Stony Brook University

Tuftsin and Its Receptor Neuropilin-1 in the Attenuation of Experimental Autoimmune Encephalomyelitis

A Dissertation Presented

by

Jillian Nissen

to

The Graduate School

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

in

Molecular and Cellular Pharmacology

Stony Brook University

May 2014

Stony Brook University

The Graduate School

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Abstract of the Dissertation

Tuftsin and Its Receptor Neuropilin-1 in the Attenuation of Experimental Autoimmune Encephalomyelitis

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2014

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by progressive demyelination and degeneration of neurons. As MS progresses, T cells that are normally excluded from the CNS cross the compromised blood-brain barrier due to recruitment by the resident CNS immune cells, microglia. Both microglia and T cells can promote either disease progression or disease resolution through polarization into pro- and anti-inflammatory subsets, respectively. Therapeutic approaches in MS focus on diminishing inflammation and promoting expansion of anti-inflammatory immune cell populations.

Tuftsin, a stimulator of macrophage/microglial activation, is a naturally occurring tetrapeptide with the sequence T-K-P-R. In the experimental autoimmune

encephalomyelitis (EAE) mouse model of MS, infusion of tuftsin attenuates disease symptoms and promotes the anti-inflammatory response. Tuftsin's binding target is neuropilin-1 (Nrp1), a protein with critical roles in the nervous, vascular, and immune systems.

In this study, we investigated whether tuftsin's beneficial effects during EAE are mediated by Nrp1. Our results show that tuftsin polarizes microglia to an anti-inflammatory phenotype specifically via Nrp1 through the canonical TGFβ pathway. Further, we report that Nrp1 promotes long, functional contacts between microglia and immunosuppressive regulatory T cells. Mice treated with tuftsin during EAE show significant reductions in disease severity, demyelination, weight loss, and inflammatory polarization in microglia. However, in mice lacking Nrp1 on macrophages/microglia, tuftsin's beneficial effects were completely abolished. Taken together, these data indicate that the Nrp1-microglial axis is the primary mediator of tuftsin's function *in vivo*. The results of this project provide us a fuller understanding of the mechanism of tuftsin's action during EAE, and may lead to the development of more effective disease modifying reagents for MS treatment

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List of Abbreviations

APC	Antigen Presenting Cell
Arg1	Arginase-1
BCG	Bacillus Calmette-Guérin
b-FGF	Basic Fibroblast Growth Factor
CFA	Complete Freund's Adjuvant
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DAN	Diaminonapthalene
DC	Dendritic Cell
DTH	Delayed-Type Hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein-Barr Virus
GWAS	Genome-Wide Association Studies
HGF	Hepatocyte Growth Factor
HLA	Human Leukocyte Antigen
IFNγ	Interferon Gamma
iDC	Immature Dendritic Cell
IL4	Interleukin 4
IL6	Interleukin 6
IL10	Interleukin 10
IL13	Interleukin 13

IL17	Interleukin 17
LPS	Lipopolysaccharide
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MHC II	Major Histocompatibility Complex Type II
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
NAWM	Normal Appearing White Matter
NCM	Neuronal Conditioned Media
NO	Nitric Oxide
Nrp1	Neuropilin-1
OPC	Oligodendrocyte Progenitor Cell
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PLP	Proteolipid Protein
PP-MS	Primary Progressive Multiple Sclerosis
PR-MS	Progressive Relapsing Multiple Sclerosis
RR-MS	Relapsing Remitting Multiple Sclerosis
TβR1	Transforming Growth Factor Beta Receptor 1
TCR	T cell Receptor
TGFβ	Transforming Growth Factor Beta
Th1	T helper type 1

Th2	T helper type 2
Th17	T helper 17
Treg	Regulatory T cell
TMEV-DD	Theiler's Murine Encephalomyelitis Virus-Induced Demyelinating Disease
TNFα	Tumor Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

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Acknowledgements

First and foremost, I would like to express my sincere thanks and gratitude to my advisor Dr. Stella Tsirka for her support and guidance. She is the best teacher, mentor, and friend that I could have asked for, and it has truly been a privilege to work with her over the years. She has encouraged me to take ownership of my project; giving me the independence to explore new avenues and ideas, but is always there to provide input and assistance when difficulties arise. I have learned from Stella far more than she has taught me directly, for by her example she has shown me what a scientist should be. Her encouragement, patience, and advice on matters both inside and out of the lab has made me into the scientist I am today, and for that I am very grateful.

I would also like to thank my committee members Dr. Nicholas Carpino, Dr. Holly Colognato, and Dr. Ando van der Velden, for their guidance throughout my project. Their valuable criticisms and comments during my committee meetings have allowed me to recognize and address weak points in my project, as well as provided me with new perspectives that have helped develop the future directions of this study.

I also want to recognize the current and former members of the Tsirka lab that I have been truly fortunate to work with over the years. I thank Dr. Muzhou Wu, who trained me during my rotation and introduced me to the project that is contained within this dissertation. I would also like to thank Dr. Zhen Gao who was a tremendous help in teaching me the EAE surgery and scoring system, as well as for her advice and input on my project. I especially want to thank the current members of the Tsirka Lab: Luisa

Torres, for her knowledge of animal experiments; Robert Bronstein, for his help in troubleshooting my western blots and for teaching me how to generate retrovirus; Jeremy Miyauchi, for rescuing me by sharing his primary microglia when my cultures failed; and Joan Danver for her insightful questions about my research. Most of all, I want to thank all of the Tsirka lab members for their friendship and for the years of wonderful memories both inside and out of the lab that we have shared.

Many faculty and staff members at Stony Brook have provided me with assistance during my studies here. I would like to thank the members of the Colognato lab for the use of their microscope as well as for sharing reagents that have contributed to this study. I would also like to thank Lynda Perdomo-Ayala, Janice Kito, Melissa Daley, and Odalis Hernandez who have helped me with administrative issues during my time at Stony Brook.

Last but not least, my sincere gratitude goes to my family. Throughout my life, my parents Nancy and Gus Cypser have provided me with unconditional love and encouraged me to pursue my dreams. Without their support, I am certain I would not be who I am today and would not have accomplished all that I have in life. I would like to thank my brother Greg Cypser for always being there for me when I needed help, and for reminding me not to take things too seriously. Most of all, I want to thank my husband Dan Nissen. He has been by my side as my greatest support, and has kept me grounded throughout all the ups and downs of my graduate career. His love and constant belief in me inspires me to seek out and achieve all of my goals in life.

Chapter I

General Introduction

Multiple sclerosis

Multiple sclerosis (MS) is a debilitating chronic autoimmune disease of the central nervous system (CNS) in which the adaptive and innate immune cells of the body aberrantly attack the protective myelin sheath on nerve fibers in both the brain and spinal cord. This progressive demyelination and degradation of neurons results in sensory and motor deficits in patients which range from blurry vision, slurred speech, and loss of balance to muscle weakness and paralysis. This disease affects 250,000-400,000 people in the US, and 2.5 million individuals worldwide. (Steinman 2001; Trapp and Nave 2008). MS typically occurs in young adulthood, between the ages of 20 to 50, and affects women twice as often as men.

Based on the symptoms observed in patients, three different disease courses have been identified in MS. In “relapsing-remitting MS” (RR-MS), patients present with discrete attacks separated by periods of recovery. This is the most common form of the disease, with approximately 80-90% of cases falling under this distinction. In 10-15% of patients, MS progresses with a “primary progressive” course (PP-MS), where patients have steadily progressive neurological deficits without intervening periods of acute relapse. The “progressive relapsing” (PR-MS) course is the most rare, only evident in about 5% of cases, in which ongoing degeneration occurs, although clear periods of attacks are evident (Thompson 2004).

Although the etiology of MS is poorly understood, there has been some evidence of genetic susceptibility. As there is greater prevalence of MS in Caucasians relative to other races (Compston 2007), and family members of patients have a significantly higher risk of developing the disease (Ebers 2005), a large number of studies to identify

disease-related genes and loci were performed. One of the earliest identified and most well confirmed alleles associated with MS is the human leukocyte antigen (HLA) locus. This is located in a region known as the major histocompatibility complex (MHC), in which the genes encode highly polymorphic cell-surface glycoproteins that are key components of the immune system. However, it has since become evident that these genes alone are far from solely responsible for the development of MS (Barcellos et al. 2003; Irizar et al. 2012). In a recent study performed by the joint efforts of the International Multiple Sclerosis Genetics Consortium and the Wellcome Trust Case Control Consortium, 29 new non-HLA genes were described as risk factors for development of MS using genome-wide association studies (GWAS). However, the function of these genes and proteins and the role they have in MS susceptibility and progression have yet to be explored (International Multiple Sclerosis Genetics et al. 2011). Furthermore, environmental factors have also been implicated in the development of MS. These include vitamin D deficiency, exposure to pathogens such as the Epstein–Barr virus (EBV) after early childhood and manifestations of infectious mononucleosis, and cigarette smoking (Ascherio and Munger 2007a; Ascherio and Munger 2007b). As described above, it is clear that there is not a defined causative factor for MS.

There are many events that occur on a cellular level during the progression of MS. The most prominent is the demyelination and disruption of remyelination of axons. Myelin is produced by oligodendrocytes in the CNS, and these cells can be targets of autoimmune attack (Franklin 2002). The precise events that lead to loss of oligodendrocytes are complex, but MS is classically characterized by the infiltration of

inflammatory T cells from the periphery (Dhib-Jalbut 2007; Fletcher et al. 2010). Although these cells are normally excluded from the CNS, in the disease state myelin reactive T cells cross the blood brain barrier and interact with target cells, resulting in the initiation of the inflammatory cascade and the degeneration of the myelin sheath on axons. However, it has been shown that MS can be initiated within the CNS in the absence of T cell infiltration (Centonze et al. 2009; van Noort et al. 2011). Excitotoxic injury to axons has been reported during the development of the disease, prior to peripheral cell infiltration (Matthews et al. 1996; Pitt et al. 2000). Additionally, activation of microglial cells and apoptosis within oligodendroglial populations has been observed in presymptomatic conditions (Barnett and Prineas 2004; van Noort et al. 2011).

MS is currently incurable, so therapies focus on minimizing CNS inflammation and immune cell infiltration, with varying levels of success (Miravalle and Corboy 2010; Palmer 2009). At present, five injectable disease-modifying drugs are available for the treatment of relapsing-remitting MS: interferon beta-1a, interferon beta-1b, glatiramer acetate, natalizumab, and mitoxantrone. The mechanism by which interferon beta functions to improve symptoms in MS are poorly understood, but it is believed to act on several levels of cellular function, from modulation of expression of anti-inflammatory cytokines to the reduced trafficking of inflammatory cells across the blood–brain barrier (Kieseier 2011). Glatiramer acetate is also a drug that does not have a clearly defined mechanism of action, but could include interactions with T cells and antigen-presenting cells, with an overall shift in the immune cytokine profile to an anti-inflammatory state mediated by T helper-2 cells (Farina et al. 2005). The migration of encephalitogenic T cells into the CNS is dependent on the interaction of adhesion molecules on the surface

of T cells with the inflamed vascular endothelium (Yednock et al. 1992). Natalizumab is a humanized monoclonal antibody directed against the α4 subunit of the α4β1 integrin dimer, and exerts its beneficial effects in MS by preventing autoreactive T cells from crossing the blood–brain barrier, which disrupts one of the early steps in MS pathogenesis. However, this comes with the risk of progressive multifocal leukoencephalopathy (Oh and Calabresi 2013; Polman et al. 2006). The cytotoxic drug mitoxantrone disrupts DNA synthesis and repair by inhibiting DNA topoisomerase and causing DNA intercalation. While this drug's effects are mediated by its suppression of humoral immunity and modulation of T cells, its potential toxic effects on patients have resulted in a lack of clinical trials (Marriott et al. 2010). Developing an understanding of the mechanisms underlying the disease and the shortfalls of current therapies is essential for development of more effective treatments.

Animal models for MS

As MS has an exceedingly complex etiology and pathology, it is difficult to develop a single animal model that accurately recapitulates the conditions and symptoms of patients as a whole. Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model of MS. EAE is induced by injection of myelin antigens, which include myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and proteolipid protein (PLP) which triggers an immune response (Hemmer et al. 2002). This immunization can either be active, by administration of myelin antigens, or passive, through administration of myelin-reactive T cells. Many features of MS are present in mice during EAE, which include paralysis, weight loss,

demyelination, and CNS inflammation. During EAE, activated myelin-specific T cells cross the compromised blood-brain barrier (Mor et al. 1996). They are recruited by CNS-resident microglia, which are activated and induce an inflammatory cascade that results in loss of axonal myelination and neurodegeneration (Steinman 1996; Steinman 2000) (Figure I-1).

Not all strains of rodent have the same susceptibility for developing MS-like symptoms during EAE, as this is largely dependent on the type of antigen used. H2-U mice, particularly the PL/J strains, are highly susceptible to MBP-induced EAE. PLP is most effective for inducing symptoms in SL/J mice, while C57BL/6 mice respond only to MOG-induced disease (Kuerten and Angelov 2008). Additionally, the disease course the mice develop is dependent on both strain and the particular myelin peptide used for induction, which can mirror several clinical subsets of MS seen in humans. For example, SJL/J mice readily develop relapsing forms of EAE (Dal Canto et al. 1995), while C57BL/6 mice have a monophasic disease course. The most commonly used strain is C57BL/6, as a majority of transgenic animals are maintained on this genetic background. However, MOG-induced EAE in this strain is an acute version of the disease without any remission or relapse, which is not a phenomenon seen in human patients (Ransohoff 2012). Thus, it is important to acknowledge that this is a limited model of a highly complex human disease.

An alternative to an antigen-induced MS model is Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-DD), where demyelination occurs as a result of viral infection. As infection is not a physiological component of MS, the response from immune cells as well as the particular timing and

the subtype of the reacting population differs significantly from EAE (Ransohoff 2012). Similarly, subcutaneous injection of BCG (bacillus Calmette-Guérin) has been shown to induce a delayed-type hypersensitivity (DTH) response. This also follows pathological hallmarks of MS through infiltration of peripheral macrophages and lymphocytes, degeneration of the blood–brain barrier and immunoreactive myelin loss (Matyszak and Perry 1996).

Understanding the remyelination process is essential for ameliorating progressive MS, but this is a process that is difficult to study in EAE. As an alternative, toxic models of demyelination have been employed which allow for precise control of both the timing and location of the injured areas, as well as controlling remyelination as the process begins once the toxin is removed. These models include cuprizone, which causes cell death in oligodendrocytes (Torkildsen et al. 2008), as well as lysolecithin or ethidium bromide which causes prompt demyelination when microinjected into white matter tracts. However, as these models do not include significant leukocyte infiltration, they lack the ability to recapitulate MS as a whole (Blakemore and Franklin 2008; Chastain et al. 2011).

An effective model for MS must satisfy at least these three criteria- that it is mediated by self-reactive T cells, that demyelination can be observed, and that the disease course is consistent among animals to facilitate the observation of differences resulting from treatments. As EAE is a system in which myelin-reactive T cells promote CNS injury and demyelination, and the disease course is highly predictable in C57BL/6 mice, this is the most relied-upon model for MS in many current studies.

Microglia

Microglia are a distinct class of glial cells that play critical roles in the innate and adaptive immune responses of the CNS. They are the resident macrophage-like immune cells of the CNS, which comprise 10-20% of the total glial cells and about 7% of all CNS cells. Due to their morphology, microglia cells are described as being in a "resting" state under healthy conditions. This term is somewhat misleading, since in vivo imaging has revealed that microglia actively scavenge and monitor their CNS microenvironment with ramified processes for pathogens and injury (van Rossum and Hanisch 2004). In contrast to neurons and macroglia (oligodendrocytes and astrocytes), microglia originate from the primitive hematopoiesis within the yolk sac and migrate to the neural tube during embryogenesis (Ginhoux et al. 2010). As microglia express major histocompatibility complex type II (MHC II) proteins, they represent immature antigen presenting cells (APCs) when in the resting state (Olson et al. 2001).

Upon injury to the CNS, microglia migrate to the site of damage and undergo a process known as activation, where they obtain a more amoeboid shape and proliferate (Kreutzberg 1996; Raivich and Banati 2004). This results in the upregulation of MHC II, which allows for further recruitment of other inflammatory cells such as neutrophils, lymphocytes, and monocytes into the injury site (Hanisch 2002). Activated microglia can release tumor necrosis factor alpha (TNFα) and nitric oxide (NO), which promote demyelination (Chao et al. 1992). Accordingly, blockage of microglial activation during EAE significantly repressed the disease course (Heppner et al. 2005). However, microglia also produce anti-inflammatory cytokines such as transforming growth factor beta (TGFβ) and interleukin 10 (IL10). IL10 administration during EAE prevents the

development of symptoms (Rott et al. 1994), while TGFβ has been shown to suppress the generation of TNFα and reduces EAE severity (Stevens et al. 1994).

These inconsistencies can potentially be explained by the presence of two subsets of microglia, identified as M1 and M2 (Colton 2009; Colton and Wilcock 2010). M1 microglia/macrophages are considered to be “classically activated,” by lipopolysaccharide (LPS) or cytokines such as interferon gamma (IFNγ), and produce pro-inflammatory cytokines and nitric oxide. However, microglia exposed to interleukin 4 (IL4), IL10, or interleukin 13 (IL13) present an “alternatively activated” phenotype, which release IL10 and TGFβ (Gordon and Martinez 2010; Michelucci et al. 2009). Neuronal regeneration in a model of spinal cord injury is significantly improved by M2 macrophages, while M1 macrophages are neurodegenerative (Kigerl et al. 2009). Furthermore, administration of M2 monocytes in EAE suppressed ongoing severe EAE (Mikita et al. 2010). Thus, EAE disease progression appears to be mediated by M1 microglia, and recovery is promoted by M2 microglia.

