Title
Toll-like receptor 7 tolerance in anti-neuroinflammation in murine experimental autoimmune encephalomyelitis

Permalink
https://escholarship.org/uc/item/1mz5t4wr

Author
Vuong, Linda

Publication Date
2010

Peer reviewed|Thesis/dissertation
Toll-like Receptor 7 Tolerance in Anti-Neuroinflammation in Murine Experimental Autoimmune Encephalomyelitis

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Linda Vuong

Committee in charge:
Professor Randall S. Johnson, Chair
Professor Dennis A. Carson, Co-Chair
Professor Steven A. Wasserman
Professor Elina I. Zuñiga

2010
The Thesis of LindaVuong is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

__________________________________________

__________________________________________

__________________________________________

Co-Chair

Chair

University of California, San Diego

2010
DEDICATION

I would like to recognize my mother Dominique Vuong and our family for their endless encouragement and dedication. I would also like to recognize my dear friends John, Travis, Raymond and Jennifer for their immense support throughout the research and writing processes.
EPIGRAPH

Il faut d’abord durer.

Ernest Hemingway
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Epigraph</td>
<td>v</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>xi</td>
</tr>
<tr>
<td>Abstract</td>
<td>xii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>16</td>
</tr>
<tr>
<td>Results</td>
<td>23</td>
</tr>
<tr>
<td>Discussion</td>
<td>27</td>
</tr>
<tr>
<td>Figures</td>
<td>33</td>
</tr>
<tr>
<td>Tables</td>
<td>45</td>
</tr>
<tr>
<td>References</td>
<td>50</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

1V136 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine
Ac acetylated N-terminus
ANOVA analysis of variance
BBB blood-brain barrier
CII collagen II
CCL cysteine-cysteine motif ligand
CFA complete Freund’s adjuvant
CNS central nervous system
CpG unmethylated cytosine-guanine
CXCL cysteine-X amino acid-cysteine motif ligand
DC dendritic cell
DMEM Dulbecco’s modified Eagle’s medium
DNA deoxyribonucleic acid
EAE experimental autoimmune encephalomyelitis
ELISA enzyme-linked immunosorbent assay
FACS fluorescence-activated cell sorting
FBS fetal bovine serum
Foxp3 forkhead box P3
H&E hemotoxylin and eosin
HBSS Hank’s balanced salt solution
IFN interferon
Ig immunoglobulin
IL interleukin
KC keratinocyte-derived cytokine
LFB Luxol fast blue
LPS lipopolysaccharide
MBP myelin basic protein
MCP monocyte chemotactic protein
MIP macrophage inflammatory protein
MOG myelin oligodendrocyte glycoprotein
MS multiple sclerosis
MyD88 myeloid differentiation primary-response protein 88
NF-κB nuclear factor κ-light chain-enhancer of activated B cells
OD optical density
P/S penicillin:streptomycin
PAMP pathogen-associated molecular pattern
PBL peripheral blood leukocyte
PLP proteolipid protein
PTX Pertussis toxin
pre-tx pre-treatment with 1V136
PRR pattern recognition receptor
qPCR quantitative polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>restim</td>
<td>restimulated with 1V136</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of measurement</td>
</tr>
<tr>
<td>SC</td>
<td>spinal cord</td>
</tr>
<tr>
<td>T reg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor homology</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>veh</td>
<td>vehicle</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1: Toll-like receptor signaling................................................................. 33
Figure 2: Structure of 1V136................................................................. 34
Figure 3: Protocol for the establishment and treatment of EAE............... 35
Figure 4: 1V136 treatment decreases disease severity in EAE mice....... 36
Figure 5: 1V136 treatment significantly reduces EAE clinical score...... 37
Figure 6: 1V136 treatment significantly reduces cell infiltration to the SC but not the brain................................................................. 38
Figure 7: Histology of the SCs of 1V136-treated EAE mice show decreased cell trafficking and demyelination......................................................... 39
Figure 8: The decrease in SC cells in 1V136-treated EAE mice corresponds to a decrease in cell trafficking................................................................. 40
Figure 9: qPCR of EAE SCs shows a decrease in cytokine and chemokine expression in 1V136-treated mice................................................................. 41
Figure 10: Microglia pre-treated with 1V136 become hyporesponsive in vitro................................................................. 42
Figure 11: 1V136 treatment reduces antigen-specific cytokine production in splenocytes................................................................. 43
Figure 12: IgG1 and IgG2a production is unaffected by 1V136 treatment.... 44
LIST OF TABLES

Table 1: Types of MS grouped by disease progression…………………………… 45
Table 2: Murine EAE models........................................................................ 46
Table 3: TLR ligands and expression.............................................................. 47
Table 4: Clinical scoring of EAE................................................................. 48
Table 5: Primer pairs used for qPCR............................................................ 49
AKNOWLEDGMENTS

I would like to acknowledge Dr. Randall S. Johnson and Dr. Dennis A. Carson for serving as the chairs of my committee and Dr. Steven A. Wasserman and Dr. Elina I. Zuñiga for serving on my committee.

I also thank Dr. Carson for the opportunity to work in his laboratory. I would also like to acknowledge the members of the Carson Lab I have worked with, past and present, for their collaboration, hard work and support. I especially thank Dr. Tomoko Hayashi for her patience, dedication and mentorship.

A special thank you is given to Dr. Maripat Corr for helping me to focus on this project and start the writing process.
ABSTRACT OF THE THESIS

Toll-like Receptor 7 Tolerance in Anti-Neuroinflammation in Murine Experimental Autoimmune Encephalomyelitis

by

Linda Vuong

Master of Science in Biology

University of California, San Diego, 2010

Professor Randall S. Johnson, Chair
Professor Dennis A. Carson, Co-Chair

Multiple sclerosis (MS) is an autoimmune disease that results in demyelination and neurodegeneration of the central nervous system (CNS). This disease has a chronic progressive or a relapsing course that is partially recapitulated in murine models such as experimental autoimmune encephalomyelitis (EAE). Toll-like receptors (TLRs) are a family of pattern-recognition receptors (PRRs) that mediates
the innate and adaptive immune responses. TLR tolerance is a phenomenon in which repeated stimulation of a TLR will lead to hyporesponsiveness. To test the potential for TLR7 hyporesponsiveness to limit CNS inflammation, SJL/J mice immunized with proteolipid protein (PLP)\textsubscript{139-151} were treated with the synthetic TLR7 agonist 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (SM360320, designated here as 1V136). Daily low-dose 1V136 treatment significantly decreased disease severity. Concordantly, the number of spinal cord (SC)-infiltrating immune cells was significantly reduced in 1V136-treated mice. A microglia-enriched cell population tested for response to TLR agonists confirmed that 1V136 treatment induces hyporeponsiveness to subsequent TLR7 stimulation. Splenocytes from 1V136-treated mice exhibited a specific decrease in interleukin (IL)-17 and interferon (IFN)-γ secretion. Serum samples from 1V136-treated mice showed no difference in the humoral immune response. In summary, chronic treatment with 1V136 can induce innate immune system hyporesponsiveness and inhibit a normal adaptive immune response. The direct effects of 1V136 on the CNS may contribute to a reduction in the clinical severity of a murine model of MS.
INTRODUCTION

Multiple sclerosis

MS is a progressive autoimmune disease in which CD4+ T cells attack the myelin sheaths of the CNS. There are about 1.5 million people affected by MS worldwide (Bhat and Steinman, 2009), with about 350,000 Americans currently diagnosed with MS. Although MS may develop in children (Duquette, et al., 1987) and the elderly (Martinelli, et al., 2004), the peak age of onset is about 27 to 30 years old (Kurtzke, et al., 1992). It is the leading cause of paralysis in young adults (Shoenfeld and Rose, 2004). Women are affected by MS two to three times more often than men (Wallin, et al., 2004).

MS is grouped into four main types based on changes in the disease symptoms over time: primary-progressive, progressive-relapsing, relapsing-remitting and secondary-progressive (Table 1). Progressive forms of MS steadily worsen while patients with relapsing forms show at least partial recovery before symptoms recur.

The hallmarks of the disease are white matter scleroses, or lesions, resulting from demyelination and neuroinflammation caused by CD4+ helper T (Th) cells that are autoreactive to CNS antigens. Devic’s disease is another autoimmune disease with similar features to MS. Patients’ optic nerves, SCs and sometimes their brains are attacked by autoantibodies specific for aquaporin 4, a protein found in the cell membranes of astrocytes (Wingerchuk, 2006). Although inflammatory demyelination of the SC is common to both MS and Devic’s disease, the lesions in Devic’s disease are more commonly found near the vasculature (Lucchinetti, et al. 2002).
**Symptomology**

MS symptoms include numbness, weakness, loss of muscle coordination and problems with vision, speech and bladder control. Lhermitte’s symptom, in which neck movements cause tingling sensations, numbness or lightening-like shooting pains, and Uhthoff phenomenon, a temporary worsening of symptoms when body temperature rises, are also characteristic of MS. All of these symptoms, however, are not disease-specific, and MS patients can suffer from almost any neurological disturbance (Compston and Coles, 2008).

