflammatory cytokines in different ways. Although there are limited studies on TLRs and C. rodentium in particular, it has been shown that both M. tuberculosis and C. rodentium stimulate TLR2 and TLR4 on immune cells (Abel et al., 2002; Gibson et al., 2008; Harding and Boom, 2010; Khan et al., 2006). We have previously shown that when C57Bl/6 mice are immunized with MOG35-55 using either a specific TLR2 or TLR4 agonists, instead of M. tuberculosis in the adjuvant, classical EAE is induced (Hansen et al., 2006). To effectively induce EAE, the choice of bacterium in the adjuvant may not matter as long as it is capable of activating essential TLR signaling pathways. Differential expression of ligands for TLRs on individual bacteria and any disparity in bacterial numbers may account for differences in IFN-γ and IL-17 expression in lymph node cells and CNS of CRA-immunized mice.

The effects of adjuvants in the EAE model support the role of infections in the development and progression of MS. Although it is still not certain if infectious agents actually cause MS, evidence suggests that they play an important role in the development of disease (Milo and Kahana, 2010). Infections in patients with MS also cause an increased chance of relapse (Confavreux, 2002). Variation in the signs and symptoms of different MS patients may be influenced by environmental factors, infectious and noninfectious, encountered by individuals. The use of C. rodentium in the adjuvant shows that the use of different bacteria in the adjuvant can change the clinical course of EAE, illustrating that different pathogens may influence susceptibility and severity of autoimmune demyelinating disease.
Figure 5.1: *C. rodentium* adjuvant (CRA) induces a late onset of EAE. (A) B10.PL Vβ8.2 TCR-transgenic mice were immunized with MBP Ac1-11 in emulsion with CFA (2mg/ml), CRA (2mg/ml), or IFA with or without co-injection of pertussis toxin (Ptx). Mice were monitored for EAE development (mean ± SEM) (P values are listed in table below). *Mice were euthanized due to EAE severity. (B) C57Bl/6 mice were immunized with MOG35-55 in emulsion with CFA (2mg/ml) and CRA (2mg/ml) with co-injection of pertussis toxin. Mice were monitored for EAE development (mean ± SEM) (P<0.0001).