The timing and type of microglial activation has been shown to play a key role in the development and recovery of MS. Activated microglia can be found both in active demyelinating regions as well as inflammatory non-demyelinating areas of the CNS, which persists through the course of the disease (Mikita et al. 2011; Wu and Tsirka 2009). They have also been shown to interact with damaged myelin sheaths, as well as phagocytose myelin debris (Koning et al. 2009; Zhang et al. 2011b). Early activation of microglia is associated with neuroprotection, and potentially can induce “protective autoimmunity” (Shaked et al. 2004). Microglial activation is also seen in very early stages of multiple sclerosis, as microglia clusters, known as microglia nodules, are

found in preactive lesions in the white matter of MS patients (Singh et al. 2013; van der Valk and Amor 2009). Further, positron emission tomography (PET) imaging showed that this activation was evident in normal appearing white matter (NAWM), which was devoid of leukocyte infiltration, demyelination, or even blood-brain barrier disruption (van Noort et al. 2011).

Thus, it is clear that microglia play a complex role in MS, which can lead to both beneficial and detrimental outcomes depending on the timing and form of activation.

T cells

T cells belong to a population of white blood cells known as lymphocytes, and are major mediators of the adaptive immune system. During the T cell maturation process within the thymus, their T cell receptors (TCRs) are generated by random genetic rearrangements. This results in TCRs that can recognize yet to be encountered foreign antigens, but also inevitably results in the generation of TCRs that are specific for self-antigens. The classic mechanism that accounts for the silencing of such potentially autoaggressive T cells is negative selection, also called central tolerance, which causes self-reactive T cells to undergo apoptosis (Gardner et al. 2009). In order for this negative selection process to successfully occur, it requires a minimum expression threshold of the concerned antigen. Naïve T cells that receive TCR signals just below this threshold undergo a differentiation process to become natural regulatory T cells which function in an immunosuppressive manner (Picca et al. 2009). The presence of autoreactive T cells in MS patients, which also can be observed in healthy

individuals, demonstrates that central tolerance is not a completely efficient process (Gallegos and Bevan 2006).

Failure of self-reactive cells to be eliminated in the thymus can then be corrected by several mechanisms in the periphery known as peripheral tolerance. Aside from TCR-MHC interactions, T cells require additional costimulatory signals to become activated. The absence of these signals, which occurs when a T cell interacts with a "quiescent" APC that has not reacted to the presence of a pathogen or injury, results in T cell anergy and death (Goverman 2011). Extrinsically, peripheral tolerance can be induced through the presence of regulatory T cells (Treg). As cells that could potentially be autoreactive during negative selection become Treg, these then serve as natural immunosuppressive cells in the case of their aberrant activation by self-antigen (Picca et al. 2009). In fact, the ablation of Treg populations in healthy animals results in rapid induction of severe autoimmunity, indicating their constant role in peripheral tolerance (Kim et al. 2007a; Sakaguchi et al. 1995).

The sum of these processes still leaves the question of how myelin-reactive T cells are capable of persisting and inducing disease, despite several rounds of selection that should have resulted in their elimination. First, it is possible that certain self antigens are expressed at levels too low to induce self-tolerance. As myelin components are normally excluded from the periphery, the negative selection process is less efficient for these antigens (Targoni and Lehmann 1998). It is also possible that certain epitopes that are never presented in the thymus, and thus not utilized for negative selection, can potentially be presented in the periphery due to aberrant induction of antigen processing enzymes (Mamula 1993). In the case of MOG-induced

EAE, while the peptide fragment injected to promote the development of autoimmunity is exposed on the extracellular surface of myelin sheaths (Brunner et al. 1989), this autoantigen is specific to CNS myelin, and absent from peripheral myelin (Lebar et al. 1986). Further, as approximately 40% of MS patients are seropositive for IgG antibodies to MOG (Reindl et al. 1999), this could potentially explain how disease develops in both humans and mouse models.

T cells consist of several subsets: T helper cells, cytotoxic T cells, memory T cells, Treg, and natural killer T cells. Naïve T cells differentiate into these various subsets depending on the cytokine milieu present as well as the timing of the initial engagement of their TCR. Three main subsets of T helper cells are Th1, Th2 (Mosmann and Coffman 1989) and the more recently described Th17 cells (Langrish et al. 2005). Pro-inflammatory Th1 cell differentiation is promoted by exposure to IFNγ, which upregulate the transcription factor Tbet and produce TNFα and IFNγ; anti-inflammatory Th2 cells differentiate in the presence of IL4, express high levels of the transcription factor GATA3 and produce IL10, IL4, and IL13. T cells are key players in the development and recovery of MS, as Th1 cells predominate in EAE, but shifting the ratio towards Th2 cells ameliorates the disease (Weaver et al. 2005; Yoles et al. 2001). Th17 cells produce interleukin 17 (IL17), and differentiate when exposed to interleukin 6 (IL6) and TGFβ. Th17 cells are implicated in autoimmune inflammation (Lohr et al. 2006), and suppression of Th17 cells results in EAE disease recovery (Hao et al. 2010). However, when naïve T cells are exposed to TGFβ alone, they convert into Treg cells, which express the transcription factor FoxP3 and the cell surface receptor CD25. Tregs have been well characterized by their ability to suppress the activation of the immune

system and promote tolerance (Kohm et al. 2002; Liu et al. 2006; McGeachy et al. 2005). During the EAE disease course, Treg have been shown to be induced just prior to the start of the recovery phase (Ephrem et al. 2008; Ochoa-Reparaz et al. 2007).

Antigen presentation by innate immune cells is an essential component of T cell activation and functional modulation. CD4 and CD8 positive helper and cytotoxic T cells, respectively, require antigen presentation from target cells via MHC II. However, this signal alone is not enough to induce activation, as engagement of co-stimulatory molecules including CD80, CD86, and CD40 on antigen-presenting cells, and CTLA-4 and CD40L on T cells are necessary as MHC-TCR binding without these additional molecules leads to T cell death (Almolda et al. 2011; Chastain et al. 2011; Dhib-Jalbut 2007; Fletcher et al. 2010). Microglia are the resident antigen-presenting cells in the CNS, and they have been shown to promote differentiation in T cells in their activated state (Fischer and Reichmann 2001; Murphy and Stockinger 2010).

Tuftsin

Tuftsin is a naturally occurring tetrapeptide (T-K-P-R) that is derived from the proteolytic degradation of IgG (Najjar and Nishioka 1970). This component represents residues 282 to 292 of the heavy chain of γ-globulin, and is generated in the spleen by several enzymes (Fridkin and Najjar 1989). It was further shown to increase phagocytic activity in a variety of cells of monocytic origin, including macrophages and microglia. Tuftsin or tuftsin-like peptides can stimulate cell migration, chemotaxis, and respiratory burst (Siemion and Kluczyk 1999). When administered to immature bone marrow cells,

tuftsin strongly promoted the development of macrophage-lineage cells (Babcock et al. 1983). Most importantly, tuftsin administration to macrophages in the presence of splenic T lymphocytes promoted the antigen presenting capabilities of cells of monocytic origin (Tzehoval et al. 1978).

Tuftsin has been characterized as having various antimicrobial and antiviral effects. In mice infected with both listeria and pneumococcus, pretreatment with tuftsin showed enhanced microbial killing by macrophages. Further, over several bacterial species, tuftsin resulted in a significant augmentation of blood clearing of pathogens (Fridkin and Najjar 1989). Deficiency of naturally-derived tuftsin in healthy individuals manifests as severe recurrent infections involving primarily the skin, lymph nodes, and lungs (Najjar 1979). Further, naturally derived tuftsin was reduced in patients suffering from AIDS or AIDS-related complex, which possibly contributes to the increased risk of bacterial infection in HIV-positive individuals (Corazza et al. 1991). As tuftsin readily interacts with macrophage and microglial populations, and is only comprised of four amino acids, many studies are conjugating tuftsin molecules to other compounds to increase the efficiency of drug delivery in various diseases, including HIV treatment (Fridkin et al. 2005; Wardowska et al. 2009).

Tuftsin administration during EAE attenuates disease symptoms and improves recovery. During EAE, the predominant T cell transcription factor present is Tbet, indicating a Th1-mediated disease. However, tuftsin infusion increases GATA3 expression, indicating a shift towards anti-inflammatory Th2 cells that promote recovery (Bhasin et al. 2007). Several candidates for cell surface receptors for tuftsin were identified (Bump et al. 1986b), including neuropilin-1 (Nrp1) (von Wronski et al. 2006).

Neuropilin-1

The binding target for tuftsin has since been definitively identified as Nrp1 (von Wronski et al. 2006). Nrp1 has been shown to have roles mediating axonal guidance in the nervous system through binding Sema3A (Gavazzi 2001; Kolodkin et al. 1997), as well as binding vascular endothelial growth factor (VEGF) in cardiovascular development (Soker et al. 1998). It is also capable of being shed from the cell surface through proteolysis by ADAM10, although the function of this action is not well understood (Swendeman et al. 2008). In the immune system, Nrp1 has been shown to be important in the initiation of the primary immune response by mediating interactions between dendritic cells (DC) and T cells (Tordjman et al. 2002). Specifically between Tregs and immature DCs (iDC), homotypic binding of Nrp-1 allows for prolonged contact (Sarris et al. 2008).

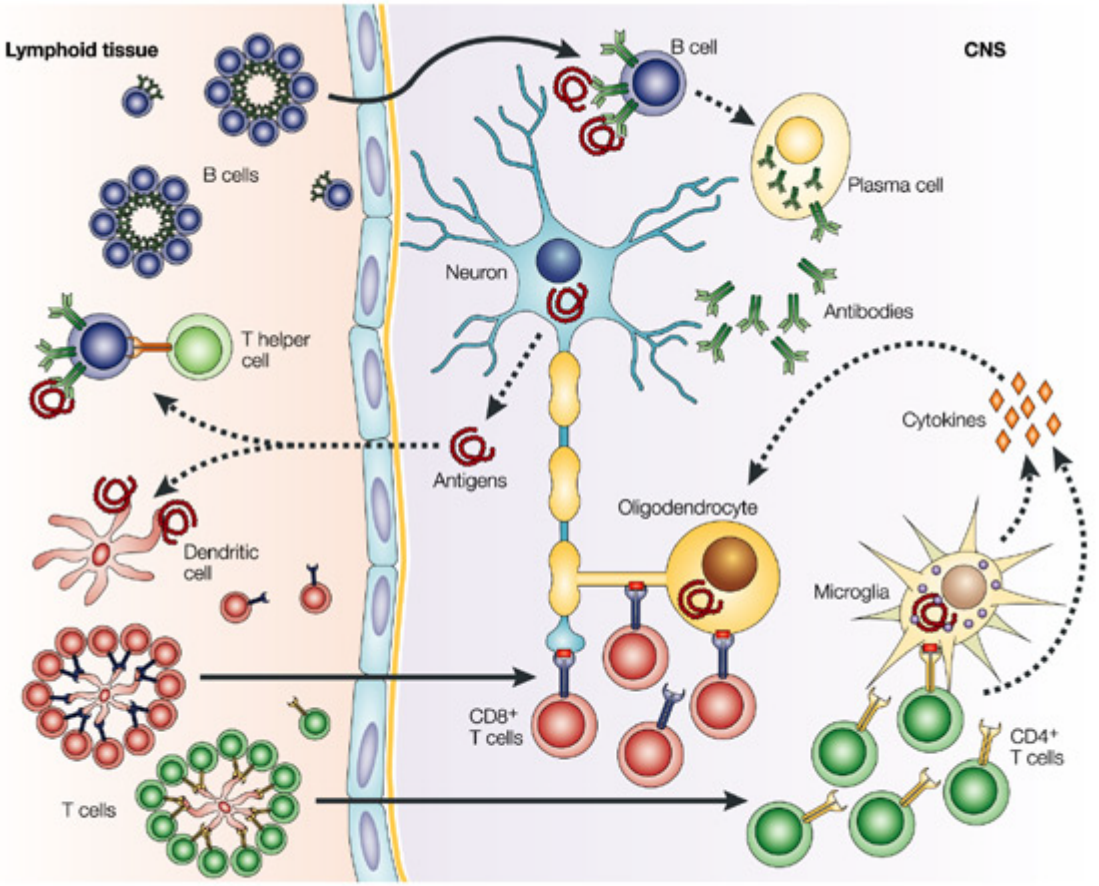
Microglia express Nrp1, which is upregulated under inflammatory conditions (Agudo et al. 2005; Majed et al. 2006). Nrp1 has also been described to be expressed on T cells, but their particular subtype distribution is debated. Tregs have been shown to express Nrp1, and exogenous production of FoxP3 in naïve T cells causes them to upregulate Nrp1 (Bruder et al. 2004; Sarris et al. 2008). However, it has recently been shown that activated T cells that are not of the Treg lineage are also capable of expressing Nrp1 (Milpied et al. 2009). Additionally, the cytokine TGFβ, in both its active and its latent form, can bind to Nrp1. Nrp1 is capable of converting this molecule to an active form, and can promote Treg proliferation and activity (Glinka and Prud'homme 2008). Nrp1 is critical for maintaining tolerance and attenuating autoreactivity in EAE (Solomon et al. 2011). As Nrp1 is a key receptor in the immune system, is expressed on

both microglia and T cells, and is a binding partner for tuftsin, it is an important prospective mediator for tuftsin's effect both *in vitro* and *in vivo*.

These observations lead to the hypothesis that tuftsin's beneficial effects during EAE are mediated through its receptor Nrp1. A potential model for tuftsin's action is that tuftsin binds to Nrp1 receptors on microglia, which causes polarization to an M2 phenotype. These microglia then produce IL10 and TGFβ, which polarize naïve T cells to a Treg/Th2 phenotype. Additionally, microglia would come in close contact with Treg through homotypic Nrp1 interactions, and promote Treg function through TGFβ production.

FIGURES

Figure I-1: Immune responses in multiple sclerosis. Figure from (Hemmer et al. 2002)



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Chapter II

General Materials and Methods

Animals

C57BL/6, Csf1R-cre (Deng et al. 2010), and $Nrp1^{fl/fl}$ (Gu et al. 2003) mice were bred in-house under specific pathogen-free conditions set by the Division of Laboratory Animal Resources at Stony Brook University. The environment was controlled for temperature (21°C), and maintained under a 12-hour light/dark cycle. Access to food and water was *ad libitum*. All procedures were approved by the IACUC committee at Stony Brook University.

MOG peptide

MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by the Yale University Peptide Synthesis Facility and purified using reverse-phase (C18) HPLC.

Induction of EAE with MOG_{35-55} peptide

EAE was induced as described previously (Bernard et al. 1997; Bhasin et al. 2007; Lu et al. 2002) by subcutaneous injection into the mouse flank on day 0 with 300 µg of MOG_{35-55} peptide thoroughly emulsified in complete Freund's adjuvant (CFA) containing 500 µg of heat-inactivated Mycobacterium tuberculosis (Difco, Detroit, MI). One week later (day 7), the mice were boosted with 300 µg of MOG_{35-55} peptide subcutaneously in the other flank. 500 ng Pertussis toxin (List Biologicals, Campbell, CA) in 200 µl of PBS was injected intraperitoneally on days 0 and 2. All EAE experiments were performed using littermate controls.

Evaluation of EAE symptoms

After immunization with MOG, mice were observed and weighed daily blindly. Symptom severity was scored on a scale of 0 to 5 with gradations of 0.5 for intermediate symptoms. The score is defined as follows (Hjelmstrom et al. 1998): 0, no detectable symptoms; 1, loss of tail tone; 2, hindlimb weakness or abnormal gait; 3, complete paralysis of the hindlimbs; 4, complete hindlimb paralysis with forelimb weakness or paralysis; 5, moribund or dead.

Time-controlled Drug Delivery

Alzet mini-osmotic pumps (Durect, Cupertino, CA) were used to ensure time-controlled drug delivery. 28-day pumps (rate of infusion 0.25 µl/hr, 250 µl total volume) were filled with either PBS or 500 µM tuftsin (Sigma) and incubated at 37C overnight before using. Adult female C57BL/6 mice (8-10 weeks old) were deeply anesthetized, and pumps were implanted subcutaneously in the back of the mice the same day as the initial MOG immunization.

Tissue collection and processing

Mice were deeply anesthetized with intraperitoneal injection of 2.5% avertin (0.02 ml/g body weight) and then transcardially perfused using PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in PBS (pH 7.4). Spinal cords were isolated, post-fixed in 4% PFA at 4^{o}C overnight, and dehydrated in 30% sucrose at 4^{o}C until the samples sank. After the meninges were removed, the spinal cord was cut into equal sections,

embedded in optimal cutting temperature compound (Tissue Tek), frozen and stored at -80^{o}C until use.

Immunoflurorescence

Cells used for immunofluorescence were fixed for 30 minutes at room temperature in 4% PFA, while slides were incubated for 5 minutes in PBS to remove residual OCT. After washing with PBS, samples were blocked in serum of the host of the secondary antibody (5% serum and 0.3% BSA in PBS with 0.2% TritonX-100), and then incubated overnight at 4°C with rabbit anti-mouse Nrp1 (Abcam), rabbit anti-mouse Iba1 (Wako), mouse anti-mouse iNOS (BD Biosciences), and mouse anti-mouse Arginase1 (BD Biosciences) in 0.3% BSA in PBS with 0.2% TritonX-100. After washing with PBS, samples were incubated with fluorescence-conjugated FITC or Cy3 goat anti-rabbit or rabbit anti-mouse secondary antibody and Streptavidin-conjugated Cy3 (to detect bound biotinylated tuftsin) for 1 hour at room temperature, washed 3 times with PBS, and mounted using Fluoromount-G with DAPI (Southern Biotech, USA). These were then visualized with a Nikon Eclipse E600 microscope (for surface tuftsin and Iba1 staining) or a Zeiss LSM 510 confocal microscope (for iNOS and Arginase1 staining).