**Etiology**

There is great debate over what initially triggers MS. Some argue that a microbe causes MS, possibly Epstein-Barr virus or a microbial brain infection that spreads to neighboring neural structures (Steinman and Oldstone, 1997). Others believe that a degenerative, biochemical disturbance is the cause. This is true of X-linked adrenoleukodystrophy, in which a mutation in an adenosine triphosphate-binding cassette transporter causes a primary biochemical disturbance in the brain. MS lesions do show increased transcription of fumarylacetoacetate hydrolase, an enzyme involved in tyrosine catabolism (Lock, et al., 2002). Another theory is that the striking similarity between microbial components and myelin sheath and neuronal components elicits an autoimmune response. Classic examples of this mimicry can be seen between Hepatitis B and myelin oligodendrocyte glycoprotein (MOG) (Fujinami and Oldstone, 1985) and Hepatitis A viral polymerase and myelin basic protein (MBP) (Wucherpfennig and Strominger, 1995).
Oligodendrocytes coat axons with sheaths of myelin that allow salutatory axonal conduction. At the onset of MS, demyelination is followed by periods of remyelination in 20% of patients (Patrikios, et al. 2006). However, partially myelinated axons still exhibit impaired function, in part because the speed at which impulses are conducted is reduced. As MS progresses, there is oligodendrocyte apoptosis in established lesions that inhibits remyelination (Mason, et al. 2004). Demyelinated axons are susceptible to cross-talk with one another as well as to uncontrolled action potential discharge. This type of axonal damage may account for many MS symptoms.

Immunopathology

Demyelination and inflammation are the hallmarks of MS. It is hypothesized that autoreactive T cells are responsible for both of these symptoms. T cells that are specific for CNS antigens including MBP, MOG and PLP attack the myelin sheaths of the CNS, leading to demyelination. These attacks trigger inflammatory processes such as the stimulation of other immune cells through pro-inflammatory cytokines and chemokines (Compston and Coles, 2002).

An additional role for T cells in MS has recently been demonstrated. It has been shown that CD4$^+$ T cells that secrete IL-17 or both IL-17 and IFN-γ infiltrate the brain before any EAE symptoms develop. This infiltration coincides with the activation of CD11b$^+$ microglia and CNS production of the pro-inflammatory cytokines IL-1β, tumor necrosis factor (TNF)-α and IL-6 (Murphy, et al., 2010).
B cells play a pivotal role in current transgenic mouse models of MS. Transgenic mice expressing a T cell receptor (TCR) specific for MOG do not develop EAE if their B cell compartments are not intact and no MOG antigens are present. The group that created the TCR transgenic mice theorize that the transgenic T cells expand B cells specific to MOG antigens and observed that the antibodies secreted by these B cells enhanced demyelination (Pöllinger, et al., 2009).

Antibodies specific for all major myelin proteins and many myelin lipids have been found in the cerebrospinal fluid of MS patients (Bhat and Steinman, 2009). However, the prevalence of antibodies specific for myelin surface antigens decreases with age at MS onset. 38.7% of patients who were less than ten years old at the time of disease onset expressed antibodies specific to MOG antigens while these antibodies were found in only 14.7% of patients whose disease onset occurred between ten to 18 years of age (McLaughlin, et al., 2009).

Although MS is classically thought of as T cell mediated, the innate immune response has recently been implicated in the disease. Patients with secondary-progressive MS have been observed to have an increased percentage of dendritic cells (DCs) that produce IL-12 and TNF-α. These DCs seem to promote the differentiation of naïve T cells to mature T cells that produce IFN-γ, a pro-inflammatory cytokine (Karni, et al., 2006). MS patients also show increased peripheral blood mononuclear cells with caspase-1 and IL-18 expression (Huang, et al., 2004).

Treatment
There are currently four major types of drugs used to treat MS. IFN-β such as Avonex, Rebif and Betaseron inhibits leukocyte proliferation and antigen presentation and acts as an anti-inflammatory agent (Murdoch and Lyseng-Williamson, 2005). Natalizumab is a synthetic monoclonal antibody that inhibits cell trafficking across the blood-brain barrier (BBB) (Polman, et al., 2006). Mitoxantrone suppresses B cell, T cell and macrophage activity through its anti-neoplastic function (Martinelli, et al., 2009). Finally, Copaxone is a synthetic polymer made up of a random combination of alanine, glutamic acid, lysine and tyrosine (Racke, et al., 2010). It works by inducing specific regulatory T cells that downregulate inflammation in the CNS and by inhibiting autoreactive T cells specific for myelin antigens (Simpson, et al., 2003). Though these drugs work to alleviate symptoms and slow disease progression, there is currently still no known cure for MS.

**Experimental autoimmune encephalomyelitis**

The three approaches to establishing a murine MS model are active immunization, adoptive transfer and transgenic mice. In active immunization models, mice can be immunized with short sequences of peptides contained in MBP, MOG or PLP (Table 2). MBP and PLP are both proteins that are part of the myelin sheath surrounding axons in the CNS with PLP being the predominant protein present. MOG, on the other hand, is believed to provide maintenance to the myelin sheets (Roth, et al., 1995). Adoptive transfer involves transferring memory T cells in the lymph nodes of an actively immunized EAE mouse to a naïve mouse. This model in particular is effective for studying the effector phase of the disease because there is no
priming phase in the naïve mouse. More recently, mice with transgenic T cell receptors specific for MOG$_{92-106}$ (Pöllinger, et al., 2009) and a mouse knock-in with an immunoglobulin (Ig) H chain specific for MOG (Litzenburger, et al., 1998) have been created.

The murine MS model used in the following studies was established through direct immunization using PLP$_{139-151}$. Immunization of mice with PLP results in a chronic relapsing EAE model in SJL/J mice (Tuohy, et al., 1988 and Brown, et al. 1981). Because the mice are immunized with myelin peptides that they then mount an autoimmune response against, EAE closely models the CNS inflammation and demyelination of human MS.

**Innate and adaptive immunity**

The higher vertebrate immune system can be divided into two response types: the innate and adaptive immunities (Pancer and Cooper, 2006). These responses act together to combat pathogens with the innate immunity providing a quick, non-specific response and the adaptive immunity providing a later, pathogen-specific response.

**Innate immunity**

Innate immunity is the first active response upon pathogen invasion. The cells that make up the innate immune response, which includes mast cells, macrophages, neutrophils and DCs (Janeway, et al., 2001), have a limited number of germ-line encoded PRRs (Janeway, 1989). PRRs include TLRs, mannose receptors and nucleotide-binding oligomerization domain-containing protein-like receptors (Jo,
PRRs do not recognize pathogen-specific antigens but instead recognize pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). PAMPs are conserved ligands common to microbes such as bacteria, viruses and fungi. These ligands include flagellin, lipopolysaccharide (LPS), single-stranded DNA and double-stranded RNA (Turvey and Broide, 2009). Upon activation, the innate immune response releases cytokines that can cause inflammation and enhance the expression of costimulatory molecules that determine the direction of the adaptive immune response.

**Adaptive immunity**

The adaptive immune response becomes active later in the course of infection and initiates immune responses that target the specific invading pathogen. This level of specificity is achieved through the random generation of a multitude of B and T cell receptors through gene rearrangement and somatic hyper-mutation (Janeway, et al., 2005). During infection, the cell expressing the receptor specific for the particular pathogen is identified and expanded, giving rise to an army of pathogen-specific T or B cells. These cells go on to identify the pathogen through the use of their specific receptors and attempt to destroy the invader.

**Toll-like receptors**

TLRs are named for the *toll* gene found in *Drosophila* that encodes a protein that protects against fungi and bacterial infections (Hoffmann, 2003). Vertebrates can have up to 12 different TLRs with humans having ten different functional TLRs (Roach, et al., 2005). TLRs are membrane-bound glycoproteins that have a Toll/interleukin-1 receptor homology (TIR) signaling domain and an antigen
recognition domain made up of leucine-rich repeats. They are located on diverse cell types including immune cells and epithelial cells. TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface while TLR3, 7, 8 and 9 are expressed on intracellular vesicle surfaces (Kanzler, et al., 2007).