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<th>CRA+Ptx</th>
<th>CRA</th>
<th>IFA+Ptx</th>
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Figure 5.2: *C. rodentium* adjuvant (CRA) induces a milder EAE disease course. (A) C57Bl/6 mice were immunized with MOG35-55 given in emulsion with CFA (2mg/ml) or CRA at 2mg/ml, 1mg/ml, and 0.5mg/ml with co-injection of Ptx. Mice were monitored for EAE development (mean ± SEM) (P<0.0001 in comparison of all treatment groups). (B) B10.PL Vβ8.2 TCR-transgenic mice were immunized with MBP Ac1-11 in emulsion with CFA (2mg/ml) or CRA at 2mg/ml and 5mg/ml with co-injection of Ptx. Mice were monitored for EAE development (mean ± SEM) (P=0.4035 in comparison of CFA group to CRA at 2mg/ml) (P≤0.0268 in comparison of CRA at 5mg/ml group to all other groups). *Mice were euthanized due to EAE severity. (C) C57Bl/6 mice were immunized with MOG35-55 given in emulsion with CRA (2mg/ml) or CFA at 2mg/ml, 1mg/ml, and 0.5mg/ml with co-injection of Ptx. Mice were monitored for EAE development (mean ± SEM) (P<0.0001 in comparison of all treatment groups).
Figure 5.3: CFA and CRA produce similar cytokine profiles dominated by IFN-γ. C57Bl/6 mice were immunized with MOG35-55 with CFA (2mg/ml; middle) or CRA (2mg/ml; right) with co-injection of Ptx. Draining lymph node cells were isolated 9 days after immunization and cultured with 2µg/ml MOG35-55 for 3 days. (A) Flow cytometric analysis was used to determine intracellular expression of IFN-γ and IL-17 on CD4⁺CD44⁹ gated cells. (B) Supernatants were collected, and IFN-γ, IL-17, IL-10 and IL-4 were measured by ELISA (mean ± SEM) (P>0.05 in all treatment groups that were biologically relevant). Data are representative of 3 independent experiments.
Figure 5.4: CFA and CRA immunization generates similar proliferative responses in draining lymph nodes. B10.PL Vβ8.2 TCR-transgenic mice were immunized with MBP Ac1-11 in emulsion with CFA (2mg/ml) or CRA (2mg/ml) with co-injection of Ptx. Draining lymph node cells were isolated 9 days after immunization and cultured with 2µg/ml MBP Ac1-11 (5, 2, 0.4, 0.08µg/ml). Proliferation was measured using a $[^{3}H]$-thymidine incorporation assay and the stimulation index (SI) was determined (mean ± SEM) (P>0.05 in comparison of CFA and CRA groups). Data are representative of 3 independent experiments.
Figure 5.5: Infiltration of activated T cells correlates with late incidence of EAE. C57Bl/6 mice were immunized with MOG35-55 with CFA (2mg/ml; middle) or CRA (2mg/ml; right) with co-injection of Ptx. CNS mononuclear cells were isolated from mice showing signs of EAE and were cultured overnight. Flow cytometric analysis was used to determine intracellular expression of IFN-γ and IL-17 on CD45+CD4+CD44hi gated cells. (A) On day 16, CNS cells were isolated and cultured with and without 2µg/ml MOG35-55. Mean clinical score of mice was CFA=3 and CRA=2. (B) On day 42, CNS cells were isolated and cultured with 2µg/ml MOG35-55. Mean clinical score of mice was CFA=2.5 and CRA=3.5. (C) Represents number of gated CD45+ cells from A and B that were CD4+CD44hi (mean ± SD).
Figure 5.6: Epitope spreading occurs in both CFA and CRA-immunized mice that develop EAE. C57Bl/6 mice were immunized with MOG35-55 with CFA (2mg/ml; middle) or CRA (2mg/ml; right) with co-injection of Ptx. Splenocytes were isolated from mice showing clinical signs of EAE 67 days after immunization and cells were cultured with MOG35-55 (10, 2, 0.4µg/ml) or bovine MBP (100, 20, 4µg/ml). Proliferation was measured using a [³H]-thymidine incorporation assay and the stimulation index (SI) was determined (mean ± SEM) (P<0.0001 in comparison of MOG35-55 stimulation of CFA and CRA groups) (P>0.05 in comparison of bMBP stimulation of CFA and CRA groups).
Table 5.1: Epitope spreading in CRA-immunized mice correlates with EAE development. C57Bl/6 mice were immunized with MOG35-55 given in emulsion with CFA (2mg/ml) or CRA (2mg/ml) with co-injection of Ptx. Mice were monitored for EAE development. Splenocytes were isolated from mice 50 days after immunization and cells were cultured with or without MOG35-55 (2µg/ml) or bovine MBP (20µg/ml). Proliferation was measured using a [³H]-thymidine incorporation assay, and precursor frequency was calculated as stated in the methods.

Table 1. Estimated frequency of autoreactive T cells

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Chapter 6: Discussion

A primary goal of this project has been to characterize IL-23R expression under the hypothesis that IL-23R signaling is critical for the development of pathogenic myelin-reactive T cells. This IL-23R characterization was then applied to both the immunization and adoptive transfer models of EAE to determine the effect of IL-23R signaling on T cell encephalitogenicity. We found that IL-6 plays a critical role in the expression of functional IL-23R, and IL-23R is also regulated by IL-23, IL-4 and IFN-γ. Each of these findings is supported by data from gene-deficient mice (Awasthi et al., 2009; Cua et al., 2003; Falcone et al., 1998; Ferber et al., 1996; Liu et al., 2008a; McGeachy et al., 2009; Mendel et al., 1998; Okuda et al., 1998; Samoilova et al., 1998; Thakker et al., 2007; Willenborg et al., 1996). IL-23R may also be regulated as to its expression on the plasma membrane the mechanism through which is unknown. Additionally, signaling through the IL-23R significantly enhances T cell encephalitogenicity, and it causes an inflammatory eye disease that resembles the optic neuritis commonly seen in MS patients. Lastly, immunizing mice with *Citrobacter rodentium* adjuvant produces late onset EAE in C57Bl/6 mice that resembles MS progression better than CFA immunizations.