Fluoromyelin staining

Slides were rehydrated in PBS for 5 minutes, incubated with fluoromyelin staining solution (1:300, Invitrogen) for 20 minutes at room temperature, washed, and mounted

with Fluoromount-G (Southern Biotech). The ImageJ freeware (NIH) was used to measure the demyelinated and total areas of WM as described below

$$Demyelinated\ area\ (\%) = \frac{Demyelinated\ area\ in\ white\ matter}{Total\ white\ matter\ area} * 100\%$$

Fluorescent image quantification

All tissues collected to be used for quantification were sectioned serially utilizing the entire tissue, such that each individual slide was representative of a complete cross section. For Iba1 and fluoromyelin detection, images of six sections per slide containing the entirety of the coronal section of the spinal cord were obtained, and quantified for intensity (Iba1) or myelinated area (fluoromyelin) as described above in ImageJ software. For arginase-1 and iNOS, slides were stained with either arginase-1/Iba1 or iNOS/Iba1. Three coronal sections per slide were selected for quantification, with six images within the white matter taken per section. These were then quantified for the total number of double positive cells/Iba1-positive cells. One representative slide was utilized for each biological replicate, with tissue from three separate mice quantified per genotype/per treatment/per timepoint.

Primary T cell culture

Primary T cells from mouse spleens were isolated using the CD4+ mouse T cell negative isolation kit (Invitrogen) by the manufacturer's protocol. Briefly, a mixture of monoclonal antibodies against unwanted cells (B cells, NK cells, monocytes, dendritic

cells, $CD8^+$ T cells, erythrocytes and granulocytes) was added to spleen cells. T cells were isolated by removing the antibody-labeled cells using mouse depletion Dynabeads and a Dynal MPC. The $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (10 µg/ml, BD Biosciences) antibody and soluble anti-CD28 (1:1000, Biolegend), serving as 'artificial APCs' (Funderburg et al. 2008).

Generation of retroviral Cre

Retrovirus containing a Cre expression construct (HR-MMPCreGFP) was generated as previously described (Silver and Livingston 2001). Phoenix cells were transfected with a plasmid containing both Cre and GFP sequences (Fig. II-1) using Lipofectamine in serum-free Opti-MEM media. After 12 hours, the media was changed to DMEM with 10% FBS and penicillin/streptomycin. After 24 hours, viral containing media were collected and used freshly in downstream application.

Regulatory T cell culture

Tregs were generated using the Millipore FlowCellect Treg differentiation kit. In short, CD4+ helper T cells were isolated from the spleen of $Nrp1^{fl/fl}$ mice as described above. These were then cultured in a CD3 coated plate, and treated with two stages of T cell activator solutions as per kit instructions. On day 4 of the seven day procedure, Tregs for Nrp1 knockout groups were treated with retroviral-Cre containing media isolated from Phoenix-Eco cells for three days to allow for incorporation of the Cre gene and the excision of the $Nrp^{fl/fl}$ sequence generating a Treg-specific Nrp1 knockout.

Mixed cortical and primary microglia cultures

Microglia were isolated as previously described (Rogove and Tsirka 1998). In short, newborn (d0-d2) pups of wild-type mice were used to isolate cortical cells. The brains were removed, and cortices were freed from meninges, hippocampi and basal ganglia, and then digested in 0.25% Trypsin/EDTA (Sigma) at 37 ^{o}C for 20 minutes. To obtain a single-cell suspension, the tissue was then triturated and filtered through a 40 μm cell strainer, and plated in mixed cortical medium (DMEM, 10% FBS, 40 μg/ml Gentamycin). Tissue culture plates used for plating mixed cortical cultures were coated overnight at 4^{o}C with 5 μg/ml poly-D-lysine (PDL, Sigma).

The medium was changed 3 days after plating, and microglial cells were harvested 10 days after plating. Briefly, lidocaine was added directly to the culture medium at a final concentration of 1 mM and the culture left at room temperature for 15 minutes. The medium containing the floating microglia was collected and centrifuged at 500g for 5 min, following which the cell pellet was resuspended in microglia medium (DMEM, 1% FBS) and cells were counted on a hemocytometer. Cultures are >98% pure, as previously described (Rogove and Tsirka 1998; Siao and Tsirka 2002; Yao and Tsirka 2010).

Inhibitors used to treat microglia were diluted in DMSO, and were EG00229, a Nrp1 inhibitor (Jarvis et al. 2010) at 15, 30 or 50 μM final concentration; LY 364947, a TβR1 inhibitor at 10 μM final concentration; and PF 04217903, a c-Met inhibitor (Tocris) at 5 nM final concentration. Microglial cells were plated at a density of 5 x 10^5 cells per 35mm plate, and neuronal conditioned medium (NCM) was utilized in a 1:1 ratio to microglial culture medium.

Primary Neuronal Cultures

Primary cortical neuronal cultures were prepared from embryonic day 14-15 mice as previously described (Siddiq and Tsirka 2004). Briefly, mouse cortices were dissected and put in Hanks solution (HBSS), and triturated to form single cell suspensions. The cells were plated at a density of 100,000 cells/cm^2 in Neurobasal medium with B27 supplements, 25μM glutamate, 0.5 mM L-glutamine and 10 g/L gentamycin sulfate. Tissue culture plates used for plating neuronal cultures were coated overnight at 4 ^{o}C with 5 μg/ml poly-D-lysine (PDL, Sigma). Cells were utilized for experiments after seven days *in vitro*.

NCM was prepared from primary cortical neurons exposed overnight to 100 μM glutamate added directly to the culture medium to induce excitotoxic injury. Prior to treating microglia with NCM, the media supernatant was spun down to remove debris.

Proteomic analysis of conditioned media

Sample Preparation:

Samples for proteomic analysis were derived from serum- and supplement-free conditioned media, and were concentrated in Amicon Ultra-15, UltraCel 3 k columns (ThermoFisher) to acquire appropriate concentrations for digestion. Protein concentration was determined by the EZQ protein quantification assay (Invitrogen, CA) according to the manufacturer suggested protocol.

Trypsin Digestion:

50 µg of proteins from the conditioned media were diluted in 50 mM NH_4HCO_3 solution for trypsin digestion. Trypsin was added to each sample at a ratio of 1:30 enzyme/protein along with 2 mM $CaCl_2$ and incubated for 16 hours at 37°C. Following digestion, all reactions were acidified with 90% formic acid (2% final) to stop proteolysis. Then, samples were centrifuged for 30 minutes at 14,000 rpm to remove insoluble material. The soluble peptide mixtures were collected for LC-MS/MS analysis.

Multidimensional chromatography and tandem mass spectrometry:

Peptide mixtures were pressure-loaded onto a 250 µm inner diameter (i.d.) fused-silica capillary packed first with 3 cm of 5 µm strong cation exchange material (Partisphere SCX, Whatman), followed by 3 cm of 10 µm C18 reverse phase (RP) particles (Aqua, Phenomenex, CA). Loaded and washed microcapillaries were connected *via* a 2 µm filtered union (UpChurch Scientific) to a 100 µm i.d. column, which had been pulled to a 5 µm i.d. tip using a P-2000 CO_2 laser puller (Sutter Instruments), then packed with 13 cm of 3 µm C18 reverse phase (RP) particles (Aqua, Phenomenex, CA) and equilibrated in 5% acetonitrile, 0.1% formic acid (Buffer A). This split-column was then installed in-line with a NanoLC Eskigent HPLC pump. The flow rate of channel 2 was set at 300 nl/min for the organic gradient. The flow rate of channel 1 was set to 0.5 µl/min for the salt pulse. Fully automated 11-step chromatography runs were carried out. Three different elution buffers were used: 5% acetonitrile, 0.1% formic acid (Buffer A); 98% acetonitrile, 0.1% formic acid (Buffer B); and 0.5 M ammonium acetate, 5% acetonitrile, 0.1% formic acid (Buffer C). In such sequences of chromatographic events, peptides are sequentially eluted from the SCX resin to the RP resin by increasing salt steps (increase in Buffer C concentration), followed by organic

gradients (increase in Buffer B concentration). The last chromatography step consists in a high salt wash with 100% Buffer C followed by acetonitrile gradient. The application of a 2.5 kV distal voltage electrosprayed the eluting peptides directly into a LTQ-Orbitrap XL mass spectrometer equipped with a nano-LC electrospray ionization source (ThermoFinnigan). Full MS spectra were recorded on the peptides over a 400 to 2000 m/z range by the Orbitrap, followed by five tandem mass (MS/MS) events sequentially generated by LTQ in a data-dependent manner on the first, second, third, and fourth most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFinnigan, San Jose, CA).

Database search and interpretation of MS/MS datasets:

Tandem mass spectra were extracted from raw files, and a binary classifier - previously trained on a manually validated data set - was used to remove the low quality MS/MS spectra. The remaining spectra were searched against a UniProt mouse protein database released on May, 3rd 2011 (Kersey et al. 2004) and 124 common contaminant proteins. To calculate confidence levels and false positive rates, we used a decoy database containing the reverse sequences of the UniProt protein database appended to the target database (Elias and Gygi 2007), and the SEQUEST algorithm to find the best matching sequences from the combined database. SEQUEST searches were done through the Integrated Proteomics Pipeline (IP2, Integrated Proteomics Inc., CA) on Intel Xeon X5450 X/3.0 PROC processor clusters running under the Linux operating system. The peptide mass search tolerance was set to 50 ppm. No differential

modifications were considered. A fully tryptic status was imposed on the database search.

The validity of peptide/spectrum matches was assessed in DTASelect2 (Eng et al. 1994) using SEQUEST-defined parameters, the cross-correlation score (XCorr) and normalized difference in cross-correlation scores (DeltaCN). The search results were grouped by charge state (+1, +2, and +3) and tryptic status (fully tryptic, half-tryptic, and non-tryptic), resulting in 9 distinct sub-groups. In each one of the sub-groups, the distribution of XCorr and DeltaCN values for (a) direct and (b) decoy database hits was obtained, and the two subsets were separated by quadratic discriminant analysis. Outlier points in the two distributions (for example, matches with very low Xcorr but very high DeltaCN) were discarded. Full separation of the direct and decoy subsets is not generally possible; therefore, the discriminant score was set such that a false discovery rate of 1% was determined based on the number of accepted decoy database peptides. This procedure was independently performed on each data subset, resulting in a false positive rate independent of tryptic status or charge state. In addition, a minimum sequence length of 7 amino acid residues was required, and each protein on the final list was supported by at least two independent peptide identifications unless specified. These additional requirements – especially the latter - resulted in the elimination of most decoy database and false positive hits, as these tended to be overwhelmingly present as proteins identified by single peptide matches. After this last filtering step, the false discovery rate was reduced to below 1%. Relative fold difference between samples was derived using the spectral counting method (Carvalho et al. 2008; Liu et al. 2004).

Immunoblotting

Cells were lysed in 50 mM Tris-HCl (pH 7.4) containing 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1% SDS, and protease inhibitor cocktail (Sigma-Aldrich). After incubation on ice for 10 minutes, the lysates were centrifuged at 13,000 RPM for 10 minutes to remove debris, and the supernatant was collected. The extracts were separated on a reducing 10% SDS-PAGE gel, and blotted to PVDF membrane (Immobilon-P; Millipore). Membranes were then incubated overnight at 4^{o} C with anti-phospho-Smad3 or anti-phospho-Akt (1:1000, cell signaling) primary antibodies. These were then incubated with HRP-conjugated anti-rabbit secondary for 1 hour at room temperature, and visualized with ECL (Pierce). After stripping, membranes were reprobed with anti-Smad3 or anti-Akt antibodies (1:1000, cell signaling).

RNA isolation and quantitative real-time PCR

To prepare RNA, primary microglia that were treated for 10 hours were washed with PBS and lysed with RNA-Bee (Tel-Test), by the manufacturer's protocol. To obtain cDNA, one microgram of RNA was reverse transcribed on a Veriti Thermocycler (Applied Biosystems) using the High Capacity cDNA Reverse Transcription kit. Amplification was performed on a StepOnePlus real-time PCR machine using a SYBR Green kit (Applied Biosystems). Primer sequences are as follows: GAPDH forward, 5'-GCACAGTCAAGGCCGAGAAT-3'; GAPDH reverse, 5'-GCCTTCTCCATGGTGGTG GA-3'; IL-10 forward, 5'-TGGCCACACTTGAGAGCTGC-3'; IL-10 reverse, 5'-TTCAGGGATGAAGCGGCTGG-3'; TNFα forward, 5'-ATGAGCACAGAAAGCATGATC-3'; TNFα reverse, 5'-TACAGGCTTGTCACTCGAATT-3'; Nrp1 forward, 5'-

GGGCAGAGACTGCAAGTATGA-3'; Nrp1 reverse, 5'-AGAAATGGCCCTGAAGACAC-3'. For gene expression analysis, the relative quantitation method was used (ΔΔCt) with GAPDH as an internal control.

Tuftsin binding assay

To observe tuftsin binding to the cell surface in the presence or absence of Nrp1 inhibitor EG00229, primary microglia cells were exposed to varying concentrations of inhibitor at 37° C for 1 hour, and then treated with 100 μg/ml of biotinylated tuftsin (GeneScript) for 30 minutes at room temperature to minimize internalization of the peptide. After thorough washing, tuftsin's presence on the cell surface was observed by fixation of the non-permeabilized cells and staining with Cy3-conjugated streptavidin.

Measurement of cytokine levels

Primary microglial cells were treated with combinations of NCM, 100 μg/mL tuftsin, 10 μM TβR1 inhibitor, 5 nM c-Met inhibitor, or 30 μM EG00229 for 10 hours. Media was collected after 4 days of co-culture in microglial-Treg interaction experiments. Prior to analysis by ELISA, media supernatants were spun down to remove debris.

To measure TGFβ levels, the Ready-Set-GO TGFβ cytokine ELISA kit from eBioscience was utilized. Briefly, 96-well plates were coated overnight with the appropriate dilution of capturing antibody in coating buffer (0.2 M sodium phosphate buffer pH 6.5). The plate was washed three times with PBS-T (PBS, 0.05% Tween-20)

and blocked with Assay Diluent for 1 hour at RT. To activate latent TGFβ1, samples were treated with 20 µl of 1N HCl per 100 µl of sample for 10 minutes, and then neutralized with 1N NaOH. After washing as before, 100 µl of sample or cytokines standard prepared in assay diluent were added followed by 2 hour incubation at RT. After 5 washes with PBS-T, 100 µl of assay diluent containing biotin-conjugated detection antibody and Avidin-HRP reagent at the appropriate dilutions were added and incubated for 1 hour at RT. Following 7 washes with PBS-T, 100 µl of Substrate Solution was added to each well. After 30 min incubation in the dark, 50 µl of Stop Solution (2N H_2SO_4) were added and absorbance at 450 nm was read within 30 minutes on a SpectraMax microplate reader using the Softmax Pro software. Final readings were calculated with a dilution factor of 1.4 to account for acid activation/neutralization.

Measurement of nitrite levels

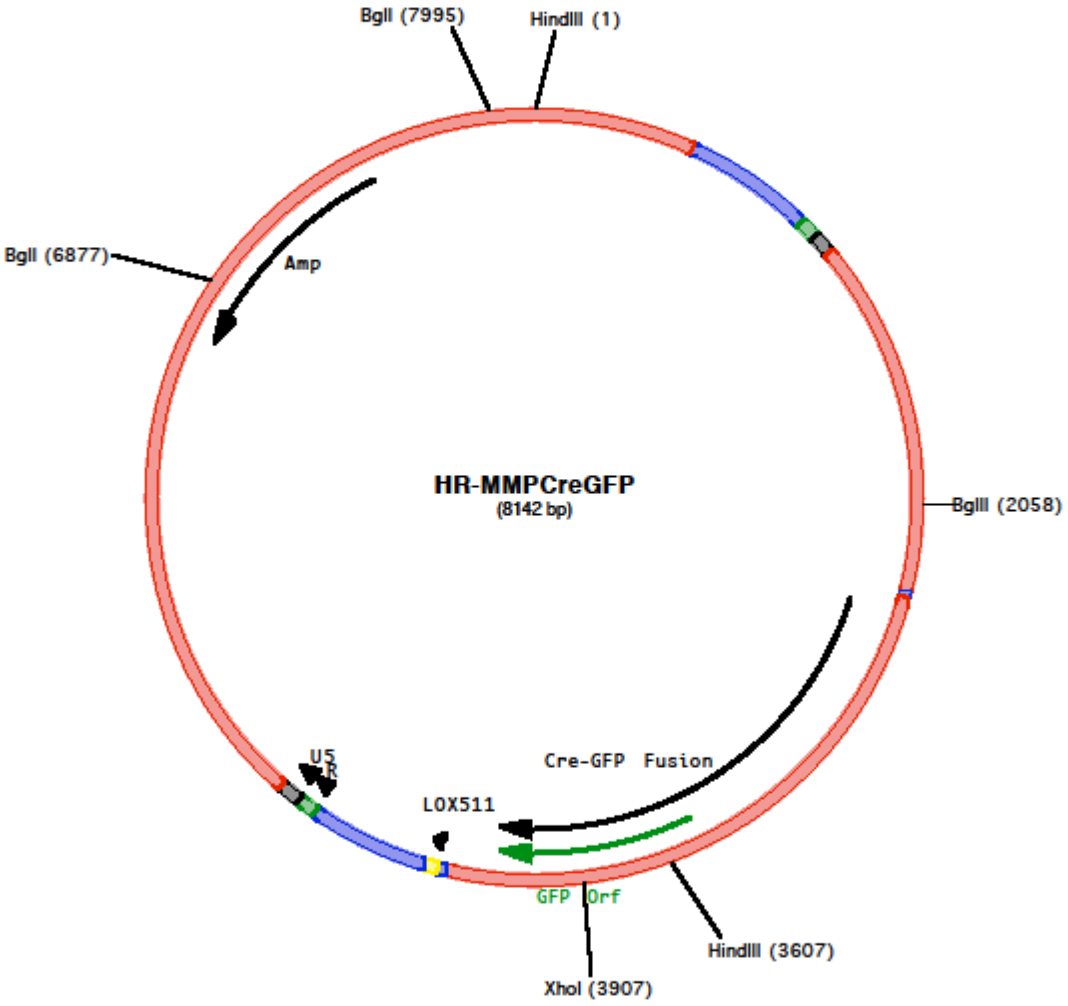
To observe nitric oxide levels, a nitrite assay was performed. Nitrite level measurement was based on the reaction of nitrites with 2,3-diaminonaphthalene (DAN) under acidic conditions, which results in 2,3-naphthyltriazole formation. Briefly, 100 µl of sample or standard ($NaNO_2$) was loaded in a black 96-well plate with a clear bottom. 20 µl of DAN (0.05 mg/ml in 0.62 M HCl) was added to each well. After proceeding at RT for 20 minutes, the reaction was terminated by the addition of 100 µl 0.28 M NaOH. After an additional 10 minute incubation at RT, fluorescence was measured on a Titertek Fluoroscan II fluorescence plate reader using a filter pair with excitation at 355 nm and emission at 460 nm.