Some differences in TLR expression exist between humans and mice. While in humans TLR2 is typically most expressed in peripheral blood leukocytes (PBLs), murine TLR2 is low to undetectable in blood cells. TLR2 is also only found on murine T cells. Human TLR3 transcripts are exclusively found in myeloid DCs but are expressed in murine macrophages. TLR4 expression is strongest in splenocytes and PBLs in humans and found in weaker levels in all other tissues except liver. Mice, on the other hand, express TLR4 strongly in lung, heart, spleen, muscle, liver and kidney tissue (Rehli, 2002). Murine TLR7 is expressed on both myeloid, or conventional, DCs as well as plasmacytoid DCs (Asselin-Paturel, et al., 2005). While human TLR7 is not expressed in myeloid DCs, it is widely expressed in plasmacytoid DCs (Ito, et al., 2002 and Schreibelt, et al., 2010). Finally, although it has long been believed that TLR8 is only functionally expressed in humans, recent studies have demonstrated TLR8 activation in the brains (Ma, et al., 2006 and 2007 and Mishra, et al., 2006), spleens (Mao, et al., 2009) and some dendritic cells (Martinez, et al., 2010) of mice.

TLRs are able to recognize bacterial, fungal and protozoan lipopeptides and proteins and viral nucleic acids. Synthetic ligands can also bind to and activate TLRs (Table 3). Upon antigen binding, TLRs recruit adaptor proteins such as myeloid
differentiation primary response protein 88 (MyD88) and TIR domain-containing adaptor protein inducing IFN-β. With the exception of TLR3 and some TLR4 signaling, all TLRs signal through MyD88. Adaptor binding leads to the downstream activation of mitogen-activated protein kinases and nuclear translocation of nuclear factor κB (NF-κB), all of which are inflammatory response pathway regulators (Akira and Takeda, 2004) (Figure 1), or the activation of IFN regulatory factors and the production of type I IFN (Stetson and Medzhitov, 2006). IFNs have anti-viral capabilities that inhibit cell protein synthesis and can lead to apoptosis of virally infected cells (Le, et al., 2004). Pro-inflammatory cytokines and chemokines, on the other hand, can recruit immune cells to the site of infection. When the recruited innate immune cells phagocytose a pathogen, its antigens can then be presented to CD4+ T cells. Signaling through TLRs enhances the maturation process of antigen presenting cells. This allows TLRs to act as a link between the innate and adaptive immune responses.

**Toll-like receptors and neuroinflammation**

The CNS is considered to be an immune privileged site in the body, protected from exaggerated inflammatory responses to prevent the permanent loss of neural cells. However, glial and neural cells have recently been identified as having a role in immune surveillance as well. In the CNS, TLRs are involved in many pathological conditions and contribute to host defense and maintenance of homeostasis in response to inflammation caused by infectious disease, neurodegenerative disease and neural injury (Lavelle, et al., 2010).
Microglia are the resident mononuclear phagocytes of the CNS that mediate neuroinflammatory responses. TLR1 through 9 are expressed on microglia (Bsbisi et al., 2002). Upon activation, microglia release pro-inflammatory cytokines and chemokines and regulate the innate and adaptive immune responses in the CNS. Murine microglia stimulated with LPS, peptidoglycan, polyinosinic:polycytidylic acid (poly I:C) and CpG DNA show increased cytokine and chemokine secretion including IFN-α, IFN-β, IL-1β, IL-6, IL-10, IL-12, IL-18, monocyte chemotactic protein (MCP)-1a, macrophage inflammatory protein (MIP)-1α and TNF-α (Olson and Miller, 2004). Astrocytes, oligodendrocytes and neurons have recently been discovered to express a wide range of TLRs and to contribute to neuroinflammation, regeneration and development (Jack, et al., 2005 and Mishra, et al., 2006).

**Viral infection**

Viral infection can induce neuroinflammation. In herpes simplex virus infection, TLR2 has been shown to mediate microglial expression of the following pro-inflammatory cytokines and chemokines: cysteine-cysteine motif ligand (CCL) 7-9, cysteine-X-cysteine motif ligand (CXCL) 1, 2, 4 and 5, IL-1β, IL-6, IL-12 and TNF-α (Aravalli, et al., 2005). TLR7 contributes to neuroinflammation caused by single-stranded RNA viruses and was found to be necessary for the production of pro-inflammatory cytokines and chemokines and for the activation of astrocytes early in disease (Lewis, et al., 2008).

**Neurodegenerative disease**
Several neurodegenerative diseases show differential expression of TLRs. Transcription of TLR genes is upregulated in Alzheimer’s disease patients (Cashman, et al., 2008). There is also increased expression of TLR4 in the substantia nigra of mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine to create a model of Parkinson’s disease (Panaro, et al., 2008). Studies have suggested that TLR activation of the innate immune response may inhibit the disease progression of scrapie, a prion disease. Activation of TLR9 through CpG DNA has been shown to delay scrapie infection by prolonging its incubation period. Additionally, C3H/HeJ mice with a TLR4 intracellular domain mutation have significantly shorter scrapie incubation periods than wild-type C3H/HeOuJ mice (Spinner, et al., 2008).

Multiple sclerosis

MS lesions in the CNS not only contain autoreactive T cells but activated innate immune cells as well. These cells include macrophages, dendritic cells and microglia (Prat and Antel, 2005). Human MS lesions also show upregulation of TLR expression (Bsibsi, et al., 2002). Additionally, there is increased TLR expression in the SCs harvested from EAE mice. During the initial stages of EAE when leukocytes infiltrate the CNS, there is an increase in TLR1, 2, 4 and 6-9 as well as MyD88, the adaptor protein for most TLRs, in the SC. At later stages of EAE, TLR7 and 9 mRNA expression increase (Prinz, et al., 2006).

It has been confirmed by two separate groups that MyD88 is necessary for EAE induction (Prinz, et al., 2006 and Marta, et al., 2008). MyD88 null mice were found to be resistant to EAE and had no neuroinflammation (Prinz, et al., 2006).
TLR2 has been linked to neuroinflammation in the secondary-progressive phase of EAE (Farez, et al., 2009). However, TLR2 deficient mice are still susceptible to EAE, suggesting that it is not required for disease progression (Prinz, et al., 2006). One recent report has indicated that the administration of the TLR7 agonist imiquimod reduces EAE severity through an increase in the production of IFN-β (O’Brien, et al., 2010).

The role of TLR4 in EAE is not as clear. TLR4 deficient mice exhibit exacerbated EAE (Marta, et al., 2008), but mice with B cells activated by TLR4 reportedly have reduced T cell activation during EAE (Lampropoulou, et al., 2008). Similarly, results have also been mixed regarding the role of TLR9 in EAE. TLR9 null C57BL/6 mice immunized with MOG$_{35-55}$ were shown to be resistant to EAE (Prinz, et al., 2006). However, the absence of TLR9 showed heightened disease when the same strain was immunized using MOG$_{1-125}$ (Marta, et al., 2008).

**Toll-like receptor tolerance**

Tolerance describes a condition wherein the immune system does not mount an attack against a given antigen. An example of tolerance is central tolerance. In healthy individuals, one’s immune system does not attack oneself because central tolerance occurs when maturing B and T cells are exposed to self-antigens. The cells that have high affinity for the self-antigens are deleted. Acquired tolerance to an external antigen is also possible. It is characterized by immune cell hyporesponsiveness when the cells are exposed to an antigen that would normally
elicit a response. This type of tolerance may be induced by repeated administration of an antigen.

TLR tolerance was first described for TLR4. After immune cells are chronically exposed to bacterial endotoxin, a TLR4 agonist, they can become hyporesponsive to subsequent endotoxin stimulation. Animals survived a normally lethal dose of endotoxin if they had been previously exposed to a sublethal injection of it (Greisman and Hornick, 1975). One of the earliest human examples of this was found in patients recovering from typhoid fever or malaria. Subsequent challenges with endotoxin resulted in a reduction of TNF-α expression leading to lower fever (Cavaillon and Adib-Conquy, 2006). Similarly, LPS tolerance occurs when cells are hyporesponsive to a second challenge with LPS. LPS tolerance is also established when the initial challenge is performed using lipopeptides or lipoteichoic acid (Sato, et al., 2002).

Monocytes have been identified as the cells being tolerized (Cavaillon and Adib-Conquy, 2006). Functionally, monocytes tolerized by endotoxin show hyporesponsiveness to subsequent stimuli, have increased phagocytic ability and have impaired antigen presentation ability (del Fresno, et al., 2009). Together, these characteristics can have a protective effect against septic shock, allow for increased pathogen clearance and alter the development of an adaptive immune response.

The mechanism of endotoxin tolerance has been widely studied. When cells become tolerized, the TLR4 signaling pathway is inhibited through reduced receptor expression, degradation of IL-1 receptor-associated kinase (IRAK) and decreased
IRAK-MyD88 association (Piao, et al., 2009). Induction of anti-inflammatory molecules including IRAK-M, suppressor of cytokine signaling 1, Src homology 2-containing inositol phosphatase-1 and activator protein-1 has also been reported (van’t Veer, et al., 2007 and Sly, et al., 2004).