IL-23 is known to play a significant role in contributing to inflammation and EAE pathogenesis, but the mechanism by which IL-23 affects inflammation is still unknown. IL-23 was initially characterized to increase proliferation of memory T cells (Oppmann et al., 2000), and IL-23 is known to increase IL-17 production on CD4+ T cells (Langrish et al., 2005). For some time, it was believed that the role of IL-23 was to stabilize the Th17 phenotype (Korn et al., 2009). This was during a time when IL-17 was believed to be a critical factor for
encephalitogenic T cells. IL-23 was frequently inefficient at generating IL-17-producing T cells especially in comparison to the combination of IL-6 and TGF-β. This was largely because it was unknown how to efficiently upregulate IL-23R expression. Nevertheless, it was known that IL-23 itself was a potent enhancer of encephalitogenicity. The dogma of IL-23 affecting encephalitogenicity through the production of IL-17 changed when IL-17 was later shown to be dispensable in EAE, and the IL-17-producing phenotype was shown to be transient and unstable (Haak et al., 2009; Hirota et al., 2011; Lee et al., 2009). Recently, there have been some newly proposed functions of IL-23 and how it contributes to T cell encephalitogenicity.

Recently Ghoreschi et al. (Ghoreschi et al., 2010) showed by mRNA analysis, that IL-23R is upregulated by IL-6 and IL-1β. They subsequently compared activated T cells cultured in the presence of IL-6, IL-1β and IL-23 to cells cultured in the presence of IL-6, IL-1β and TGF-β. Both treatment groups generated similar frequencies of IL-17⁺ T cells, yet the IL-23 treatment group generated highly encephalitogenic T cells and the TGF-β treatment group did not. Microarray analysis was performed on each treatment group comparing the relative expression of different genes. The IL-23 treatment group had an increase in expression of a number of cytokines and chemokines, but only one of the targets is known to be critical in EAE, and that is the transcription factor T-bet. Our lab and others have found that T-bet is a critical transcription factor for generating encephalitogenic T cells (Bettelli et al., 2004; Yang et al., 2009). IL-23 could be affecting the T cell encephalitogenic potential at least to some degree through T-bet. It is also important to note that T-bet contributes to the transcription of IL-23R suggesting a positive feedback loop (Gocke et al., 2007). We are currently working on finding potential links between IL-23 signaling and T-bet expression to answer this question.
Two other groups recently proposed another mechanism by which IL-23 contributes to T cell encephalitogenicity, and that is through the secretion of the T cell cytokine granulocyte macrophage colony-stimulating factor (GM-CSF). Both groups state that IL-23 enhances secretion of GM-CSF and that GM-CSF, secreted by encephalitogenic T cells, is essential to induce EAE. El-Behi et al. (El-Behi et al., 2011) used *in vitro* cultures activating cells under nonencephalitogenic Th17 conditions (IL-6 and TGF-β), resting the cells and then reactivating the cells in the presence of either IL-6 and TGF-β or IL-23. Indeed they found that IL-23 enhanced expression of GM-CSF; however, they also found that if the cells were activated under encephalitogenic Th17 conditions (IL-6, IL-1β and IL-23), rested and reactivated, the addition of IL-23 did not enhance GM-CSF expression. They concluded the cells differentiated this way have a unique phenotype that requires further characterization. These findings as well as findings from Ghoreshi et al. (Ghoreschi et al., 2010) suggest that there are different methods to develop IL-17 producing T cells, and each of these methods results in a different phenotype. The relevance of each method thus relies in which one is more physiologically relevant. Given that T cells differentiated in the presence of IL-6 and TGF-β typically require significant manipulation involving multiple activation steps and the presence of multiple specific cytokines at each step in order to become encephalitogenic, their relevance is questionable in their application to MS. However IL-6, IL-1β and IL-23 contribute to T cell encephalitogenicity without the need of significant manipulation (Yang et al., 2009); therefore this method of activation may likely be more physiologically relevant.

Codarri et al. (Codarri et al., 2011) also claimed to link IL-23 to the expression of GM-CSF, but their data was insufficient to draw an accurate conclusion. First, they measured GM-CSF mRNA expression in cultures treated with increasing concentrations of IL-23. Although they did
see a modest increase in GM-CSF mRNA expression, their concentrations of IL-23 ranged from 10ng/ml to 500ng/ml. Considering that common measurements of IL-23 production from in vitro cultures never exceed 1ng/ml, this titration is likely inaccurate due to the addition of excessively high concentrations of IL-23. An appropriate titration would probably utilize at least 10-100-fold lower concentrations of IL-23. This data is also suspect because neither FACS analysis nor ELISA was performed despite the availability of effective commercial reagents. This group also did an adoptive transfer of cells from immunized GM-CSF-deficient and wild-type mice activated in the presence of IL-23, and the GM-CSF-deficient cells were unable to transfer disease. However, GM-CSF could have affected the original T cell differentiation or the ability of T cells to express IL-23R, and this group did not take this into consideration. Lastly this group generated and immunized bone marrow chimeras using IL-12Rβ1 and IL-12Rβ2-deficient cells suggesting that a deficiency in the IL-12Rβ1 would be linked to both IL-12 and IL-23 whereas a deficiency in the IL-12Rβ2 would be linked strictly to IL-12. Any difference in the two responses would represent an IL-23 response. Despite observing only a modest increase in the percentage of cells that express GM-CSF, they ignore the fact that the IL-12Rβ1 subunit is also linked to the IL-12(p40) homodimer, and the IL-12Rβ2 subunit may also be linked to other cytokines such as IL-35 (Wang et al., 1999). The IL-12(p40) homodimer in particular is known to be critical in the response to M. tuberculosis potentially making it a very relevant protein in any situation where CFA is used (Khader et al., 2006). Although GM-CSF is a critical cytokine for the induction of EAE, more work will need to be done to confirm any link between GM-CSF and IL-23.