Statistics

For multiple comparisons within a group, statistical analysis was performed using one-way ANOVA followed by a Bonferroni-Dunn test. For comparisons between groups, a two-tailed t-test was used, as indicated by the figure legends. For all figures, $p < 0.05$ was considered significant and is marked by *; $p < 0.01$ and $p < 0.001$ were considered very significant and are marked by ** and *** respectively. All results are represented as average with error bars indicating the standard error of the mean. In all experiments, n refers to the number of biological replicates used for each condition.

FIGURES

Figure II-1: Composition of the HR-MMPCreGFP plasmid.


BglI (7995)
HindIII (1)
BglI (6877)
Amp
HR-MMPCreGFP
(8142 bp)
BglII (2058)
U5
LR
Cre-GFP Fusion
LOX511
GFP Orf
HindIII (3607)
XhoI (3907)

Chapter III

Tuftsin polarizes microglia to an anti-inflammatory, M2 phenotype

INTRODUCTION

Microglia are macrophage-like innate immune cells normally resident in the CNS. During MS and EAE, they become activated and contribute to the inflammatory process through several mechanisms, including phagocytosis and production of various factors such as cytokines, free radicals, and metalloproteinases (Chao et al. 1992). However, the effects of microglia are not solely deleterious; microglia also produce anti-inflammatory cytokines, such as TGFβ and IL10 (Rott et al. 1994), which are associated with inhibition or prevention of EAE. The timing and strength of these protective and neurotoxic outputs determine which overall effect predominates. Microglia are capable of polarizing into pro- and anti-inflammatory subsets known as M1 and M2. Classically activated M1 microglia, which are neurodegenerative in a model of spinal cord injury, produce TNFα and NO. On the other hand, alternatively activated, neuroprotective M2 microglia release IL10 and TGFβ (Gordon and Martinez 2010; Kigerl et al. 2009; Michelucci et al. 2009). Additionally, administration of M2 monocytes in EAE suppressed ongoing severe symptoms (Mikita et al. 2010).

T cells undertake the primary role in modulating the outcome of MS/EAE. Naïve T cells can differentiate into helper (Th) and regulatory cells (Tregs). There are three subsets of T helper cells: Th1, Th2 (Mosmann and Coffman 1989) and the more recently described Th17 cells (Langrish et al. 2005). Similarly to microglia, Th1 cells produce proinflammatory cytokines (e.g. TNFα) and mediate proinflammatory responses during MS/EAE, whereas Th2 cells secrete anti-inflammatory cytokines [e.g. IL4, IL10, and IL13] and participate in prevention or remission of MS/EAE. Th17 cells produce IL17 and play a pathogenic role in inducing autoimmune tissue inflammation

(Lock et al. 2002; Lohr et al. 2006; Wilson et al. 2007). In the presence of TGFβ, naive T cells become Tregs, which express FoxP3 and suppress immune system activation (Kohm et al. 2002; Liu et al. 2006; Lohr et al. 2006; McGeachy et al. 2005; Olivares-Villagomez et al. 1998).

Tuftsin is a naturally occurring tetrapeptide (threonine-lysine-proline-arginine) that was described originally as a phagocytosis-stimulating factor derived from the proteolytic processing of IgG (Nishioka et al. 1973). Tuftsin promotes phagocytic activity for cells of monocytic origin, such as neutrophils, macrophages and microglia, all of which are thought to express tuftsin receptors. Tuftsin or tuftsin-like peptides also exert other stimulatory effects, including enhanced migration/chemotaxis and antigen presentation and can affect T cell function as well (Siemion and Kluczyk 1999). Moreover, tuftsin may have direct effects on the nervous system, including induction of analgesia (Herman et al. 1981) and inhibition of axonal regeneration (Thanos et al. 1993).

Previous work in our laboratory revealed that modification of the status of microglia affected the timing and symptoms of EAE (Bhasin et al. 2007). In particular, the microglial activator tuftsin, which readily crosses into the CNS (Paul et al. 2000), decreased the severity of EAE symptoms and drastically improved recovery in wild-type mice. Real-time PCR data revealed that wild-type EAE mice exhibited prevalent T-bet expression, which is a transcription factor that promotes Th1 lineage development and cytokine production. Conversely, tuftsin infusion into wild-type mice subjected to EAE resulted in increased GATA3 expression, which is a transcription factor that drives Th2 lineage development and release of anti-inflammatory cytokines.

Recent studies in our lab (Wu et al. 2012) investigated the role of tuftsin in polarizing the T cell response *in vitro*. When T cells were incubated directly with tuftsin, they showed an increase in TNFα production by ELISA. This result indicated a Th1 response, which did not correspond with prior *in vivo* data (Bhasin et al. 2007). However, if primed microglia were exposed to tuftsin and their conditioned media was transferred to T cells, the T cells showed decreased TNFα production and increased IL-10 production, which points towards a Th2 mediated polarization. These data strongly indicate that microglial modulation of tuftsin is responsible for the beneficial effects seen *in vivo*. In this study, we used both *in culture* and *in vivo* methods to investigate the mechanism through which tuftsin modulates the immune response in EAE. Our results show that *in culture*, modulating microglial activity with tuftsin affects cell fate by downregulating their proinflammatory responses and upregulating anti-inflammatory cytokines. *In vivo* during EAE, tuftsin infusion promotes dominance of an M2 phenotype, as compared to saline infused animals that predominantly have M1 microglia. Finally, we show that tuftsin promotes the upregulation and release of its receptor Nrp1 from microglial cells.

RESULTS

Tuftsin-activated microglia exposed to excitotoxic media polarize to an anti-inflammatory, M2 phenotype.

Previously, we have shown that T cells shift to a Th2 subset when treated with conditioned media from microglia exposed to neuronal conditioned media (NCM) and tuftsin (Wu et al. 2012). This culture system was devised to mimic *in vitro* the cellular events that occur during MS and EAE. Primary neurons were cultured overnight to generate NCM in the presence of 100 μM glutamate, which mimics the early excitotoxic events of EAE (Pitt et al. 2000). Here, we proposed that the combination of tuftsin and NCM would cause microglia to shift to an anti-inflammatory, M2 phenotype, which could explain the prevalence of Th2 cytokines in our earlier work.

As microglial involvement is necessary for mediating tuftsin's beneficial effects on T cells *in culture* (Wu et al. 2012), we analyzed the changes that occur in the treated microglia themselves. To observe the effect of tuftsin and NCM on microglial subtype, we treated primary microglia for 10 hours with NCM and increasing concentrations of tuftsin as previously performed on T cells (Wu et al. 2012). From these cells we harvested RNA and performed quantitative real-time PCR to determine microglial phenotype based on TNFα levels for M1, and IL10 levels for M2. The combination of NCM and tuftsin at all concentrations significantly affected M1 and M2 specific genes in the microglia, with a 3-fold increase in IL10 and a 3-fold decrease in TNFα (Figure III-1A,B). More dramatically, when observing the ratio of the change in M2 to M1 genes, there is greater than a 10-fold shift towards neuroprotection (Figure III-1C). Surprisingly, NCM alone shows a slight shift towards neuroprotection, with a decrease in TNFα and

an increase in IL10 gene expression. However, the combination of tuftsin and NCM shows a significantly higher upregulation of IL10 than NCM alone, and the degree of neuroprotection represented by the M2/M1 ratio for NCM alone is significantly less than the combination of NCM and tuftsin. Additionally, it has been shown that moderate neuronal cell death can cause microglia to assume a more protective phenotype, which could explain this phenomenon (Lai and Todd 2008). Thus, these experiments indicate that the combination of NCM and tuftsin promotes an anti-inflammatory, M2 phenotype in microglia, which is the likely cause for the polarization of T cells towards a Th2 phenotype as shown above.

Tuftsin infusion during EAE promotes an M2 shift in microglial cells *in vivo*.

Although we showed that microglia exposed to tuftsin and NCM polarize to an M2 phenotype *in culture*, this system does not accurately recapitulate the totality of the cellular events that occur during EAE *in vivo.* To determine whether microglia polarize to M2 *in vivo*, we infused mice with tuftsin via osmotic pump for 28 days during the EAE disease course.

iNOS, a marker for M1 microglia was detectable starting at day 14 after EAE induction in saline infused mice, which continued through day 28. The expression level of iNOS was most pronounced at day 21, the peak of the disease course, in control mice. However, this was only detectable at very low levels on day 21 in tuftsin infused mice (Figure III-2). When observing arginase-1 levels, a marker for M2 microglia, both tuftsin and control mice showed similar levels of expression, particularly during the recovery phase on day 28. (Figure III-3). As both tuftsin and saline infused mice have

comparable M2 microglial levels, but saline infused mice have a strong M1 upregulation at day 21 in particular, control mice have an overall M1 predominance while tuftsin infused mice have an overall M2 predominance. As MS/EAE is an M1-mediated disease, this could explain why tuftsin infused mice have attenuated disease scores (Bhasin et al. 2007).

Tuftsin promotes the upregulation of its receptor Nrp1 in microglia under activating conditions.

Receptors for tuftsin have long been discussed in the literature. Originally, using affinity chromatography, two binding activities were reported in peritoneal granulocytes which indicated a potential for two receptors (Bump et al. 1986a). More recently, the single-pass transmembrane glycoprotein Nrp1 was identified as a receptor for tuftsin (von Wronski et al. 2006). Nrp1 has been shown to play key roles in development, immunity, and cancer (Gu et al. 2003; Sarris et al. 2008).

To investigate whether microglial cells upregulate Nrp1 in response to tuftsin, we exposed primary microglia to tuftsin or NCM alone, or a combination of both, for 10 hours. While untreated cells, as well as singly treated cells, had similar expression levels of Nrp1, those exposed to the combination treatment showed a visible increase in Nrp1 staining intensity. Additionally, all treatment conditions excluding the untreated control appeared to have promoted activation in microglia, apparent through the change in their morphology from a ramified, resting state to a more amoeboid shape (Figure III-4).

Microglia shed factors in response to tuftsin treatment.

To determine what components present in the microglial conditioned medium could act on T cells, we repeated the experiment described in Figure III-1, collected and concentrated media from treated microglia. The control microglial conditioned media, which was derived from microglia exposed to NCM alone (MgCM), and samples obtained from microglia treated with NCM and tuftsin (MgCM + tuftsin) were concentrated and digested with trypsin and subjected to shotgun proteomic analysis. Several proteins were identified whose expression was upregulated with tuftsin treatment, a short list of which is shown (Table III-1). The presence of some of these factors (e.g., PLD3, Nrp1) were confirmed by western blot analysis (Figure III-5). Most interestingly, Nrp1, tuftsin's receptor (von Wronski et al. 2006), was upregulated in the MgCM+Tuf treatment. The presence of this factor in the analyzed conditioned media, despite its role as a cell surface receptor, is likely due to its shedding from the cell surface by the protease ADAM10 (Crawford et al. 2009), which was also present in the analyzed conditioned media.

DISCUSSION

Tuftsin's broad activities on phagocytotic cells, especially microglia and macrophages, make the peptide a potential candidate for immunotherapy. In our previous work, we reported that early activation of microglia/macrophages by tuftsin abrogated clinical symptoms in EAE mice, and this correlated with a switch towards increased expression of a Th2 transcription factor (Bhasin et al. 2007). Additionally, using an *in culture* system that mimicked cellular events that occur during EAE, we showed that media isolated from tuftsin-modulated microglia polarized T cells to their anti-inflammatory subsets (Wu et al. 2012). Here, we have found that after tuftsin treatment, markers of the M2 phenotype are upregulated, suggesting that there is activation and expansion of the protective M2 subset. In MS/EAE, the balance between the pro-inflammatory Th1 response and the anti-inflammatory Th2 response, together with the beneficial effect of the regulatory T cells, is thought to determine the disease outcome. As tuftsin was incapable of promoting a Th2 response in T cells directly, and only could achieve this through microglial modulation, it is clear that microglia are essential mediators of this drug.

The concept of protective autoimmunity suggests that in the CNS, autoimmunity is not only a result of immune dysfunction, but is a mechanism by which the body protects itself against destructive self compounds (Schwartz and Kipnis 2005). This concept was demonstrated in a model of optic nerve crush injury, where spontaneous T cell-mediated neuroprotection was correlated with early onset of phagocytic activity and antigen presentation in microglia, which is characteristic of the M2 subset (Shaked et al. 2004). Early activation of microglia in EAE by tuftsin rather than their activation by

inflammatory cytokines and injury could potentially promote their preferential interaction with anti-inflammatory T cell subsets prior to severe demyelinating disease. Our current studies confirmed the idea that early activation of microglia by tuftsin results in a coordination of the immune response that favors protective autoimmunity as opposed to autoimmune disease.

Microglia have been shown to play an important role in the demyelination process, both through the induction of the inflammatory cascade by releasing pro-inflammatory cytokines (Kigerl et al. 2009), and through interactions with MBP. In the CNS, tissue plasminogen activator recruits and activates microglia, which then promote demyelination through contact with MBP peptides in the myelin sheath (Abe et al. 2003; Veeravalli et al. 2009). On the other hand, microglia have been shown to play important roles in the remyelination process, both through the phagocytosis of debris and support of myelinating oligodendrocytes (Olah et al. 2012), and also through promotion of axonal regeneration. From the data presented here, it is likely that the dual roles of microglia in the myelination process is also explained by the presence of M1 and M2 microglial subsets, of which we have shown tuftsin to be capable of promoting the M2, anti-inflammatory phenotype in EAE.

Many treatments for MS aim at repression of the immune response. Some reagents work by inhibiting activation of microglia and T cells and cause a downregulation of the production of proinflammatory cytokines (Stanislaus et al. 2005). These treatments attenuate MS symptoms through their non-specific “anti-inflammatory effects.” Tuftsin, however, appears to affect MS/EAE symptoms in a different manner, by not only suppressing inflammation, but also by shifting the immune response towards

an anti-inflammatory phenotype. Tuftsin functions by modulating microglial behavior towards the M2 phenotype, which promotes a downstream shift of T cells to their immunoprotective Th2 subsets (Wu et al. 2012). Thus tuftsin exerts its effect through a more broadly based “modulation of the immune system.”

Despite its 45-year history, the mechanism of action of tuftsin remains unknown. However, based on the broad range of action of tuftsin in both the immune and nervous systems, it is expected that the tuftsin receptor(s) should be common in both systems. Cultured human aortic and umbilical vein endothelial cells were shown to express tuftsin receptors and that the identity of the receptor is Nrp1 (von Wronski et al. 2006). Our proteomic screen identified several factors shed in the media in the presence of tuftsin. Some of these factors were common for the activated microglial secretome (Kim et al. 2007b), while others were unique to the tuftsin treatment. Notably, we have showed that the combination of NCM and tuftsin was capable of promoting the upregulation and release of its receptor Nrp1 from microglial cells. Because Nrp1 plays critical roles in the immune, vascular, and nervous systems and interacts with a number of different ligands, cell surface receptors and adhesion proteins (Gavazzi 2001; Tordjman et al. 2002; Wang et al. 2003), it is possible that some of the previously reported effects of tuftsin are mediated through Nrp1.

FIGURES

Figure III-1: Treatment of microglia with tuftsin and excitotoxic media causes an M2 shift. Quantitative RT-PCR was performed to analyze changes in gene expression of the M1 marker TNFα (**A**) and the M2 marker IL10 (**B**) in response to tuftsin and NCM treatment for 10 hours. Primary microglia were treated with increasing concentrations of tuftsin or tuftsin and NCM prepared from primary cortical neurons exposed to 100 μM glutamate overnight. (**C**) The ratio of the fold change of IL10 (M2) to the fold change of TNFα (M1). n= 3, *, $p<0.5$; **, $p<0.01$; ***, $p<0.001$. In (**A**) and (**B**) the comparisons are between tuftsin and tuftsin/NCM at each concentration. In (**C**) the comparisons are between NCM and tuftsin/NCM.