**Toll-like receptor 7 agonists**

Since the 1980s, research groups have observed that synthetic guanine derivatives and analogs can activate innate immunity. Thiazolo[4,5-\(d\)]pyrimidine (Nagahara, et al., 1990), pyrazolo[3,4-\(d\)]pyrimidine (Bontems, et al., 1990), purine (Michael, et al., 1993), 7-deazapurine (Smee, et al., 1991) and 9-deazapurine rings (Girgis, et al., 1990) were all found to be active in eliciting an innate immune response involving the rapid induction of IFN production (Smee, et al., 1990, Smee, et al., 1991 and Smee, et al., 1991). After the discovery of TLRs, this immune activation was shown to be through TLR7 (Lee, et al., 2003).

Later research focused on adenine derivatives as potential IFN inducers. By probing the efficacy of different functional groups at the 2-position of the adenine ring, several highly potent, highly bioavailable novel IFN inducers were synthesized. For example, 1V136 (Figure 2) was found to be at least ten times more effective at activating TLR7 to produce cytokines in mouse splenocytes than R848, a human TLR7 and 8 agonist. Activation of TLR7 at such a high level was shown to inhibit the replication of hepatitis C virus in hepatocytes (Lee, et al., 2006). Because IFN-\(\beta\) is currently used as an MS treatment, 1V136’s ability to be a potent IFN inducer may allow it to inhibit MS disease progression as well.
TLR7 has recently been implicated in autoimmune responses, especially those involved in lupus (Pisitkun, et al., 2006, Subramanian, et al., 2006 and Christensen, et al., 2006). This makes TLR7 a good candidate for further induced hyporesponsiveness studies in other autoimmune diseases such as MS. One such agonist is 1V136. 1V136 initiates a TLR7-specific immune response (Chan, et al., 2009 and Kurimoto, et al., 2003), and TLR7 tolerance, or hyporesponsiveness, induced by chronic administration of 1V136 has been shown to prevent EAE symptoms (Hayashi, et al., 2009).
MATERIALS AND METHODS

Mice

Six- to eight-week-old female SJL/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and six- to eight-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). They were fed PicoLab Rodent Diet 20 and water ad libitum and maintained in the University of California, San Diego Animal Facility under standard conditions including a 12 hour light and 12 hour dark cycle. All mice were studied in accordance with the NIH Guidelines for the Care and Use of Laboratory animals, and the Institutional Animal Care and Use Committees of the University of California, San Diego approved all protocols used.

Induction of EAE

The SJL/J mice were immunized on day 0 with 200 µg PLP139-151 (Genemed Synthesis, Inc., San Antonio, TX) and complete Freund’s adjuvant (CFA) containing 400 µg Mycobacterium tuberculosis H37Ra (Chondrex, Inc., Redmond, WA) per mouse. 100 µL of the PLP-CFA emulsion were injected subcutaneously on either side of the hind flank so that each mouse was injected with a total of 200 µL. The mice were then inoculated with 325 ng Bordetella pertussis toxin (PTX) (List Biological Laboratories, Inc., Campbell, CA) per mouse intraperitoneally immediately following the immunization and again on day 2. These mice are referred to here as EAE mice. Non-immunized mice were kept as a naïve control.

Induction of TLR7 hyporesponsiveness
On days five through 18, the EAE mice were treated subcutaneously daily with 150 nmol 1V136 in 1.5% dimethyl sulfoxide in saline, synthesized as previously described in Chan, et al., 2009. Control EAE mice were injected subcutaneously with 100 µL vehicle during the same period. Naïve mice were either injected with 100 µL vehicle or 150 nmol 1V136 according to the same timeline (Figure 3).

**Clinical score**

From day 5 onward, EAE mice were evaluated for signs of disease. A scale from 0 to 5 was used that corresponds to increasing disease severity (Table 4) (Davis, 1999).

**Isolation of cells**

On day 19 of short-term experiments or day 64 of long-term experiments, blood was collected and the mice were sacrificed. The organs were transcardially perfused using normal saline. The serum was removed from the blood and stored at -20°C.

The spleens were harvested and manually disaggregated using forceps. A single cell suspension of splenocytes was prepared using 100 µm cell strainers. A portion of the splenocytes was used for FACS analysis. The remaining cells were seeded at a concentration of 5 x 10^6 cells/mL and cultured with PLP_{159-151}, PLP_{178-191} (Genemed Synthesis Inc., San Antonio, TX) or collagen II_{263-272} (CII) (Genemed Synthesis Inc.) in RPMI-1640 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 100 units/mL penicillin and 100 µg/mL streptomycin.
The days 2 and 3 supernatants were collected, and cytokine expression was determined by enzyme-linked immunosorbent assay (ELISA).

The brains and spinal cords (SCs) were also harvested and pooled by group. SCs and brains were minced using a razorblade and then digested in HBSS (Irvine Scientific, Santa Ana, CA) containing 5 µg/mL collagenase I (Worthington Biochemical Corporation, Lakewood, NJ) and 20 µg/mL DNase I (Worthington Biochemical Corporation) for one hour at 37°C. Mononuclear cells were isolated from the resulting homogenates using a 30-70% Percoll Plus gradient (GE Healthcare, Waukesha, WI). Total cell number was determined by Guava ViaCount assay (Millipore, Billerica, MA) or Trypan Blue exclusion assay (Gibco). These cells were used for FACS analysis and quantitative PCR (qPCR).

**Microglial culture**

A mixed glial culture was prepared from the brains of neonatal C57BL/6 mice using a modified protocol from Current Protocols in Cell Biology (Viviani, 2006). On day 0 the brains were harvested and minced using a razor blade. The brains were then digested for one hour at 37°C in HBSS containing 5 µg/mL collagenase I and 20 µg/mL DNase I. The resulting homogenate was strained through a 100 µm cell strainer and seeded onto T-75 flasks (BD Biosciences, San Jose, CA) coated with 0.1 mg/mL poly-L-lysine (Sigma, St. Louis, MO). The cells were fed with DMEM supplemented with 10% FBS and 1% P/S. On the following day, all non-adherent cells were removed. On day 10 the flasks were shaken at 37°C and 250 rotations per minute. After one hour of shaking, the floating microglia-enriched cells were
collected. In the preliminary FACS evaluation, more than 90% of these cells were CD11b⁺ (data not shown).

**Induction of TLR7 hyporesponsiveness in microglia**

Once the microglia were collected, they were seeded at 5 x 10⁶ cells/mL on 96-well flat bottom plates. The microglia were allowed to adhere to the bottom of the wells overnight. On the next day, the cells were pre-treated with 10 µM 1V136 or not and left in 100 µL vehicle. On the following day, the microglia were restimulated with 10 µM 1V136 or not and left in 100 µL vehicle. The supernatant was collected after pre-treatment and restimulation.

**FACS analysis**

Portions of the CNS cells and splenocytes collected were FACS stained with the following fluorescence-conjugated antibodies: B220-FITC, CD3-PE-Cy7, CD8-PE, CD25-PE (BD Biosciences), CD11b-APC and Gr1-PE (eBioscience, San Diego, CA). The stained cells were analyzed by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest Pro software (BD Biosciences, San Jose, CA) and FlowJo analysis software (Tree Star, Inc., Ashland, OR).

**Cytokine ELISA**

Sandwich ELISAs were performed on the sera collected as well as the supernatants from the splenocytes cultured with various peptides. Antibodies for IL-1β (R&D Systems kit, Minneapolis, MN), IL-6 (BD Biopharmingen, San Jose, CA) or IL-12 antibodies (BD Biopharmingen) were used to coat half-area 96 well plates. The samples were diluted 1:2. Biotinylated rat anti-mouse antibodies (BD Biopharmingen
and R&D Systems), horseradish peroxidase-conjugated streptavidin (BD Biopharmingen and R&D Systems) and tetramethylbenzemidine (KPL, Gaithersburg, MD) were used for detection. The absorbance was measured at 450 nm – 650 nm using a microtiter plate reader.

**Antigen-specific immunoglobulin ELISA**

MOG\textsubscript{35-55}, PLP\textsubscript{139-151} or PLP\textsubscript{178-191} were used for coating at a concentration of 10 µg/mL. Sera were diluted 1 : 100. Anti-mouse IgG1 and IgG2a alkaline phosphatase antibodies (SouthernBiotech, Birmingham, AL) and Sigma FAST p-nitrophenyl phosphate tablets (Sigma) were used for detection. The absorbance was measured at 405 nm – 650 nm using a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA).

**Bead-based Luminex assay**

Multiplex Bead-based Luminex assays were performed on the supernatants from the microglia-enriched cell population. The beads were covalently bonded with antibodies against the following cytokines and chemokines: IL-1β, IL-6, IL-12, IL-17, IFN-γ, IFN-inducible protein (IP)-10, keratinocyte-derived cytokine (KC), MCP-1, MIP-1α and TNF-α (Invitrogen, Carlsbad, CA). The plates were read using a Luminex IS 100 plate reader (Luminex Corporation, Austin, TX). The data were analyzed using Upstate BeadView v. 1.0.4.15303 software (Upstate Biotech, Temecula, CA).