From the time this study started, IL-23R has been linked to several additional human diseases through genetic analysis including Crohn’s disease, psoriasis and ankylosing spondylitis thus confirming the importance of IL-23R signaling in human disease (2007; Burton et al., 2007;
Cargill et al., 2007; Duerr et al., 2006; Liu et al., 2008b; Nair et al., 2008; Rahman et al., 2008; Rueda et al., 2008). The genetic association between IL-23R and MS has also been reported, but the link may not be as strong as shown in the previous mentioned diseases (Illes et al., 2008; Roos et al., 2008). However, Nunez et al. suggested a stronger link may be associated with the more aggressive primary progressive MS (Nunez et al., 2008). Genetic links have also been made to products of IL-23R signaling such as Stat3 and Tyk2 further supporting a role of IL-23R signaling in MS pathogenesis (2009; Jakkula et al., 2010; Parham et al., 2002). In addition to genetic analysis, samples from MS patients have also been analyzed identifying increases in IL-23 and IL-17 in blood, cerebral spinal fluid and within lesions of MS patients (Krakauer et al., 2008; Lock et al., 2002; Matusevicius et al., 1999; Vaknin-Dembinsky et al., 2006). Thus IL-23 and IL-23R signaling are critical factors in several human diseases, and more work needs to be done to determine what role IL-23 and IL-23R signaling play in MS pathogenesis.

A fully human neutralizing antibody to p40 (ustekinumab) has been developed neutralizing both IL-12 and IL-23. This therapy has been effective in the treatment of severe psoriasis and inflammatory bowel disease (Leonardi et al., 2008; Papp et al., 2008; Sandborn et al., 2008). Ustekinumab was also tested in phase II clinical trials for the treatment of relapsing-remitting MS, and unfortunately no significant benefit was observed in any of the four dosing regiments tested (Segal et al., 2008). This outcome does not necessarily indicate that IL-23 is not involved in the pathogenesis of MS however. Longbrake et al. (Longbrake and Racke, 2009) proposed several mechanisms as to why ustekinumab therapy was not successful in the treatment of MS. First, the clinical trial enrolled patients with both early stage disease and long established MS. Thakker et al. (Thakker et al., 2007) showed that IL-23 may be critical at the T cell differentiation stages and not at the effector phase of EAE. They did this with adoptive...
transfers of myelin-specific T cells from either wild-type or IL-23(p19)-deficient immunized mice into both wild-type and IL-23(p19)-deficient mice. They found that T cells from wild-type immunized mice could transfer EAE equally into either wild-type or IL-23(p19)-deficient mice, but adoptive transfer of myelin-specific T cells from IL-23(p19)-deficient mice could not efficiently transfer EAE. Another proposed reason for the ineffective clinical trial was the possible inability of the neutralizing antibodies to cross the blood-brain barrier. This was evidenced by the fact that only 50% of patients showed evidence of blood-brain barrier permeability as shown by gadolinium-enhancing lesions. Lastly, there exists the possibility that although this therapy may not be effective in relapsing-remitting MS, it may prove effective in primary-progressive MS. Therefore, a greater understanding of IL-23 and IL-23R signaling may still provide successful therapeutic targets in MS.

IL-23 and its receptor are essential proteins necessary for the development of EAE, and both of these molecules likely play a critical role in the development of MS as evidenced by data from this project. IL-23R signaling can significantly enhance T cell encephalitogenicity, and IL-23R signaling can promote the development of optic neuritis, one of the most common symptoms of MS patients. These findings suggest there is a link between IL-23R signaling and MS pathogenesis, and further study of IL-23 and IL-23R signaling can lead to effective treatments for this disease.
References


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