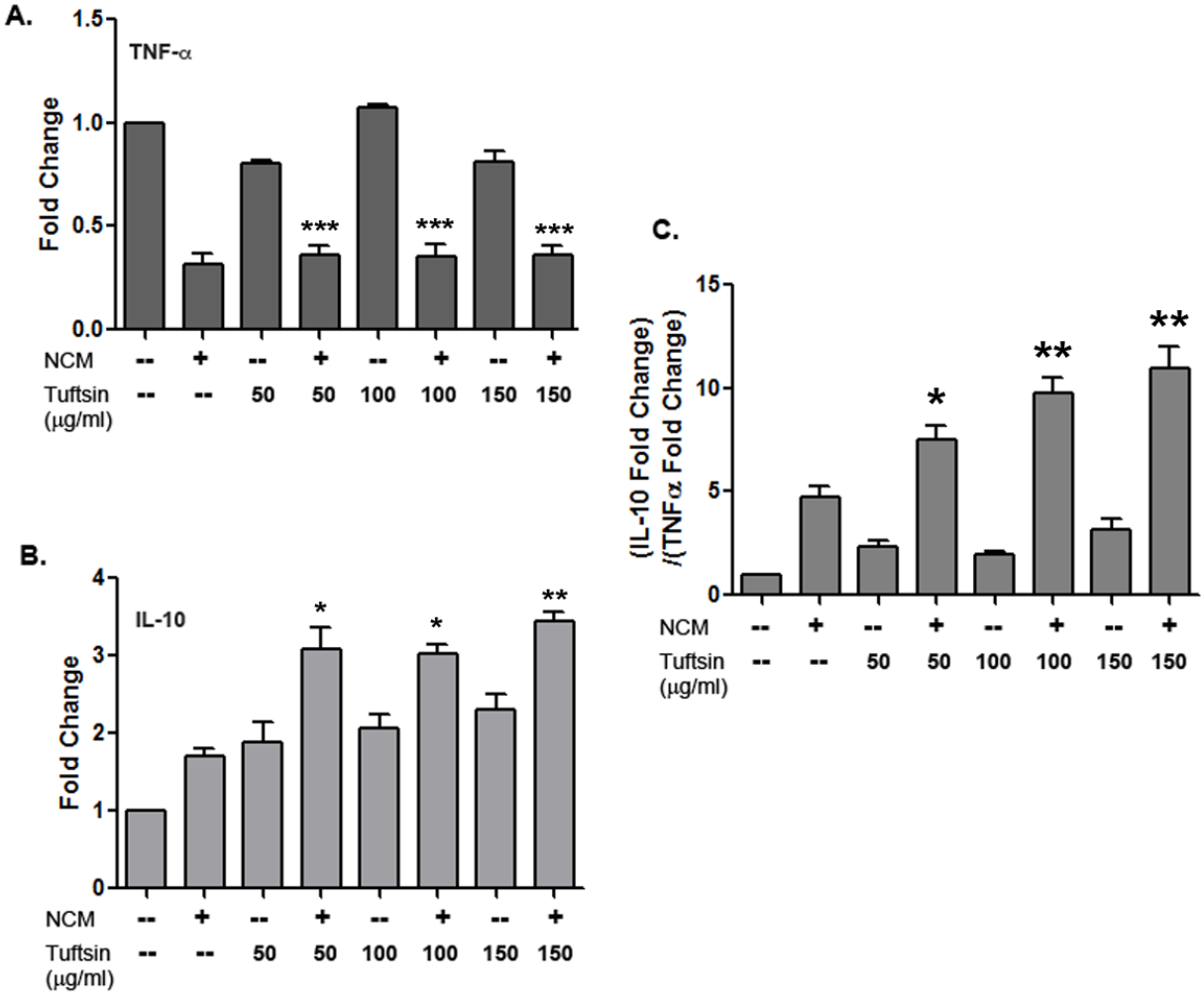

A.
TNF-α
Fold Change
1.5
1.0
0.5
0.0
***
***
***
NCM -- + -- + -- + -- +
Tuftsin (μg/ml) -- -- 50 50 100 100 150 150
B.
IL-10
Fold Change
4
3
2
1
0
*
*
**
NCM -- + -- + -- + -- +
Tuftsin (μg/ml) -- -- 50 50 100 100 150 150
C.
(IL-10 Fold Change) /(TNFα Fold Change)
15
10
5
0
*
**
**
NCM -- + -- + -- + -- +
Tuftsin (μg/ml) -- -- 50 50 100 100 150 150

Figure III-2: Tuftsin prevents the upregulation of iNOS during EAE. C57BL/6 mice were infused with either saline or 500 μM tuftsin during the course of EAE. Frozen cross-sections of spinal cords isolated at different time points during the EAE disease course were stained for Iba1 and iNOS to observe M1 microglial populations. Scale bar: 50 μm.

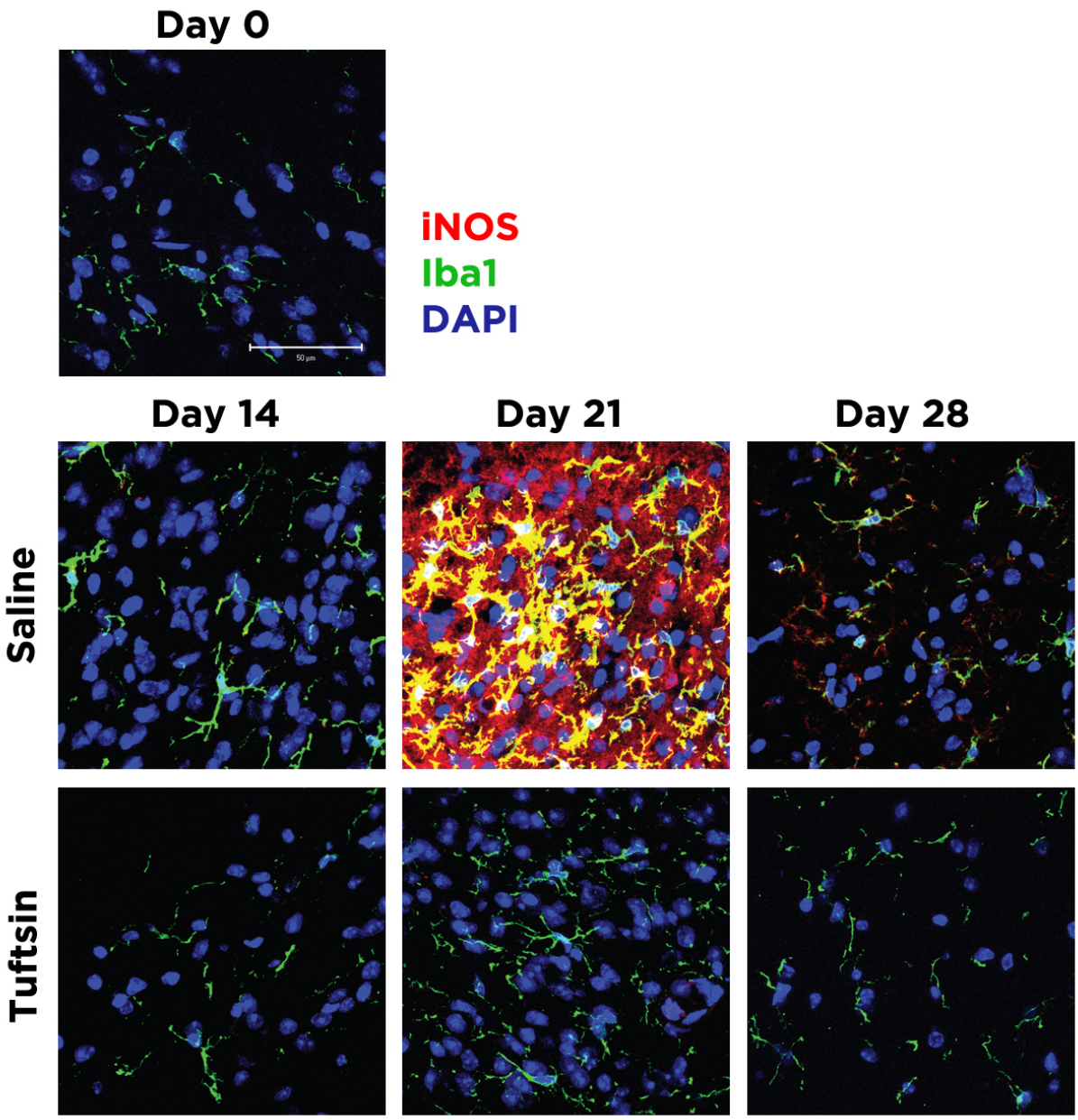

Day 0
iNOS
Iba1
DAPI
Day 14
Day 21
Day 28
Saline
Tuftsin

Figure III-3: Arginase expression is increased during the recovery phase of EAE. C57BL/6 mice were infused with either saline or 500 μM tuftsin during the course of EAE. Frozen cross-sections of spinal cords isolated at different time points during the EAE disease course were stained for Iba1 and Arginase1 to observe M2 microglial populations. Scale bar: 50 μm.

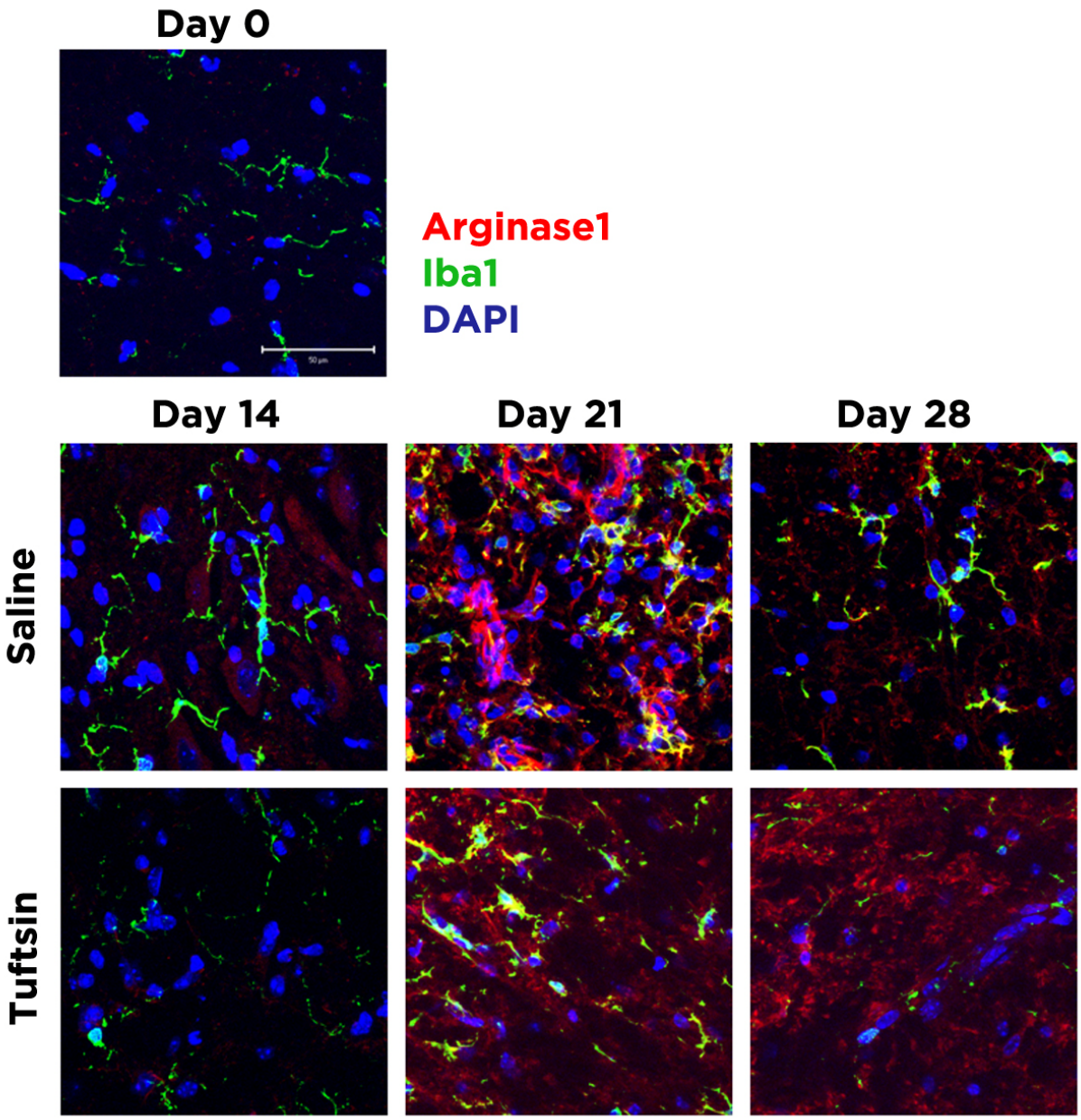

Day 0
Arginase1
Iba1
DAPI
Day 14
Day 21
Day 28
Saline
Tuftsin

Figure III-4: Microglia treated with NCM and tuftsin upregulate Nrp1. Primary microglial cells were treated for 10 hours with either NCM, 100 µg/ml tuftsin, or a combination of both, with untreated cells as controls. These were stained for Iba1 to visualize cell morphology as well as Nrp1. Scale bar: 10 µm

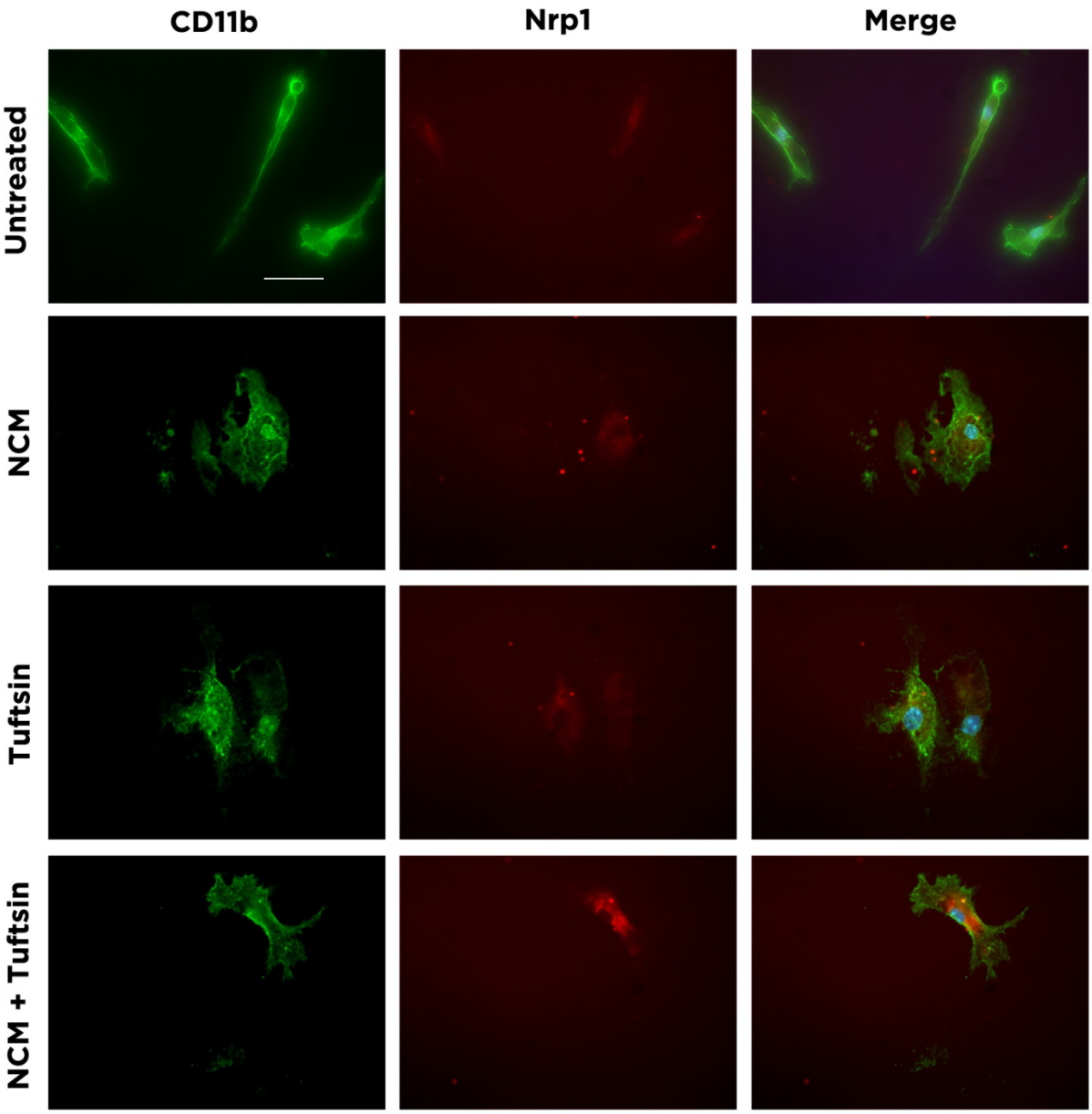
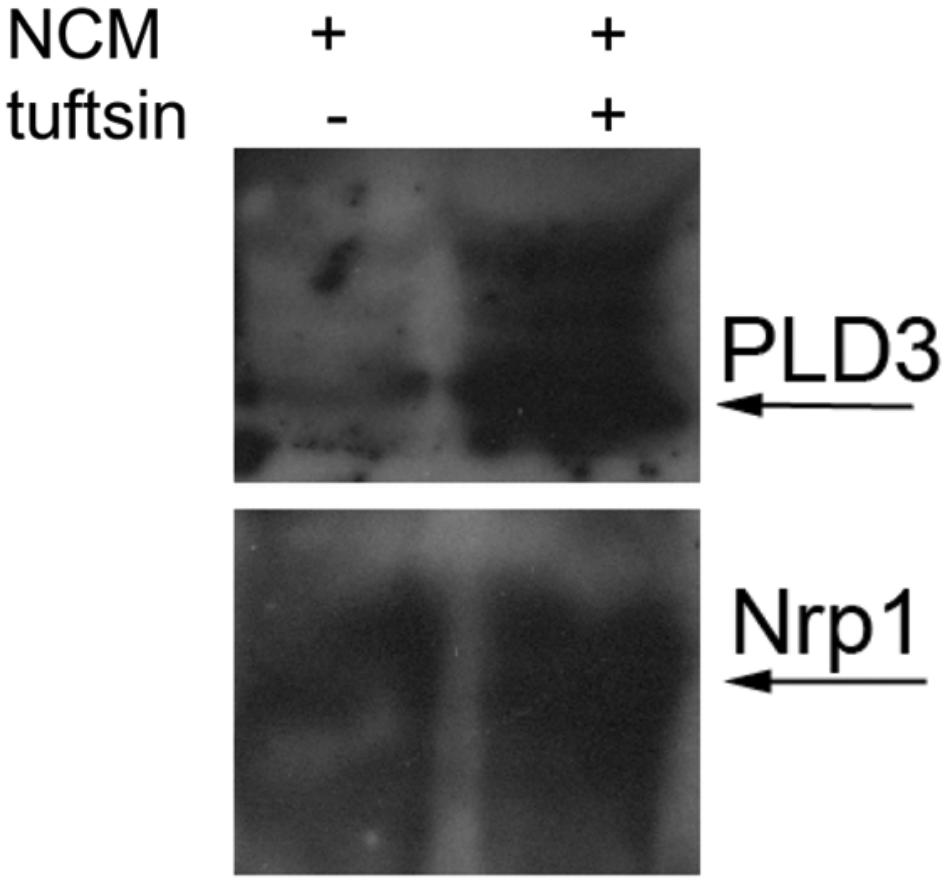

CD11b
Nrp1
Merge
Untreated
NCM
Tuftsin
NCM + Tuftsin

Table III-1: Treatment of microglia with NCM and tuftsin induced the release of immunomodulatory factors. To characterize the factors released from microglia under EAE-like conditions, microglia were treated for 10 hours with NCM isolated from neurons undergoing excitotoxic cell death in the presence or absence of tuftsin. Concentrated conditioned media were analyzed by multidimensional chromatography and tandem mass spectrometry. Partial list of the secreted proteins is shown. Spectral counts (MS/MS) of proteins identified from the media of microglia treated with NCM alone (MgCM) or a combination of NCM and 100 µg/ml of tuftsin (MgCM + Tuf) for 10 hours are listed in the respected columns. Ratios derived from spectral counts were used to predict the trend of differential expression.