**Quantitative PCR**
A portion of the mechanically disaggregated SCs was immediately flash frozen in liquid nitrogen and stored at -80°C for use in qPCR. The SCs were treated with 1 µL TURBO DNase (Ambion, Inc., Austin, TX) per 10 µg RNA. After DNase treatment, total RNA was isolated using RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA). Quantity and purity were determined using a NanoDrop spectrophotometer. Three µL RNA from each sample were used to synthesize cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Reactions amplifying the DNA sequences for IL-1β, IP-10, KC, MCP-1 and MIP-1α were prepared using 5 ng cDNA for each reaction. The 18S ribosomal RNA gene was also amplified as an endogenous control. Real-time PCR using TaqMan Universal PCR Master Mix and TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA) and an iCycler IQ (Bio-Rad) was employed to assay all genes of interest. The annealing temperature of the primers was 60°C. The primers and probes used for each gene are listed in Table 5.

**Histology**

Some SCs were preserved in the vertebral column and used for histological staining. The SCs in the vertebral columns were fixed in formalin (Fisher Scientific, Pittsburgh, PA) for two days. The vertebral columns were then decalcified using Cal-Ex II (Fisher Scientific) for three days. After decalcification, the vertebral columns were placed in 70% ethanol. Removal of the SC from the vertebral column, paraffin embedding, sectioning, hemotoxylin and eosin (H&E) staining and Luxol fast blue (LFB) myelin staining were performed by the University of California, San Diego Histology Core.
Statistical analysis

The data are presented as mean ± SEM. Significance was assigned for p < 0.05 by Student’s t-test or one- or two-way ANOVA as appropriate with Bonferroni’s post hoc test (GraphPad Prism, GraphPad Software, Inc., La Jolla, CA).

These studies were performed in the laboratory of Dr. Dennis A. Carson and were supported by funding from Telormedix, Inc. (Bioggio, Switzerland).
RESULTS

Low dose administration of 1V136 decreases disease severity in EAE

Experimental mice were divided into four groups: vehicle-treated naïve, 1V136-treated naïve, vehicle-treated EAE and 1V136-treated EAE. Mice receiving treatment were subcutaneously injected with 100 µL saline (vehicle) or 1V136, as appropriate, daily from day 5 through day 18. During this period, the mice were also scored for clinical signs of disease each day using the criteria in Table 4.

Most vehicle-treated EAE mice began to exhibit clinical signs of disease such as flaccid tail and severe hind limb paralysis on days ten through 12. 1V136-treated EAE mice only developed the preliminary signs of disease such as clumsy gait and flaccid tail. The 1V136-treated EAE mice generally remained unparalyzed. Vehicle-treated naïve mice and 1V136-treated naïve mice showed no signs of disease (Figure 4).

When the difference in EAE severity is quantified, the 1V136-treated EAE mice consistently had lower clinical scores than the vehicle-treated EAE mice. The decrease in disease severity was significant ($p < 0.0001$) on days 12 through 16 as compared to vehicle-treated EAE mice. In long-term studies, though the difference was non-significant, the clinical scores of the 1V136-treated EAE mice did remain lower than those of the vehicle-treated EAE mice during relapses in disease (Figure 5).

1V136-treated mice show decreased spinal cord cell infiltration
Increased EAE clinical score severity is known to correlate with abnormal immune cell infiltration to CNS tissue (Elhofy, et al., 2002). On day 19 the CNS from each mouse was harvested to evaluate this cell infiltration. Histological observation through H&E staining showed decreased cell infiltration and LFB staining showed decreased demyelination in the SCs of 1V136-treated EAE mice (Figure 6 A-C) as compared to vehicle-treated EAE mice (Figure 6 D-F).

In order to quantify the CNS cell infiltration, mononuclear cells were isolated from the brains and SCs. The infiltrating cell count was significantly reduced (p < 0.05) in the 1V136-treated EAE mice as compared to the vehicle-treated EAE mice. There was very little cell infiltration into the SC in both groups of naïve mice (Figure 7 A). The brain, on the other hand, showed no difference in cell infiltration between vehicle-treated and 1V136-treated mice (Figure 7 B).

FACS analysis of the SC cells implied that the difference in cell trafficking could be due to a decrease in the number of infiltrating immune cells. Fewer B220+ B cells, CD11b+ monocytes and CD4+ T cells were found in the 1V136-treated EAE group. There was no clear difference in the number of CD8+ T cells or CD4+ CD25+ lymphocytes (Figure 8).

**Decreased SC cell infiltration corresponds to decreased SC chemoattractant molecule expression**

To examine a possible mechanism for why there is decreased cell trafficking to the SCs of 1V136-treated EAE mice as compared to vehicle-treated EAE mice, the SCs from both groups were examined using qPCR for cytokines and chemokines
involved in immune cell trafficking. On day 19, SCs from EAE mice treated with 1V136 showed significantly decreased levels of IL-1β, IP-10, KC, MCP-1 and MIP-1α as compared to those of EAE mice treated with vehicle alone (Figure 9). This indicates that the decrease in immune cell trafficking to the SC during the first peak of EAE disease severity corresponds to a decrease in cytokine and chemokine production by SC cells.

**Treatment with 1V136 induces microglial hyporesponsiveness *in vitro***

Activated microglia can be found in human MS lesions (Gay, 2007). Microglia are the CNS resident macrophages and thus act as the innate immune cell in neuroinflammatory disorders. Accordingly, they express many TLRs including TLR7. To study the direct effect of 1V136 on resident CNS immune cells, microglia were used for *in vitro* studies. The microglia were either pre-treated with 1V136 or cultured in vehicle alone for 18 hours. The following day, the microglia were restimulated with 1V136 or remained in vehicle alone.

The supernatants from after the 1V136 pre-treatment and from after the 1V136 restimulation were collected and analyzed via bead-based Luminex assay. The results revealed a significant decrease in the levels of IL-1β, IL-6, KC and MIP-1α production upon restimulation in the 1V136 pre-treated cells as compared to vehicle pre-treated microglia (Figure 10 A-C and E). There was no difference in MCP-1 expression between the two groups (Figure 10 D). As expected, there was no significant change in cytokine and chemokine levels between non-restimulated vehicle pre-treated microglia and non-restimulated 1V136 pre-treated microglia (Figure 10).
These data indicate that 1V136 may act directly upon microglia in the CNS and induce hyporesponsiveness to further stimulation.

**1V136 treatment reduces antigen-specific IFN-gamma and IL-17 production in the spleen**

The innate immune response is critical in determining the direction of the adaptive immune system. To study if 1V136 treatment affects the adaptive immune system in regards to CNS auto-antigens, PLP\textsubscript{139-151} specific splenocyte responses were assessed.

In EAE mice treated with 1V136, PLP\textsubscript{139-151}-specific splenocyte secretion of IFN-\(\gamma\) and IL-17 was significantly reduced. However, splenocytes specific for PLP\textsubscript{178-191}, another CNS auto-antigen, and those specific for CII\textsubscript{263-272}, a collagen peptide irrelevant to EAE, did not exhibit decreased IFN-\(\gamma\) and IL-17 secretion (Figure 11). The total spleen cell numbers were not affected by 1V136 treatment (data not shown).

**Anti-CNS antigen immunoglobulin levels are unaffected by 1V136 treatment**

To further explore the role of 1V136 treatment on the humoral response, sera collected from EAE mice sacrificed on day 19 were used for sandwich ELISAs to determine the levels of IgG1 and IgG2a expression in vehicle-treated versus 1V136-treated EAE mice. In particular, immunoglobulin specific for the CNS peptides PLP\textsubscript{139-151} and PLP\textsubscript{178-191} were assayed. The sera were incubated overnight on plates coated with these antigens. There was no significant difference in the amount of anti-CNS antigen immunoglobulin as measured by optical density (OD) between the two EAE groups (Figure 12).
DISCUSSION

The hallmark characteristics of MS include demyelination, oligodendrocyte apoptosis and axonal injury caused by inflammation triggered by adaptive memory T cells specific to CNS antigens (Weiner, 2004). However, the role of the innate immune response has recently been reevaluated. When patients progress from relapsing-remitting MS to secondary-progressive MS, the peripheral innate immune system is activated to a pro-inflammatory state (Karni, et al., 2006). The DCs from MS patients exhibit an increased proportion of IFN-γ, IL-6 and TNF-α-secreting monocyte-derived DCs compared to those from healthy controls (Huang, et al., 1999). Additionally, MS patients’ blood myeloid DCs show increased expression of the chemokine receptor C-C motif receptor 5 (Pashenkov, et al., 2002).

TLR7, a PRR that plays a key role in the innate immune response, has been implicated in autoimmune disease due to its role in pro-inflammatory signaling. Upon ligand-binding, TLR7 is activated and signals through MyD88 to cause the nuclear translocation of NF-κB and the production of pro-inflammatory cytokines by innate immune cells. Mice that are genetically deficient in MyD88 are resistant to EAE disease induction (Prinz, et al., 2006). TLR7 can also signal through the IFN regulatory factor 7 pathway to produce type I IFN (Takeda and Akira, 2004).

It was previously reported by this laboratory that 1V136-induced hyporeponsiveness in the MyD88 signaling pathway has an anti-inflammatory effect in C57BL/6 mice immunized with MOG35-55 (Hayashi, et al., 2009). In the current study, SJL/J mice immunized with PLP138-151 were used to elucidate the mechanism of
this anti-neuroinflammatory effect. The data presented here demonstrate that 1V136 treatment reduced SC immune cell infiltration in EAE mice. The reduction in cell infiltration correlated with a decrease in SC expression of pro-inflammatory cytokines and chemokines. It was also demonstrated that repeated *in vitro* TLR7 agonist treatment could induce hyporeponsiveness in a microglia-enriched cell population. This indicates that 1V136 may have a direct effect on CNS cells.

Studies in Lewis rats immunized with MBP (Kim, et al., 2010) and C57BL/6 mice immunized with MOG<sub>35-55</sub> (Li, et al., 2009) have demonstrated that the severity of clinical symptoms correlates with increasing cell infiltration. Synthetic IFN-β, a current MS treatment, and minocycline, a drug used primarily in skin infections that has been tested in EAE, both function to decrease the immune cell infiltration to the CNS that causes demyelination (Vosoughi and Freedman, 2010 and Nikodemova, et al., 2010). This is consistent with the histological data presented here. SCs harvested from vehicle-treated EAE mice showed demyelination in sites that corresponded to areas of high mononuclear and polynuclear cell infiltration (Figure 7 A-C). 1V136-treated mice, on the other hand, exhibited a decrease in total SC cell infiltration (Figure 6 A) with specific reductions in B cells, T cells and monocytes (Figure 8). It should be noted that there was no increase in CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells (T regs) in the SCs harvested from 1V136-treated mice.

The observed decrease in SC cell infiltration in 1V136-treated EAE mice could be involved in reducing the chemoattractant concentration gradient at inflammatory sites within the CNS. qPCR was used to investigate the effect of chronic TLR7
agonist administration on SC chemokine production. The following chemoattractant were studied: IL-1β, a known neutrophil chemotactic mediator (Oliveira, et al., 2008), KC, a neutrophil chemoattractant (Kobayashi, 2008), IP-10, a chemoattractant for activated T cells (Dufour, et al., 2002), MCP-1, a myeloid and memory T cell chemoattractant (Carr, et al., 1994) and MIP-1α, a chemoattractant for neutrophils and monocytes (Menten, et al., 2002). The SCs harvested from EAE mice treated with 1V136 had decreased expression of these factors as compared to vehicle-treated mice at the mRNA level (Figure 9).

Another possible mechanism for the anti-neuroinflammatory effects observed is that 1V136 could directly influence the peripheral immune cells, reducing expression and/or responsiveness of chemotaxis receptors that are responsible for mobilizing immune cells. In fact, repeated administration of LPS is known to cause NF-κB hyporesponsiveness in neutrophils (Medvedev, et al., 2001 and Parker, et al., 2005).

To explore the possibility of 1V136 having a direct effect on the CNS, a microglia-enriched cell population was prepared from the brains of neo-natal mice. Despite their generally non-immune cell functions, neurons, astrocytes, oligodendrocytes and microglia express PRRs (Bsibsi, et al., 2002). Microglia act in concert with the innate immune response to mediate the progression of MS through epitope spread, a condition in which autoreactive cells recognize novel CNS antigens (Dörries, 2001). 1V136 can induce hyporesponsiveness to TLR stimulation in microglia in vitro (Figure 10), implying that 1V136 treatment in vivo may cause
microglia to become insensitive to stimulation by novel CNS antigens and prevent the secretion of pro-inflammatory cytokines and chemokines. This would be consistent with the report that TLR7 agonists have a direct suppressive effect on TLR9 innate immune responses in microglia (Butchi, et al., 2009). Although there is no data to support the ability of 1V136 to enter the CNS through the BBB, it is likely that small molecules such as 1V136 could pass through an abnormal BBB that has lost its normal integrity due to EAE-associated inflammation (Ladewig, et al., 2009).

In the current study, splenocytes harvested from EAE mice treated with 1V136 secreted significantly less IFN-γ and IL-17 than those from vehicle-treated EAE mice after *ex vivo* restimulation with CNS antigens. This may be the result of decreased costimulatory molecule expression by splenic DCs caused by chronic 1V136 treatment. These reduced expression levels could lead to ineffective antigen presentation upon *ex vivo* restimulation. It should be noted that there was no significant difference in IL-10 secretion between vehicle-treated and 1V136-treated EAE mice.

An increase in the number or function of T regs could be another possible mechanism by which chronic 1V136 treatment can reduce the antigen-specific adaptive immune response. T regs are defined as a CD25⁺ subset of CD4⁺ T cells that suppress potentially deleterious activities of Th cells. They also play a crucial role in the regulation of peripheral memory T cell activation (Corthay, 2009). Recent reports indicate that TLR7 signaling enhances the immunosuppressive effect of T regs. Additionally, combined treatment with imiquimod, a TLR7 agonist, and IL-2 led to
increased T reg expression of forkhead box P3 (Foxp3) (Forward, et al., 2010), a transcription fact that appears to mediate T reg development (Curiel, 2007). The T reg population in the SCs of EAE mice was assayed for using the surface markers CD4 and CD25. The population of CD4<sup>+</sup> CD25<sup>+</sup> cells made up a similar proportion of cells in both the vehicle-treated and the 1V136-treated EAE groups. Further studies employing Foxp3 as a marker to identify T regs in the splenic DC population are currently being conducted.

Under certain conditions, the CNS is capable of undergoing repair and remyelination after inflammation and/or injury (Albrecht, et al., 2003 and Murray, et al., 2001). Little is known of the role of TLRs in this process of neural repair. However, TLR2 and 3 are expressed on oligodendrocytes (Bsibsi, et al., 2002), and TLR2-induced inflammation has been shown to promote myelination of oligodendrocyte precursor cells (Setzu, et al., 2006). Currently, TLR7 is not known to be expressed on oligodendrocyte precursor cells (Bsibsi, et al., 2002), although microglial TLR7 signaling may play an indirect role in remyelination.

A major concern with the use of 1V136 in MS therapy would be that the anti-neuroinflammatory effects seen in murine models might not be reproducible in humans because of differential TLR expression between species (Campbell, et al., 2009). In humans, TLR7 is expressed in plasmacytoid DCs and not in myeloid DCs. Recent reports indicate that human plasmacytoid DCs, like myeloid DCs, can process and present antigen to T cells (Tel, et al., 2010).
Of course, safety is another concern in the clinical use of TLR agonists like 1V136. However, TLR agonists have been used safely in treatments for allergies, cancer and infectious diseases as well as in vaccines. Chronic administration of 1V136 may result in overstimulation of the immune system, but less potent TLR7 agonists may give the same clinical effects without causing overstimulation. On the other hand, when compared to other immunosuppressants such as dexamethasone or methotrexate, TLR7 agonist treatment is unique in that it targets cells expressing the corresponding receptor. This specificity could avoid broad-spectrum immunosuppression and prevent increased susceptibility to opportunistic infection.