Description	Levels in MgCM	Levels in MgCM+tuf	MgCM+tuf/MgCM
Uncharacterized protein Igf1	1	8	8
Uncharacterized protein Tnfalp8	1	8	8
Phospholipase D3	2	10	5
Glia maturation factor Gmfb	2	9	4.5
Mannosidase alpha 1C	1	4	4
Procathepsin H	6	22	3.7
Monocyte differentiation antigen CD14	8	26	3.3
Macrophage scavenger receptor	1	3	3
Neuropilin2	1	3	3
Neuropilin1	10	13	1.3
Disintegrin and metalloproteinase domain-containing protein 10, Adam10	0	4	>4

Figure III-5: Microglia shed factors in conditioned media. To confirm the results of the proteomic screen aiming to determine what factors are released by stimulated microglia, conditioned media from primary microglia treated with NCM alone or a combination of NCM and 100 µg/ml tuftsin for 10 hours were concentrated and analyzed by western blot for the abundance of PLD3 (A) or Nrp1 (B). This western blot was repeated three times on media isolated from three separate *in vitro* experiments. PLD3 fold change: 5.37 (5 on mass spec), Nrp-1 fold change: 1.23 (1.3 on mass spec)


NCM + +
tuftsin - +
PLD3
Nrp1

Chapter IV

Tuftsin signals through its receptor neuropilin-1 via the transforming growth factor beta pathway

INTRODUCTION

Tuftsin is a small, naturally occurring tetrapeptide with the sequence threonine-lysine-proline-arginine. It was originally described at its discovery in 1970 as a phagocytosis-stimulating factor derived from the proteolytic degradation of IgG (Najjar and Nishioka 1970; Nishioka et al. 1973), which represents residues 282 to 292 of the heavy chain of γ-globulin, and is generated in the spleen by several enzymes (Fridkin and Najjar 1989). Aside from its more well-known function as a stimulator of microglial activity (Siemion and Kluczyk 1999; Wu et al. 2012), tuftsin has been shown to have various antimicrobial and antiviral effects (Fridkin and Najjar 1989). In many animal disease models, such as sepsis (Wardowska et al. 2009) and multiple sclerosis (Bhasin et al. 2007), tuftsin treatment has been associated with anti-inflammatory effects, particularly among microglial cell populations (Wu et al. 2012).

Receptors for tuftsin have been described in the literature; using affinity chromatography, two binding activities were reported in peritoneal granulocytes corresponding to 250 and 500 kDa molecular masses consisting of two subunits of 62 and 52 kDa (Bump et al. 1986a). More recently Nrp1 was identified as a receptor for tuftsin (von Wronski et al. 2006). Nrp1 is a single-pass transmembrane glycoprotein that plays important roles in development, immunity, and cancer. Nrp1 has 44% homology with neuropilin-2, with which it shares many structural and biological properties, however tuftsin has been shown to only bind Nrp1 (von Wronski et al. 2006). During angiogenesis, Nrp1 binds VEGF and promotes vessel formation (Gu et al. 2003; Soker et al. 1998), which is exploited by tumor cells to provide a blood supply to cancerous tissues (Klagsbrun et al. 2002). On the other hand, during neural development Nrp1

binds class 3 semaphorins (Sema3), which provide inhibitory signals in axonal guidance (Gu et al. 2003; Kitsukawa et al. 1995). Nrp1 also plays an important role in the immune system by promoting long contacts between dendritic cells and immunosuppressive Treg (Sarris et al. 2008; Tordjman et al. 2002), and can bind and activate TGFβ, an anti-inflammatory cytokine (Glinka and Prud'homme 2008). Recently, Tregs have been shown to be recruited to tumors by Nrp1 in a VEGF dependent manner, which contribute to immune system evasion by tumor cells (Hansen 2013).

A notable feature of Nrp1's structure is that it has a short cytoplasmic domain that is only about 44 amino acids long, and contains no known signaling motif (Vander Kooi et al. 2007). Although there have been several studies indicating that this domain is capable of signaling independently, the mechanism has not been well defined (Evans et al. 2011; Fantin et al. 2011). However, Nrp1 can associate with various co-receptors via which downstream signaling can occur; PlexinA1 for Sema3, VEGFR (VEGF Receptor) for VEGF, TβR1 (TGFβ receptor 1) for TGFβ, and c-Met for hepatocyte growth factor (HGF) (Figure IV-1)(Prud'homme and Glinka 2012). Tuftsin competes with VEGF for binding on the Nrp1 molecule, as it shares sequence homology with the C-terminus of VEGF (von Wronski et al. 2006). The structure of VEGF allows its N-terminus to interact with VEGFR while bound to Nrp1 (Wiesmann et al. 1997), promoting co-receptor dimerization and signaling, which is not the case with tuftsin due to its small size. Although tuftsin has been used in various studies for over forty years, its mechanism of action and signaling pathway are still unknown.

In this study, we show that the Nrp1 inhibitor EG00229 prevents tuftsin binding to the cell surface, and effectively blocks the anti-inflammatory shift induced by tuftsin in

microglial cells. We also demonstrate that an inhibitor of TβR1 function, but not of c-Met function, disrupts tuftsin's downstream effects similarly to EG00229. Moreover, we show that tuftsin signals through the canonical TGFβ signaling pathway.

RESULTS

Neuropilin-1 inhibitor EG00229 prevents tuftsin binding to the cell surface.

Tuftsin has been shown to bind to Nrp1 on endothelial cells (von Wronski et al. 2006). To examine if tuftsin is acting through Nrp1 on microglia, we used a small molecule inhibitor for VEGF binding to Nrp1 designated as EG00229. This inhibitor was designed around the final C-terminal residues of the VEGF molecule (DKPRR), as these were the residues that interacted with the Nrp1 binding pocket (Jarvis et al. 2010). Since tuftsin shares a strong homology with the C-terminus of VEGF (TKPR), EG00229 functions effectively as an inhibitor of tuftsin binding to Nrp1 as well (Figure IV-2).

Microglia readily express Nrp1, as shown in Figure IV-3. As there are no antibodies that can stain for tuftsin due to its small size, and conjugation with GFP could potentially disrupt its binding properties, we used biotinylated tuftsin that was then detected by streptavidin-conjugated Cy3 antibody. Primary microglia were treated with a combination of EG00229 and biotinylated tuftsin. At all concentrations tested, the inhibitor seems to not activate microglia as they remained in a ramified, resting state. Tuftsin binding to Nrp1 was significantly decreased by EG00229 (Figure IV-3A), and appears to act in a dose-dependent manner (Figure IV-3B). This result demonstrates that tuftsin's function on microglia is mediated by Nrp1 and not by some alternative receptor, as its binding is prevented by a highly specific Nrp1 inhibitor.

EG00229 blocks the anti-inflammatory shift in microglia induced by tuftsin.

When activated, microglia can be polarized to either a pro- or anti-inflammatory subset known as M1 or M2, respectively. M1 microglia, which were shown to have neurodegenerative properties in a model of spinal cord injury, produce TNFα and NO, while neuroprotective M2 microglia release IL10 and TGFβ (Gordon and Martinez 2010; Kigerl et al. 2009; Michelucci et al. 2009). As shown above, a 'two-hit' treatment with a combination of NCM, isolated from neurons treated overnight with 100 μM glutamate to induce excitotoxic injury, and tuftsin reduced the release of TNFα and promoted the release of IL10 in primary microglial cells, indicating an M2 shift in response to tuftsin treatment (Wu et al. 2012).

We examined whether EG00229 could prevent this tuftsin-mediated M2 microglial shift. We treated microglial cells for 10 hours with NCM in the presence or absence of tuftsin and increasing concentrations of EG00229, choosing the inhibitor concentrations based on previous studies (Jarvis et al. 2010; Jia et al. 2010). We then harvested RNA and performed quantitative real-time PCR to observe microglial phenotype based on TNFα levels to indicate M1 polarization, and IL10 levels to indicate M2 polarization. While the combination of NCM and tuftsin reduced TNFα levels and increased IL10, as we have previously shown (Wu et al. 2012), EG00229 reversed these effects (Figure IV-4A, B). Further, while tuftsin and NCM alone significantly increase IL10 levels by about 3-fold, EG00229-treated cells at all concentrations showed no similar increase in IL10 levels, which remained comparable to control levels (Figure IV-4B). Similarly, while cells treated with tuftsin and NCM resulted in a reduction in TNFα, the opposite was observed in groups treated with EG00229, which showed a slight increase in TNFα levels over control (Figure IV-4A). Moreover, when the overall

shift to an anti-inflammatory state in microglial cells was assessed, noted by the ratio of M2 to M1 gene expression, the EG00229 treatment resulted in reversion of these cells to a state similar to untreated controls (Figure IV-4C). Thus, these experiments indicate that EG00229 can effectively prevent tuftsin's actions on microglial cells by blocking the M2 shift.

Blockade of TβR1 prevents the tuftsin-induced anti-inflammatory shift in microglia.

Nrp1 employs different co-receptors which signal following ligand binding (Prud'homme and Glinka 2012). We investigated which one of these co-receptors is involved in mediating tuftsin signaling. A likely candidate is TβR1, since its classic ligand TGFβ has been extensively associated with anti-inflammatory effects. Nrp1 can bind and activate the latent form of TGFβ, which is associated with immunosuppressive Treg function (Karpanen et al. 2006; Wei et al. 2007), and also essential in the development of alternatively activated M2 microglia (Zhou et al. 2012).

To examine if TβR1 is the co-receptor involved in tuftsin signaling, we used a specific inhibitor capable of blocking the kinase activity of TβR1 at 10 μM as previously described (Shiou et al. 2006). For comparison, we also used an inhibitor of c-Met kinase activity at 5 nM, in line with prior studies (Zou et al. 2012), which is an alternative co-receptor that Nrp1 could signal through (Prud'homme and Glinka 2012). Similarly to the experiments in Figure IV-4, microglia were treated for 10 hours with combinations of tuftsin and NCM, in the presence or absence of c-Met inhibitor or TβR1 inhibitor (Shiou

et al. 2006; Zou et al. 2012). After isolating RNA, qPCR was performed to quantify the expression of M1 and M2 markers. The ratio of M2/M1 in c-Met inhibitor treated samples was comparable to controls, with a 3-fold decrease in TNFα and 3-fold increase in IL10 in tuftsin and NCM-treated samples. However, in cells treated with TβR1 inhibitor a slight increase in TNFα levels and no change in IL10 levels were observed, as was the case for EG00229-treated microglia (Figure IV-5A,B). Furthermore, while there was a significant anti-inflammatory switch in both control and c-Met inhibitor treated cells when exposed to NCM and tuftsin, this M2 shift was abolished in TβR1 treated samples (Figure IV-5C). Taken together, these data indicate that tuftsin signals through the TGFβ signaling pathway via TβR1.

Tuftsin signals through the canonical TGFβ signaling pathway.

We extended these observations by further investigating the downstream effectors of TβR1. TGFβ is characterized by signaling through two separate pathways, the canonical and non-canonical pathways, which have disparate effects. The canonical signaling pathway is associated with Smad2/3 phosphorylation, which then forms a complex with Smad4 that translocates to the nucleus, binds DNA, and regulates transcription. This series of events has been linked with inhibition of immune responses and cell proliferation (Prud'homme and Glinka 2012; Rahimi and Leof 2007). On the other hand, non-canonical TGFβ signaling occurs through various molecules such as PI3k, Akt, or Erk (Zhang 2009). The two pathways have been shown to be involved in complex cross talk, as the canonical and non-canonical pathways can antagonize each

other. For example, hyperactivation of Akt can reduce canonical signaling, particularly through inhibition of Smad3 (Tian et al. 2011; Zhang 2009).

To investigate whether tuftsin signals through the canonical or non-canonical TGFβ pathway, we performed immunoblots to examine Smad3 and Akt phosphorylation levels. Microglial cells treated with combinations of NCM and tuftsin for 10 hours in the presence or absence of TβR1 inhibitor or c-Met inhibitor were re-stimulated at hour 9 to allow for phosphorylation changes to be more readily observable. Control samples showed a significant increase of Smad3 phosphorylation in response to tuftsin and NCM, indicating that tuftsin signals through the canonical pathway. This increase was also observed in samples treated with c-Met inhibitor, but was abolished in those treated with TβR1 inhibitor (Figure IV-6A, B). Conversely, microglia treated with NCM and tuftsin revealed significantly decreased levels of Akt phosphorylation, indicating a decrease in non-canonical TGFβ signaling similar to that in samples treated with c-Met inhibitor. However when the activity of TβR1 was blocked, the levels of Akt phosphorylation remained unchanged in the microglial samples (Figure IV-6C,D). Samples treated with 30 μM EG00229 resulted in phosphorylation levels comparable to those exposed to TβR1 inhibitor, for both p-Smad3 and p-Akt (data not shown). These experiments suggest that tuftsin signals via the canonical TGFβ signaling pathway.

Tuftsin promotes TGFβ release from cells, which is prevented by TβR1 blockade.

To further confirm polarization to an M2 subset as a result of tuftsin treatment, as well as the effect of c-Met, TβR1, and EG00229 inhibitors, we performed an ELISA

assay for TGFβ. We show that tuftsin and NCM promote a significant release of TGFβ from microglial cells, similarly to those treated with 5 nM of c-Met inhibitor. However, this does not occur when cells are exposed to TβR1 inhibitor, or EG00229 (Figure IV-7A). To observe the release of reactive nitrogen species from cells as a means of determining a shift away from the M1 phenotype in cells, we quantified nitrite release by DAN reagent. However, there was no significant change between all treatments (Figure IV-7B). Thus, blockade of tuftsin binding to Nrp1 by EG00229 or disruption of TβR1 signaling prevents the tuftsin-mediated M2 shift in microglia.

DISCUSSION

In this study, we provide several pieces of evidence that support the hypothesis that tuftsin signals exclusively through its receptor Nrp1 via the canonical TGFβ signaling pathway. Our previous work showed that tuftsin was capable of promoting an anti-inflammatory, M2 shift in microglia (Wu et al. 2012). In this report we show that blockade of tuftsin binding to Nrp1 prevents this shift, and that inhibition of TβR1 similarly blocks microglial polarization to an anti-inflammatory phenotype. As the canonical TGFβ pathway is associated with immunosuppression (Rahimi and Leof 2007), our current findings support our previously published work in the experimental autoimmune encephalomyelitis model of multiple sclerosis where tuftsin enhanced an M2 shift and promoted the expression of immunosuppressive Tregs (Wu et al. 2012).

It is notable that neither tuftsin treatment nor NCM treatment alone promotes an M2 shift in microglial cells (Wu et al. 2012). This is consistent with the idea that macrophages and microglia are often activated in a “two-hit” process. This model describes that a weaker, nonspecific initial signal produces a priming response in target cells, which, when faced with a second activation signal, are rapidly activated and produce a robust reaction (Hains et al. 2010; O'Leary et al. 2011). In our system, NCM would function as the initial signal, and tuftsin would then produce a strong anti-inflammatory response in “primed” microglial cells. We postulate that the priming signal of NCM in concert with tuftsin binding to Nrp1 on microglia promotes canonical TGFβ pathway signaling, which results in TGFβ cytokine release. The cytokine release then feeds back on the microglial cells, and fully polarizes them to the M2 phenotype resulting in the release of IL10 (Figure IV-8).

As EG00229 was originally generated as an inhibitor of VEGF binding to Nrp1 (Jarvis et al. 2010), it is possible that tuftsin is in fact signaling through the VEGF pathway. There are data both supporting and refuting this theory. It has been shown that treatment of cells with TGFβ can promote the induction of VEGF (Shao et al. 2009), and that VEGF treatment promotes TGFβ production in macrophages (Luo et al. 2012). However, VEGF binds to both Nrp1 and its co-receptor VEGFR concomitantly, which aids in the recruitment of VEGFR for signaling (Wiesmann et al. 1997). This would not occur for tuftsin, as it is comprised of only 4 amino acids and binds deep within the VEGF pocket on the Nrp1 molecule (Vander Kooi et al. 2007), preventing its interaction with any other receptors. The fact that treatment of microglia with EG00229 resulted in small increase in TNFα over control cells may possibly be due to the fact that EG00229 does not only block tuftsin binding, but prevents VEGF binding as well (Jarvis et al. 2010), so it can result in different behavior in microglia rather than just loss of tuftsin interaction alone.

Another immune cell type that readily expresses Nrp1 is the regulatory T cells, Tregs (Weiss et al. 2012). Tregs cells play an essential role in suppressing ongoing inflammatory immune responses (Kohm et al. 2002; McGeachy et al. 2005). These cells differentiate in response to, as well as bind and activate, TGFβ (Glinka and Prud'homme 2008; Lohr et al. 2006). It would thus be pertinent to investigate whether tuftsin also signals through the canonical TGFβ signaling pathway in these cells as well.