In summary, the current study indicates that 1V136 treatment ameliorates the clinical severity of EAE through downregulation of both the innate and adaptive immune systems. 1V136 treatment resulted in reduced cell infiltration into the SC and reduced SC expression of chemoattractants. It was also demonstrated that 1V136 treatment inhibited antigen-specific activation of peripheral memory T cells in the spleen. In a healthy individual, a balance exists between pro- and anti-inflammatory responses so that immune surveillance is maintained without continuous inflammation. TLR agonist-induced hyporesponsiveness may desensitize abnormally activated immune cells and correct the balance of inflammatory responses. This could be a new platform for the treatment of human autoimmune diseases.
Figure 1. **Toll-like receptor signaling.** TLR ligand binding leads to a signaling cascade resulting in the nuclear translocation of the transcription factor NF-κB. Once in the nucleus, NF-κB upregulates the expression of pro-inflammatory cytokines and chemokines.
Figure 2. Structure of 1V136. 1V136 (C_{15}H_{17}N_{5}O_{3}) has a molecular weight of 315.33 g/mol and is a known IFN inducer.
Figure 3. **Protocol for the establishment and treatment of EAE.** SJL/J mice were immunized with 200 µL of an emulsion containing 200 µg PLP (HSLGKWLGHDPDF) and 400 µg CFA per mouse. This was immediately followed by an injection of 325 ng PTX per mouse. An additional 325 ng PTX per mouse was injected two days later. Treated mice were given 150 nmol 1V136 daily from day five through 18. Control mice were injected with vehicle. Mice were sacrificed day 19 for short term studies and day 64 for long term studies.
Figure 4. 1V136 treatment decreases disease severity in EAE mice. SJL/J mice were immunized with a PLP_{139-151} and CFA emulsion to induce EAE. At 12 days after PLP immunization, vehicle-treated EAE mice more commonly experience paralysis in one or both hind limbs (A). 1V136-treated mice exhibited limp tails but were generally unparalyzed although their gaits were affected (B). The pictures shown are from one experiment and are comparable to four other independent experiments.
Figure 5. 1V136 treatment significantly reduces EAE clinical score. EAE mice were treated with either 100 µL vehicle or 150 nmol 1V136 per mouse daily from day 5 through 18. During the first peak of disease (day 9 through 18), vehicle-treated EAE mice had significantly higher clinical scores than 1V136-treated EAE mice on days 12-16. Vehicle-treated EAE mice generally continued to have higher clinical scores than 1V136-treated EAE mice during subsequent relapses, but the difference was not significant. Data shown are from one experiment and were comparable to at least two other independent experiments. (n = 5-10 mice per group per experiment. * denotes p < 0.0001 by two-way ANOVA.)
Figure 6. 1V136 treatment significantly reduces cell infiltration to the SC but not the brain. The SCs, but not the brains, of EAE mice had a significant increase in the number of infiltrating cells as compared to naïve controls (A and B). However, there were significantly fewer cells in the SCs of 1V136-treated EAE mice than vehicle-treated EAE mice (A). 1V136 treatment had no effect on cell infiltration in the brain (B). Data shown are from at least two independent experiments. (n = 5-10 mice per group per experiment. * denotes p < 0.001 by one-way ANOVA.)
<table>
<thead>
<tr>
<th>Vehicle</th>
<th>1V136</th>
<th>Staining (Magnification)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="A" /></td>
<td><img src="image" alt="D" /></td>
<td>H&amp;E (x40)</td>
</tr>
<tr>
<td><img src="image" alt="B" /></td>
<td><img src="image" alt="E" /></td>
<td>H&amp;E (x200)</td>
</tr>
<tr>
<td><img src="image" alt="C" /></td>
<td><img src="image" alt="F" /></td>
<td>LFB (x40)</td>
</tr>
</tbody>
</table>

**Figure 7.** Histology of the SCs of 1V136-treated EAE mice show decreased cell trafficking and demyelination. Total cell counts of the brains and SCs of vehicle-treated and 1V136-treated EAE mice showed a decrease in cell infiltration in the SCs of 1V136-treated mice. This decrease was confirmed through histological staining of SC specimens. The SCs of vehicle-treated EAE mice showed much more cell infiltration (dotted arrow) and demyelination (solid arrow) (A-C) than those of 1V136-treated EAE mice (D-F). The pictures shown are from one experiment and were comparable to results from two other independent experiments. (n = 5-10 mice per group per experiment.)
Figure 8. The decrease in SC cells in 1V136-treated EAE mice corresponds to a decrease in cell trafficking. The decrease in infiltrating observed in SCs harvested from 1V136-treated EAE mice as compared to vehicle-treated mice corresponds to a decrease in immune cell trafficking. SC cells from both groups were isolated through a Percoll gradient and analyzed using FACS. The cells were stained for B cell, T cell and granulocyte markers (A). EAE mice treated with 1V136 had fewer CD4$^+$ T cells (B), B220$^+$ B cells (C) and Gr1$^+$ granulocytes (D). Data shown are from one experiment and were comparable results to two other independent experiments. (n = 5-10 mice per group per experiment.)
Figure 9. qPCR of EAE SCs shows a decrease in cytokine and chemokine expression in 1V136-treated mice. A portion of the SCs from each treatment group was immediately frozen for qPCR analysis. The decrease in immune cell trafficking to SCs from EAE mice treated with 1V136 correlates to a decrease in IL-1β, IP-10, KC, MCP-1 and MIP-1α expression. Data shown are from one experiment and were comparable to results from one other independent experiment. (n = 5 mice per group per experiment.)
Figure 10. Microglia pre-treated with 1V136 become hyporesponsive in vitro. Microglia were either pre-treated with 1V136 (pre-tx +) or remained in vehicle alone (pre-tx —). After 18 hours the microglia were restimulated with 1V136 (restim +) of remained in vehicle alone (restim —). Cells that were pre-treated with 1V136 showed significantly decreased levels of IL-1β (A), IL-6 (B), KC (C) and MIP-1α (E) when restimulated with 1V136. Data shown are from one experiment and were comparable to results from three other independent experiments. (* denotes p < 0.01 and ** denotes p < 0.0001 by one-way ANOVA.)
Figure 11. 1V136 treatment reduces antigen-specific cytokine production in splenocytes.
Splenocytes harvested from vehicle-treated and 1V136-treated EAE mice were cultured in vehicle alone (veh) or with the following peptides: PLP\textsubscript{139-151}, the peptide used to induce EAE, PLP\textsubscript{178-191}, another CNS auto-antigen and CII\textsubscript{262-273}, a collagen peptide unrelated to EAE. The supernatants were collected and analyzed for cytokine production through ELISA. The splenocytes from 1V136-treated mice exhibited reduced IFN-\gamma and IL-17 production specific to the PLP\textsubscript{139-151} culture. Data shown are from one experiment and were comparable results to three other independent experiments. (* denotes p < 0.01 and ** denotes p < 0.0001 by one-way ANOVA.)
Figure 12. IgG1 and IgG2a production is unaffected by 1V136 treatment. Microtiter plates were coated with PLP_{139-151} or PLP_{178-191} for ELISAs assessing levels of CNS antigen specific immunoglobulins in sera harvested from EAE mice. There was no significant difference in IgG2a (A and B) or IgG1 (C and D) production between EAE mice treated with vehicle alone (veh) or 1V136. Data shown are from one experiment and were comparable to results from two other independent experiments.
Table 1. Types of MS grouped by disease progression. MS can be divided into four subsets based on changes in the severity of disease symptoms over time (Confavreux, et al., 1980, Andersson, et al., 1999, Tullman, et al., 2004 and Kargiotis, et al., 2010).