As tuftsin has been used extensively over its 45-year history, the lack of knowledge of its mechanism of action has been a notable void in the information regarding this useful molecule. Our work is novel as although it has been shown that

tuftsin binds to Nrp1 (von Wronski et al. 2006), Nrp1 utilizes a wide variety of co-receptors that function in disparate pathways for signaling (Glinka and Prud'homme 2008). Downstream signaling as a result of tuftsin binding to cells is an area that has not been investigated prior to our study. Here we present data that identifies the canonical TGFβ signaling pathway as the means by which tuftsin exerts its anti-inflammatory effects.

FIGURES

Figure IV-1: Nrp1 interacts with a variety of co-receptors. (A) The general domain structure of neuropilin family members Nrp1 and Nrp2. There are five extracellular domains, a single-pass TMD domain, and a short cytosolic tail lacking tyrosine kinase activity. **(B)** Nrp1 interacts with the signaling receptors for a variety of soluble mediators, which they can also bind. This demonstrates a hypothetical model of how these signaling pathways may interact, which includes TGF-β1, growth factors such as VEGF, HGF and PDGF-BB; and the SEMA3 family. Figure from (Prud'homme and Glinka 2012).

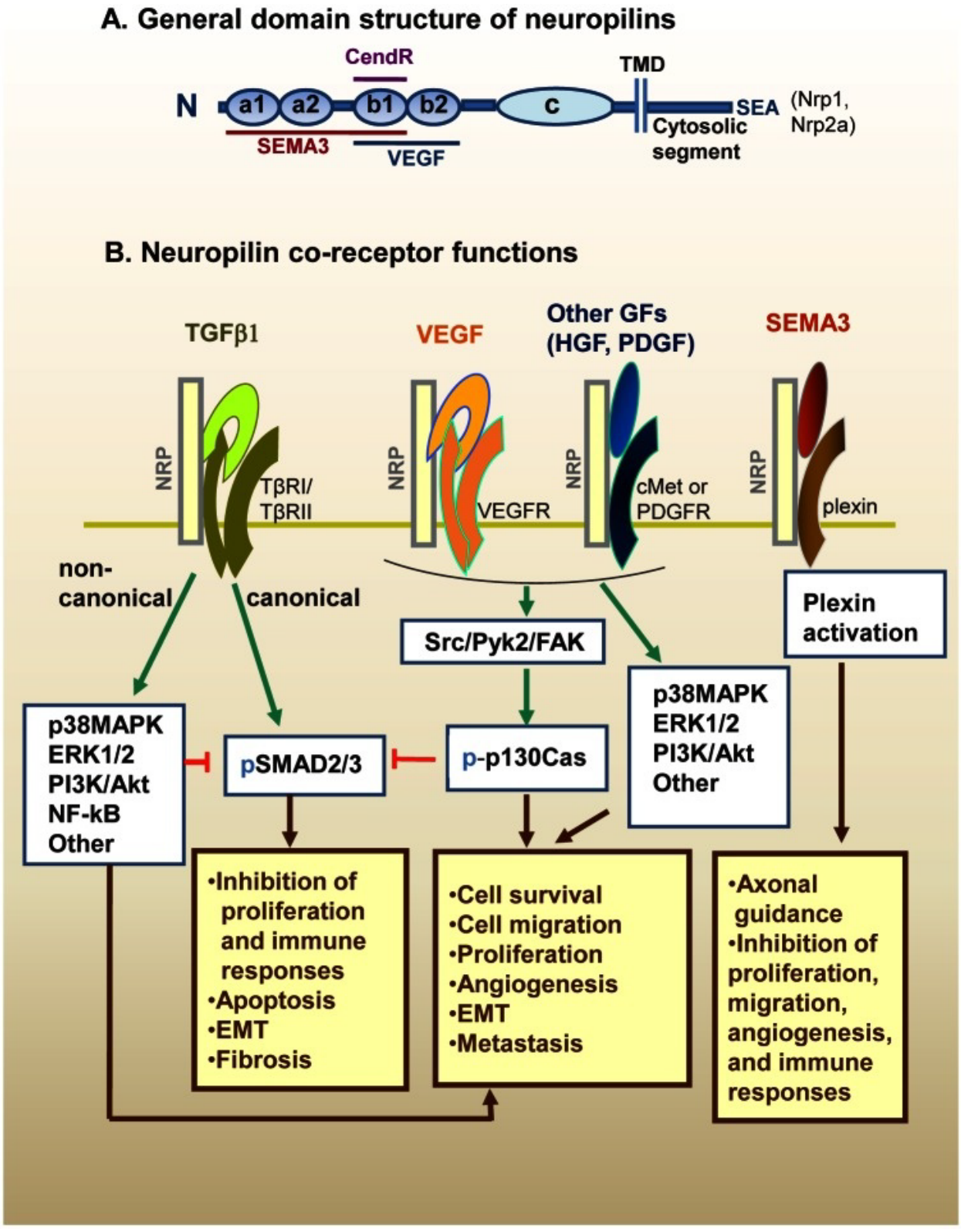

A. General domain structure of neuropilins
CendR
TMD
N
a1
a2
b1
b2
c
SEA
(Nrp1, Nrp2a)
Cytosolic segment
SEMA3
VEGF
B. Neuropilin co-receptor functions
TGFβ1
VEGF
Other GFs (HGF, PDGF)
SEMA3
NRP
TβRI/ TβRII
VEGFR
cMet or PDGFR
plexin
non-canonical
canonical
Src/Pyk2/FAK
Plexin activation
p38MAPK ERK1/2 PI3K/Akt NF-kB Other
pSMAD2/3
p-p130Cas
p38MAPK ERK1/2 PI3K/Akt Other
•Inhibition of proliferation and immune responses
•Apoptosis
•EMT
•Fibrosis
•Cell survival
•Cell migration
•Proliferation
•Angiogenesis
•EMT
•Metastasis
•Axonal guidance
•Inhibition of proliferation, migration, angiogenesis, and immune responses

Figure IV-2: Structure of EG00229, and its binding in tuftsin's interaction site on Nrp1. **(A)** Structure of small molecule neuropilin inhibitor EG00229. **(B)** NMR analysis of tuftsin binding to the b1 domain of Nrp1. **(C)** X ray analysis of EG00229 binding to the b1 domain of Nrp1. Adapted from (Jarvis et al. 2010).

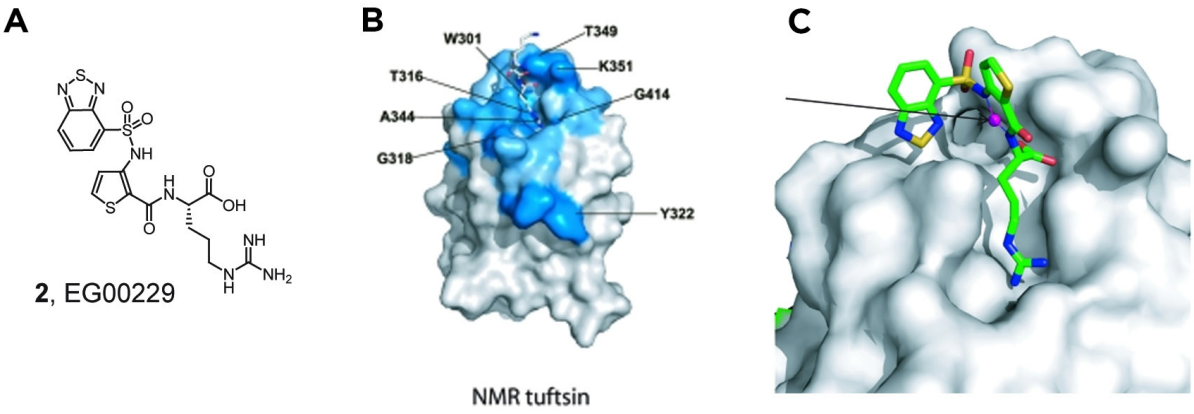

A
B
C
W301
T349
K351
T316
G414
A344
G318
Y322
2, EG00229
NMR tuftsin

Figure IV-3: EG00229 reduces tuftsin binding to the surface of microglia in a dose-dependent manner. Microglial cells were pre-blocked with the indicated concentrations of EG00229, and incubated with biotinylated tuftsin at room temperature as described in Methods. Cells were then fixed and stained for Nrp1 (FITC) and with streptavidin-conjugated Cy3 to visualize biotinylated tuftsin on the cell surface (**A**). Fluorescence intensity of tuftsin staining was quantified in (**B**). n= 3, ***, $p<0.001$.

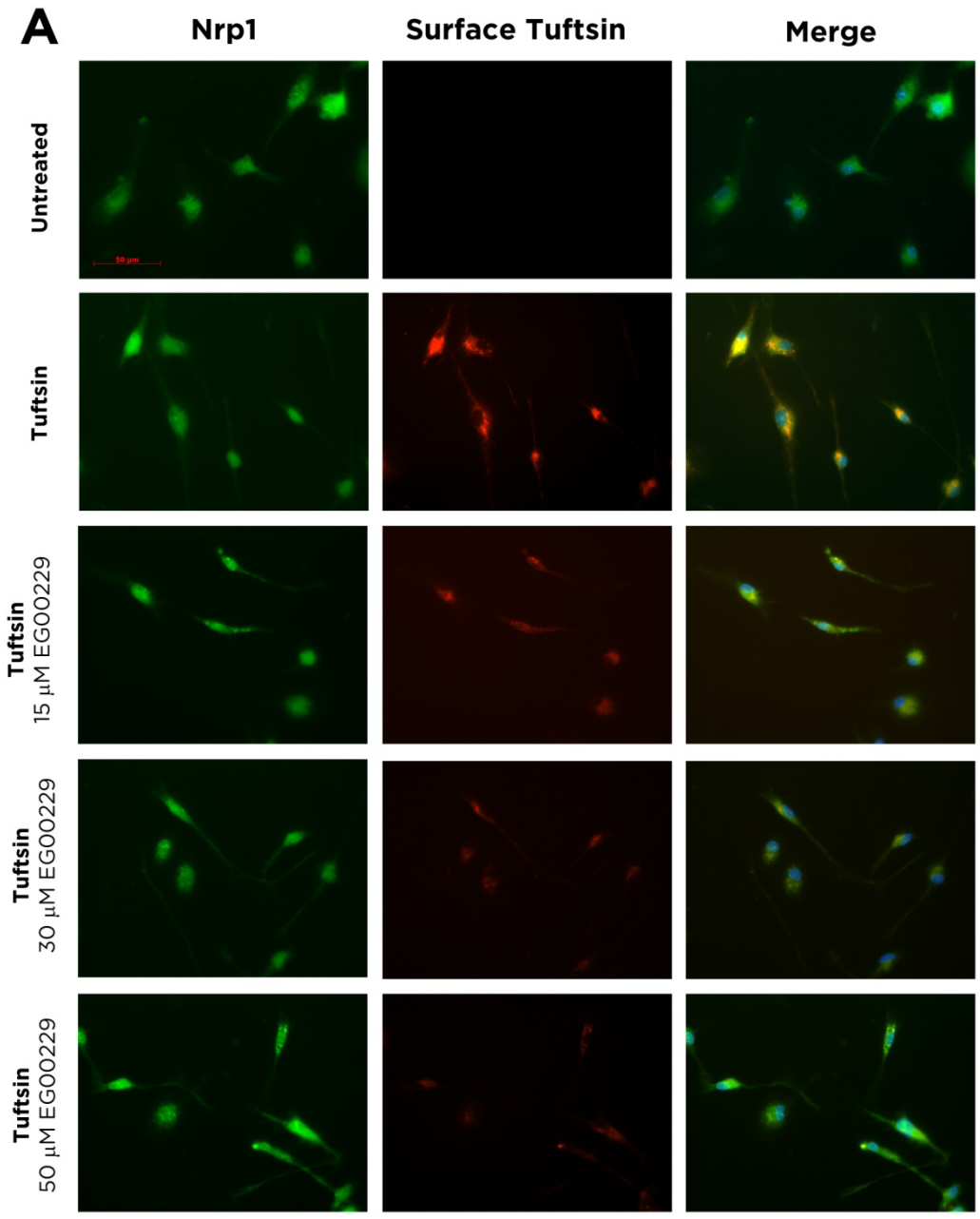

A
Nrp1
Surface Tuftsin
Merge
Untreated
Tuftsin
Tuftsin
15 μM EG00229
Tuftsin
30 μM EG00229
Tuftsin
50 μM EG00229
B
Fold Change
1.5
1.0
0.5
0.0
***
***
***
Tuftsin
EG00229
(μM)
+
-
+
15
+
30
+
50

Figure IV-4: The tuftsin-mediated M2 shift in microglia is disrupted by EG00229. Quantitative RT-PCR was performed to analyze changes in gene expression of the M1 marker TNFα (**A**) and the M2 marker IL10 (**B**). Primary microglia were treated 100 μg/ml tuftsin or 100μg/ml tuftsin and NCM. Some groups were additionally treated with increasing concentrations of EG00229 as shown. (**C**) The ratio of the fold change of IL10 (M2) to the fold change of TNFα (M1). n= 3, *, $p<0.05$

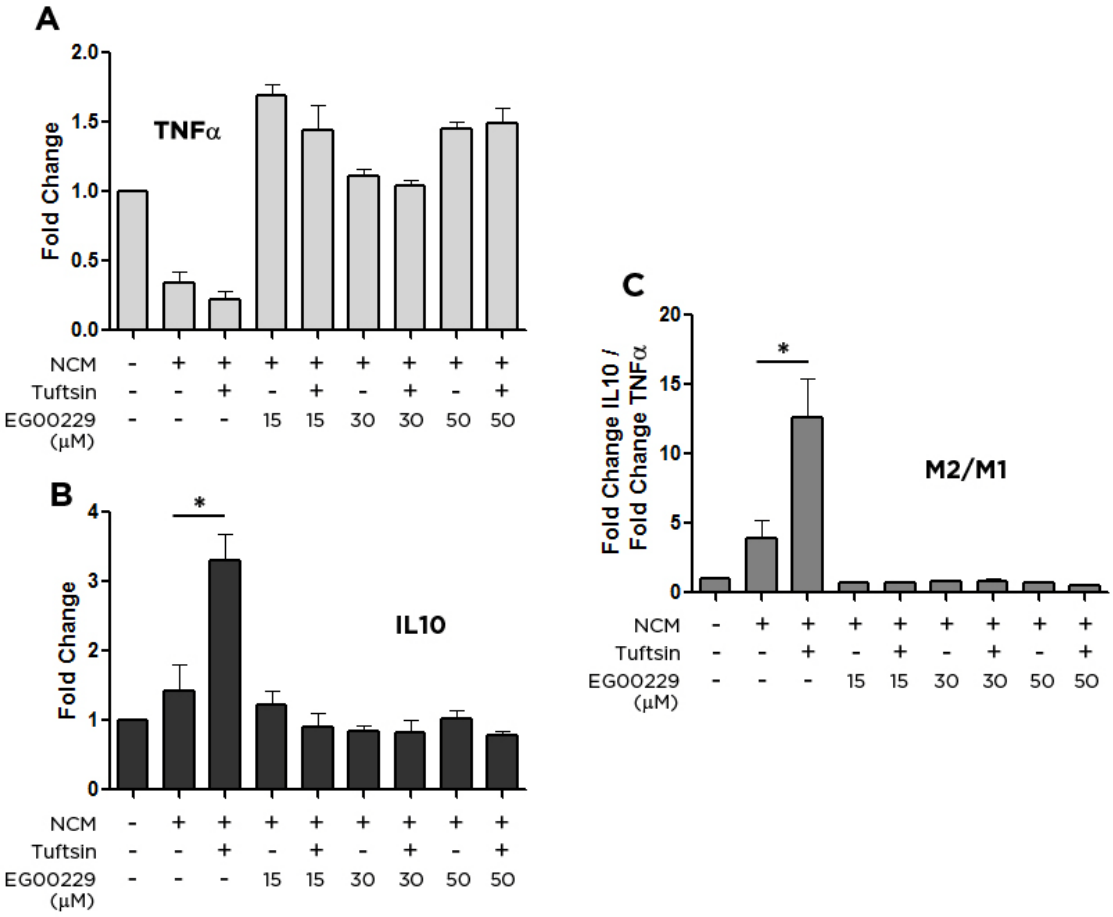
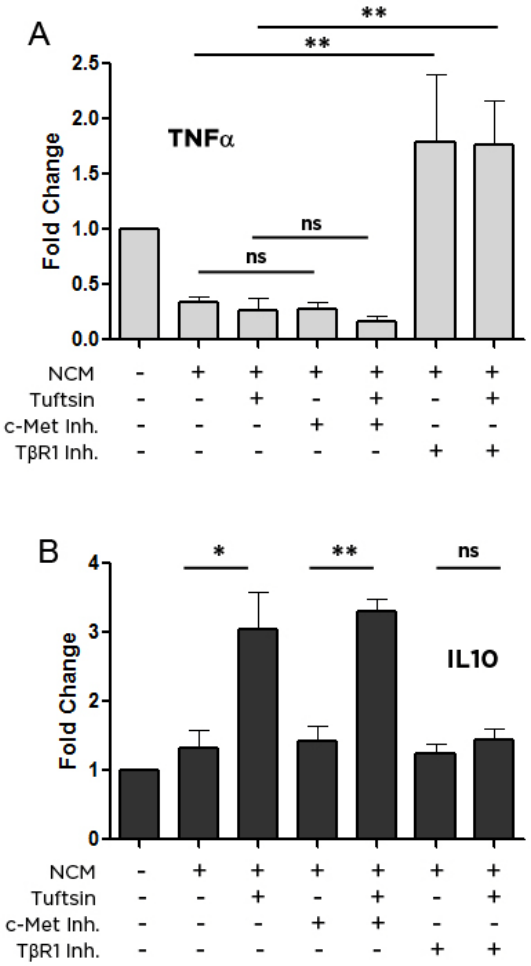

A
TNFα
Fold Change
2.0
1.5
1.0
0.5
0.0
NCM - + + + + + + + +
Tuftsin - - + - + - + - +
EG00229 (μM) - - - 15 15 30 30 50 50
B
IL10
Fold Change
4
3
2
1
0
*
NCM - + + + + + + + +
Tuftsin - - + - + - + - +
EG00229 (μM) - - - 15 15 30 30 50 50
C
M2/M1
Fold Change IL10 / Fold Change TNFα
20
15
10
5
0
*
NCM - + + + + + + + +
Tuftsin - - + - + - + - +
EG00229 (μM) - - - 15 15 30 30 50 50

Figure IV-5: Blockade of TβR1 prevents the tuftsin-mediated M2 shift in microglia. Quantitative RT-PCR was performed to analyze changes in gene expression of the M1 marker TNFα (**A**) and the M2 marker IL10 (**B**). Primary microglia were treated 100 μg/ml tuftsin or 100 μg/ml tuftsin and NCM. Some groups were additionally treated with 5 nM c-Met inhibitor or 10 μM TβR1 inhibitor. (**C**) The ratio of the fold change of IL10 (M2) to the fold change of TNFα (M1). n= 3-5, *, $p<0.05$; **, $p<0.01$; ns, not significant.