<table>
<thead>
<tr>
<th>Type of MS</th>
<th>Initial Symptoms</th>
<th>Symptoms over Time</th>
<th>% of MS Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary-progressive</td>
<td>Slow steady worsening of symptoms</td>
<td>Symptoms steadily worsen, some plateaus in disease progression or slight remissions possible</td>
<td>10%</td>
</tr>
<tr>
<td>Progressive-relapsing</td>
<td>Steady worsening of symptoms</td>
<td>Continued worsening of symptoms with no remission</td>
<td>5%</td>
</tr>
<tr>
<td>Relapsing-remitting</td>
<td>Symptomatic periods followed by remissions</td>
<td>More severe symptomatic periods, less complete recoveries</td>
<td>85% of initial diagnoses</td>
</tr>
<tr>
<td>Secondary-progressive</td>
<td>Begins with a period of relapsing-remitting MS</td>
<td>Symptoms worsen steadily, attacks and remissions may or may not occur</td>
<td>50% of relapsing-remitting cases progress to secondary-progressive within ten years</td>
</tr>
</tbody>
</table>
Table 2. Murine EAE models. Active immunization models of EAE require a short peptide sequence from a myelin protein to be injected subcutaneously. The peptide sequence used varies depending on mouse strain.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Mouse Strain</th>
<th>H-2 Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP&lt;sub&gt;Ac1-11&lt;/sub&gt;</td>
<td>Ac-ASQKRPRQHG</td>
<td>PL/J, B10.PL, PL/J x SJL F1, SJL x B10.PL F1</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;, H-2&lt;sup&gt;b&lt;/sup&gt;, H-2&lt;sup&gt;u&lt;/sup&gt;</td>
<td>Zamvil, et al., 1986, Miller, et al., 2010</td>
</tr>
<tr>
<td>MBP&lt;sub&gt;35-47&lt;/sub&gt;</td>
<td>TGILDSIGRFFSG</td>
<td>PL/J, B10.PL</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;, H-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Zamvil, et al., 1988</td>
</tr>
<tr>
<td>MBP&lt;sub&gt;34-104&lt;/sub&gt;</td>
<td>VHFFKNIVTPRTPPSQGKGR</td>
<td>SJL</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tan, et al., 1992</td>
</tr>
<tr>
<td>MBP&lt;sub&gt;84-104&lt;/sub&gt;</td>
<td>VHFFKNIVTPRTPPSQGKGR</td>
<td>C57BL/6</td>
<td>H-2&lt;sup&gt;b&lt;/sup&gt;, H-2&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Mendel, et al., 1995, Slavin, et al., 1998</td>
</tr>
<tr>
<td>MOG&lt;sub&gt;35-55&lt;/sub&gt;</td>
<td>MEVGWYRSPFSRVVHLYRNGK</td>
<td>C57BL/6, NOD</td>
<td>H-2&lt;sup&gt;b&lt;/sup&gt;, H-2&lt;sup&gt;g7&lt;/sup&gt;</td>
<td>Mendel, et al., 1995, Slavin, et al., 1998</td>
</tr>
<tr>
<td>MOG&lt;sub&gt;92-106&lt;/sub&gt;</td>
<td>DEGGYTCCFRDHSYQ</td>
<td>SJL</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Amor, et al., 1994</td>
</tr>
<tr>
<td>PLP&lt;sub&gt;41-64&lt;/sub&gt;</td>
<td>EKLIETYFSKNYQDYEYLINVI</td>
<td>PL/J, B10.PL, PL/J x SJL F1</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;, H-2&lt;sup&gt;b&lt;/sup&gt;, H-2&lt;sup&gt;u&lt;/sup&gt;, H-2&lt;sup&gt;q&lt;/sup&gt;</td>
<td>Whitham, et al., 1991, Miller, et al., 2010</td>
</tr>
<tr>
<td>PLP&lt;sub&gt;66-70&lt;/sub&gt;</td>
<td>DYEYLINVIHAFAQYV</td>
<td>NOD</td>
<td>H-2&lt;sup&gt;b7&lt;/sup&gt;</td>
<td>Girvin, et al., 1996</td>
</tr>
<tr>
<td>PLP&lt;sub&gt;67-70&lt;/sub&gt;</td>
<td>YEYLINVIIHAFQYV</td>
<td>SJL</td>
<td>H-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Greer, et al., 1996</td>
</tr>
<tr>
<td>PLP&lt;sub&gt;103-116&lt;/sub&gt;</td>
<td>YKTTCGKGLSATV</td>
<td>C3H</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tuohy, et al., 1988a</td>
</tr>
<tr>
<td>PLP&lt;sub&gt;104-117&lt;/sub&gt;</td>
<td>KTTICGKGLSATVT</td>
<td>SJL</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tuohy and Thomas, 1993</td>
</tr>
<tr>
<td>PLP&lt;sub&gt;109-111&lt;/sub&gt;</td>
<td>HSLGKWLGHPDKF</td>
<td>SJL, PL/J x SJL F1, SJL x B10.PL F1</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;, H-2&lt;sup&gt;b&lt;/sup&gt;, H-2&lt;sup&gt;u&lt;/sup&gt;, H-2&lt;sup&gt;q&lt;/sup&gt;</td>
<td>Tuohy, et al., 1989, McRae, et al., 1992, Miller, et al., 2010</td>
</tr>
<tr>
<td>PLP&lt;sub&gt;178-191&lt;/sub&gt;</td>
<td>NTWTTCQSIAFPSK</td>
<td>PL/J, B10.PL, SJL, C57BL/6, BALB/cPt, SJL x B10.PL F1</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;, H-2&lt;sup&gt;b&lt;/sup&gt;, H-2&lt;sup&gt;u&lt;/sup&gt;, H-2&lt;sup&gt;q&lt;/sup&gt;</td>
<td>Miller, et al., 2010, Greer, et al., 1992, Tompkins, et al., 2002, Greer, et al., 1992, Miller, et al., 2010</td>
</tr>
<tr>
<td>PLP&lt;sub&gt;190-209&lt;/sub&gt;</td>
<td>SKTASIGSLCADARMYGVSL</td>
<td>SJL x C3H/HeJ F1</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Muller, et al., 2000</td>
</tr>
<tr>
<td>PLP&lt;sub&gt;215-232&lt;/sub&gt;</td>
<td>PGKVCGLNLSIICKTAEF</td>
<td>SWR, SJL x C3H/HeJ F1</td>
<td>H-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Endoh, et al., 1990, Greer, et al., 1996</td>
</tr>
</tbody>
</table>
Table 3. **TLR ligands and expression.** TLRs recognize a diverse set of PAMPs and are expressed on various innate immune cells (Waltenbaugh, et al., 2008, Sallusto and Lanzavecchia, 2002 and Lehnardt, 2010).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Common Ligands</th>
<th>Source</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacylated lipoproteins</td>
<td>Bacteria</td>
<td>Monocytes&lt;br&gt;Dendritic cell subset&lt;br&gt;B cells</td>
</tr>
<tr>
<td>TLR2</td>
<td>Glycolipids, Lipoproteins, Lipoteichoic acid, Zymosan</td>
<td>Bacteria, Bacteria, Gram-positive bacteria, Yeast</td>
<td>Monocytes&lt;br&gt;Myeloid dendritic cells&lt;br&gt;Mast cells</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double-stranded RNA, Poly I:C</td>
<td>Viruses, Synthetic</td>
<td>Dendritic cells&lt;br&gt;B cells</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS</td>
<td>Bacteria</td>
<td>Monocytes&lt;br&gt;Myeloid dendritic cells&lt;br&gt;Mast cells&lt;br&gt;Intestinal epithelium</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>Gram-negative bacteria</td>
<td>Monocytes&lt;br&gt;Dendritic cell subset&lt;br&gt;Intestinal epithelium</td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacylated lipopeptides, Lipoteichoic acid</td>
<td>Mycobacteria, Gram-positive bacteria</td>
<td>Monocytes&lt;br&gt;Mast cells&lt;br&gt;B cells</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded RNA, Small compounds</td>
<td>Viruses, Synthetic</td>
<td>Monocytes&lt;br&gt;Plasmacytoid dendritic cells&lt;br&gt;B cells</td>
</tr>
<tr>
<td>TLR8</td>
<td>Single-stranded RNA, Small compounds</td>
<td>Viruses, Synthetic</td>
<td>Monocytes&lt;br&gt;Dendritic cell subset&lt;br&gt;Mast cells</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG</td>
<td>Bacteria, viruses</td>
<td>Monocytes&lt;br&gt;Plasmacytoid dendritic cells&lt;br&gt;B cells</td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Monocytes&lt;br&gt;B cells</td>
</tr>
<tr>
<td>TLR11</td>
<td>Uropathogenic bacteria, Profilin</td>
<td>Bacteria, Protozoa</td>
<td>Monocytes&lt;br&gt;Liver cells&lt;br&gt;Kidney cells&lt;br&gt;bladder epithelium</td>
</tr>
</tbody>
</table>
Table 4. Clinical scoring of EAE. EAE mice were scored daily for signs of disease from day 5 onward.

<table>
<thead>
<tr>
<th>Score</th>
<th>Clinical sign</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No clinical signs</td>
</tr>
<tr>
<td>0.5</td>
<td>Erect tail, clumsy gait</td>
</tr>
<tr>
<td>1</td>
<td>Flaccid tail</td>
</tr>
<tr>
<td>1.5</td>
<td>Flaccid tail, extremely clumsy gait, no paralysis</td>
</tr>
<tr>
<td>2</td>
<td>Flaccid tail, one hind leg paralyzed</td>
</tr>
<tr>
<td>3</td>
<td>Flaccid tail, both hind legs paralyzed</td>
</tr>
<tr>
<td>4</td>
<td>Quadriparalysis</td>
</tr>
<tr>
<td>5</td>
<td>Moribund</td>
</tr>
</tbody>
</table>
Table 5. Primer pairs used for qPCR. The following primers were designed for use in analyzing gene expression for IL-1β, IP-10, KC, MCP-1 and MIP-1α.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Position</th>
<th>Amplicon size (nt)</th>
<th>Universal Probe Library #</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>AAATCAGTTATGGTCCCTTTGGTC AACCTATTGACACCATAAGATCTCG</td>
<td>102-125 143-168</td>
<td>67</td>
<td>55</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGTAATGAAAGACGGCACACC GGTGCTTTATGGGTTTCTTCT</td>
<td>632 – 652 679 – 699</td>
<td>68</td>
<td>78</td>
</tr>
<tr>
<td>IP-10</td>
<td>GCTGCCGTCATTTTCTGC CTACTGCCCCGTCACTCT</td>
<td>53 – 70 146 – 163</td>
<td>111</td>
<td>3</td>
</tr>
<tr>
<td>KC</td>
<td>AGACTCCAGCCACACTCCAA GTTACTCGACGCGACAGT</td>
<td>3 – 22 115 – 132</td>
<td>130</td>
<td>83</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CATCCACGTTGGCTCA TGAGTAAGTGTCCTCTACTAG</td>
<td>139 – 156 192 – 214</td>
<td>76</td>
<td>62</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CAAGTCTTCTCAGCGCCTATA GGATGTCGGCCTTCTAAGG</td>
<td>159 – 178 211 – 229</td>
<td>71</td>
<td>40</td>
</tr>
</tbody>
</table>
REFERENCES


Andersson, P.B., E. Waubant, L. Gee and D.E. Goodkin. 1999. Multiple sclerosis that is progressive from the time of onset: Clinical characteristics and progression of disability. *Arch Neurol* 56(9): 1138-42.