A
TNFα
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
**
**
ns
ns
NCM - + + + + + +
Tuftsin - - + - + - +
c-Met Inh. - - - + + - -
TβR1 Inh. - - - - - + +
B
IL10
Fold Change
4
3
2
1
0
*
**
ns
NCM - + + + + + +
Tuftsin - - + - + - +
c-Met Inh. - - - + + - -
TβR1 Inh. - - - - - + +

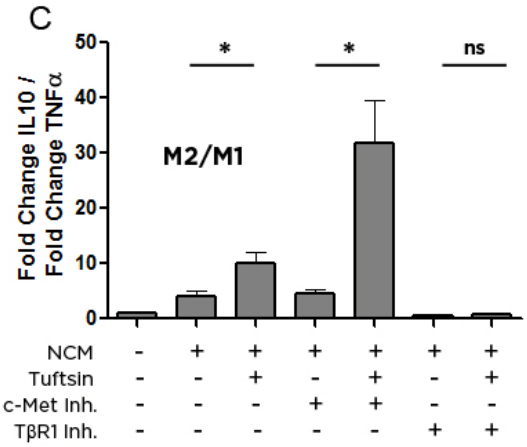

C
M2/M1
Fold Change IL10 / Fold Change TNFα
50
40
30
20
10
0
*
*
ns
NCM - + + + + + +
Tuftsin - - + - + - +
c-Met Inh. - - - + + - -
TβR1 Inh. - - - - - + +

Figure IV-6: Tuftsin signals through the canonical TGFβ signaling pathway, which is prevented by TβR1 inhibition. Western blots were performed on primary microglia extracts that were exposed to NCM, 100 µg/ml tuftsin, 5 nM c-Met inhibitor or 10 µM TβR1 inhibitor for 10 hours. 1 hour prior to harvesting, cells were restimulated with their designated treatment to better observe phosphorylation changes. Blots were probed for Smad3 and phospho-Smad3 **(A)**, and fold change relative to NCM treatment alone was quantified in (**B**), and adjusted to reflect changes in total Smad3 levels. Blots were probed for Akt and phospho-Akt in (**C**), and fold change relative to NCM alone is quantified in (**C**), and adjusted to reflect changes in total Akt levels. n= 3, *, $p<0.05$; **, $p<0.01$; ns, not significant.

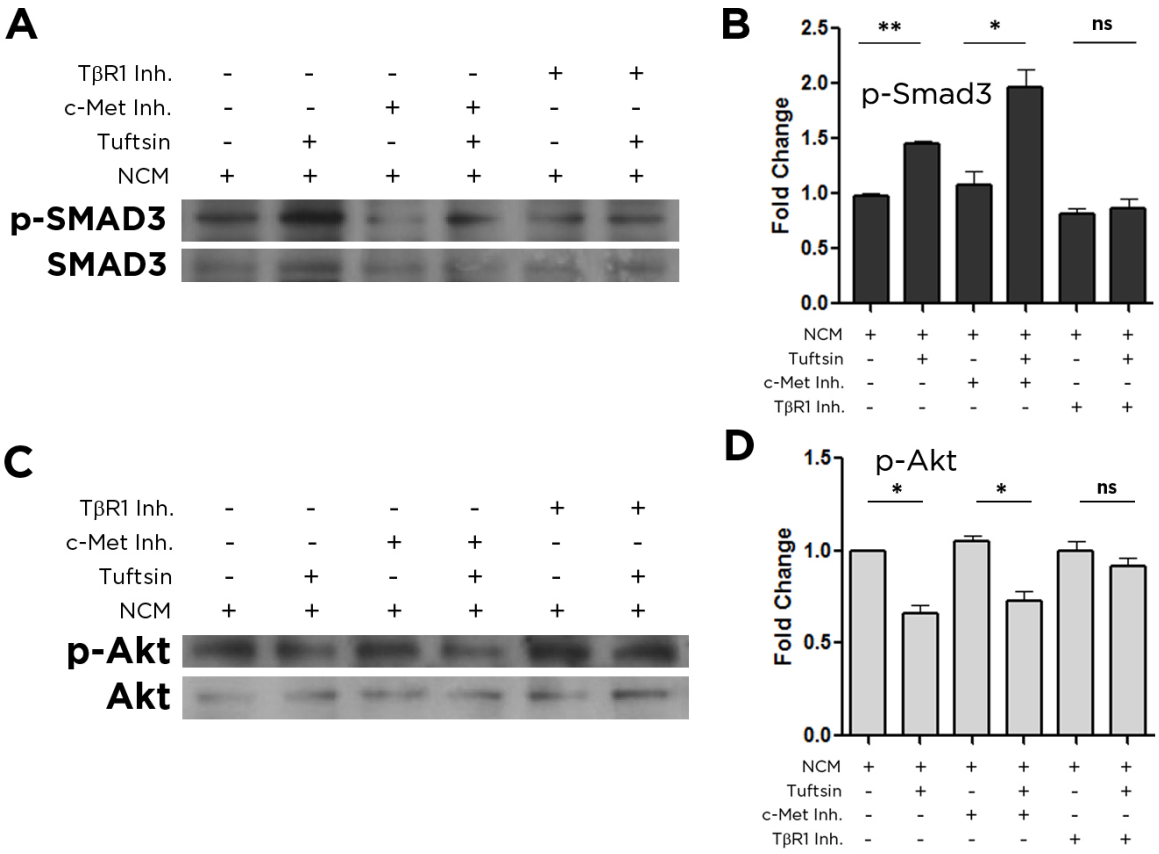

A
TβR1 Inh. - - - - + +
c-Met Inh. - - + + - -
Tuftsin - + - + - +
NCM + + + + + +
p-SMAD3
SMAD3
B
p-Smad3
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
** * ns
NCM + + + + + +
Tuftsin - + - + - +
c-Met Inh. - - + + - -
TβR1 Inh. - - - - + +
C
TβR1 Inh. - - - - + +
c-Met Inh. - - + + - -
Tuftsin - + - + - +
NCM + + + + + +
p-Akt
Akt
D
p-Akt
Fold Change
1.5
1.0
0.5
0.0
* * ns
NCM + + + + + +
Tuftsin - + - + - +
c-Met Inh. - - + + - -
TβR1 Inh. - - - - + +

Figure IV-7: Blockade of TβR1 prevents tuftsin-mediated TGFβ upregulation. An ELISA assay probing for TGFβ levels (**A**) or a nitrite assay using DAN reagent (**B**) was performed on media isolated from primary microglial cells treated with 100 μg/ml tuftsin or 100 μg/ml tuftsin and NCM for 10 hours. Some groups were additionally treated with 5 μM c-Met inhibitor, 10 μM TβR1 inhibitor, or 30 μM EG00229, also for 10 hours. n= 3, **, $p<0.01$; ***, $p<0.001$, ns, not significant.

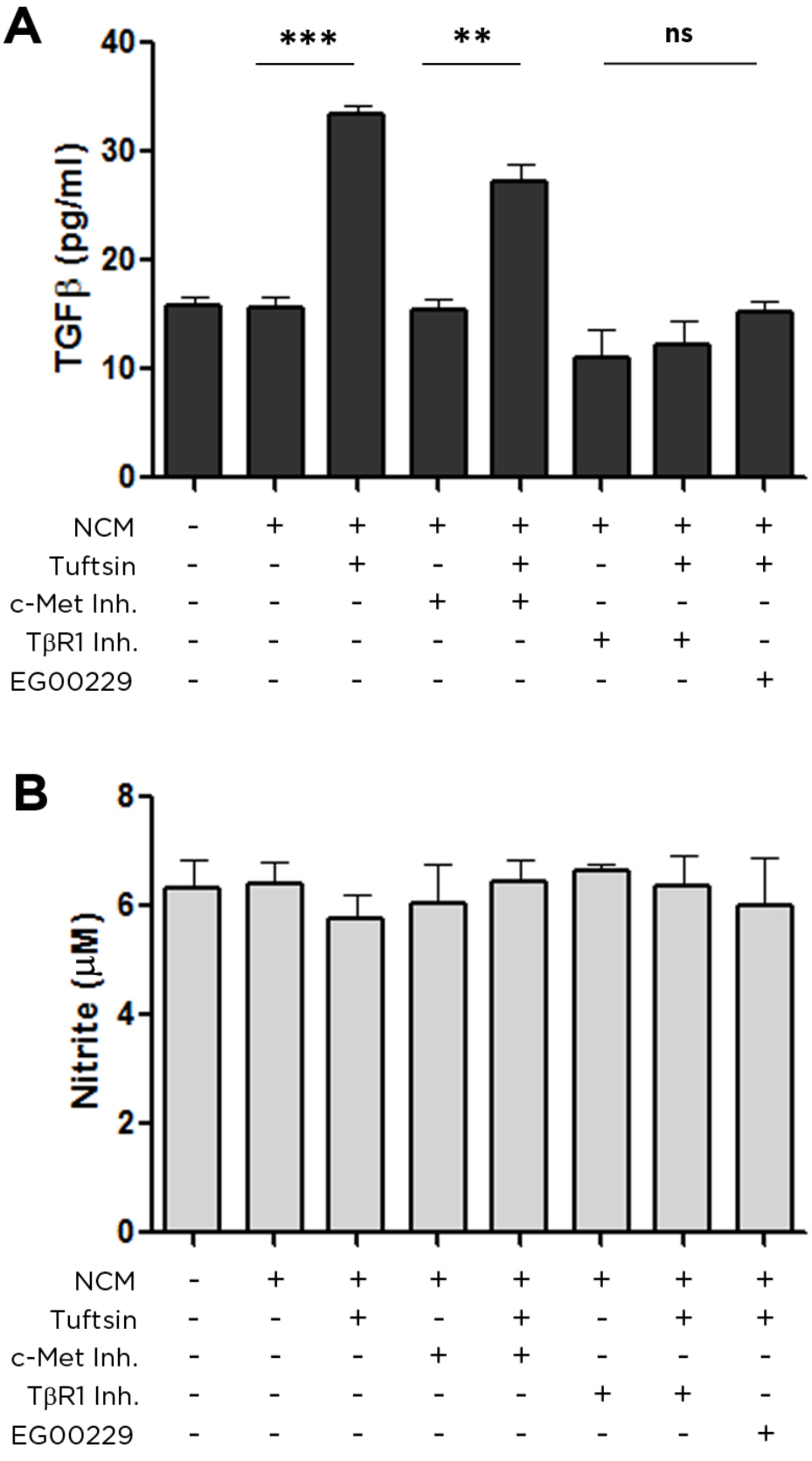
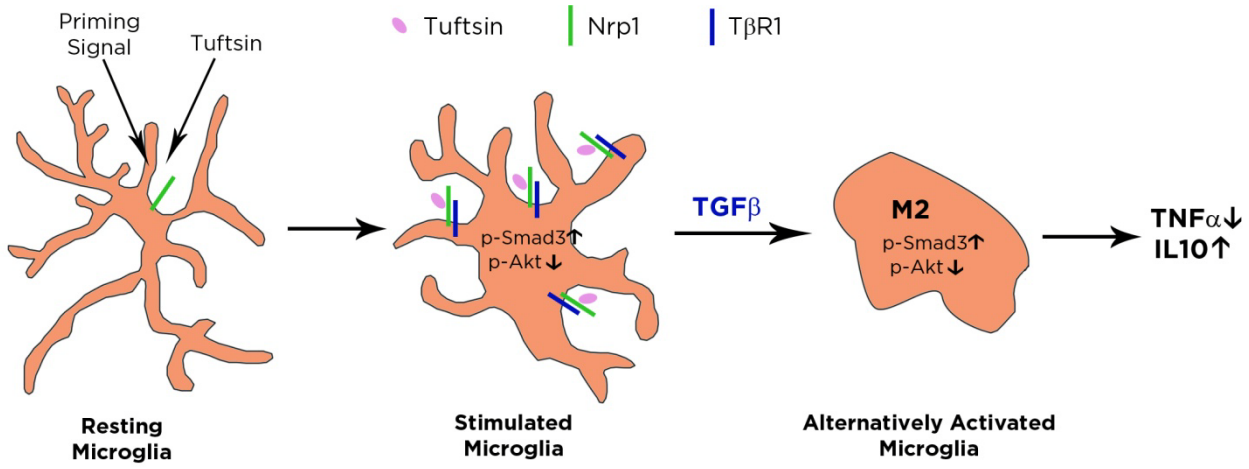

A
***
**
ns
TGFβ (pg/ml)
40
30
20
10
0
NCM - + + + + + + +
Tuftsin - - + - + - + +
c-Met Inh. - - - + + - - -
TβR1 Inh. - - - - - + + -
EG00229 - - - - - - - +
B
Nitrite (μM)
8
6
4
2
0
NCM - + + + + + + +
Tuftsin - - + - + - + +
c-Met Inh. - - - + + - - -
TβR1 Inh. - - - - - + + -
EG00229 - - - - - - - +

Figure IV-8: Model of Nrp1 signaling in microglia.


Priming Signal
Tuftsin
Tuftsin
Nrp1
TβR1
p-Smad3↑
p-Akt↓
TGFβ
M2
p-Smad3↑
p-Akt↓
TNFα↓
IL10↑
Resting Microglia
Stimulated Microglia
Alternatively Activated Microglia

Chapter V

Nrp1 mediates long functional contacts between microglia and Treg

INTRODUCTION

Treg are a subset of T cells that function in an immunosuppressive manner (Kohm et al. 2002; Liu et al. 2006; McGeachy et al. 2005). They differentiate in response to TGFβ, which they can also bind and convert to its active form (Glinka and Prud'homme 2008; Lohr et al. 2006). Tregs play an important role in the suppression of autoimmunity (Sakaguchi 2005) and maternal-fetal tolerance (Aluvihare et al. 2004), among others. Once activated, Treg cells are capable of suppressing the function and activation of many other immune cells, including helper T cells, B cells (Miyara and Sakaguchi 2007), and microglia (Reynolds et al. 2007).

Antigen presentation by innate immune cells is an essential component of T cell activation and functional modulation (Almolda et al. 2011). In the periphery, interaction of Treg with DC is required for their activation under physiological conditions (Tarbell et al. 2006). Within the CNS, microglia function as the resident innate immune cells. As microglia express MHC II proteins, they represent immature APCs when in the resting state (Olson et al. 2001). Recently, it was shown that microglia interact with Treg on an antigen-specific basis (Ebner et al. 2013).

The receptor Nrp1 has been shown to be important in the initiation of the primary immune response by mediating interactions between DCs and T cells (Tordjman et al. 2002). Specifically between Tregs and iDC, homotypic binding of Nrp1 allows for prolonged contact (Sarris et al. 2008). Outside the immune system, Nrp1 is expressed on many cell types and has roles in mediating axonal guidance in the nervous system through binding Sema3A (Gavazzi 2001; Kolodkin et al. 1997), as well as binding VEGF

in cardiovascular development (Soker et al. 1998). Microglia express Nrp1, which is upregulated under inflammatory conditions (Agudo et al. 2005; Majed et al. 2006). Although Nrp1 is expressed in T cell populations, the distribution of this expression among various subtypes has been debated. However, the consensus within the literature is that Nrp1 is typically found only within Treg populations, and exogenous production of FoxP3 in naïve T cells causes them to upregulate Nrp1 (Bruder et al. 2004; Sarris et al. 2008).

Based on these observations, we hypothesized that Nrp1 could mediate long, functional contacts between Treg and microglia. To investigate this possibility, we visualized and quantified the interactions between these cells via time-lapse video microscopy, and assessed Treg activation based on TGFβ release. Here, we show that Treg cells interact with microglia in a Nrp1-dependent manner. Loss of Nrp1 expression on either microglial cells, Treg, or both, significantly reduces the incidence of long contact times between the two cell types. Additionally, when this Nrp1-based interaction is lost, microglia are less capable of promoting Treg survival and activity as determined by a reduction in the release of TGFβ.

RESULTS

Frequent long contacts between microglia and Treg are Nrp1-dependent.

Many molecules are involved in the functional interaction between T cells and APCs. This interaction has been investigated extensively within the periphery, where DCs are the primary antigen-presenting immune cells. The contact zone between these cells is often referred to as the “immunological synapse” (Friedl et al. 2005). Within this interface, TCR molecules become centrally located where they interact with MHC II on dendritic cells, while several adhesion molecules occupy the periphery of the synapse (Monks et al. 1998). Recognition of MHC II peptide complexes locks LFA-1 into its high-affinity conformation, which increases its interaction with ICAM-1 on DCs. These additional interactions serve to stabilize the contact between T cells and APCs (Dustin et al. 1997).

However, it has been shown that iDCs preferentially interact with Treg over other T cell subtypes (Sarris et al. 2008). This is not due to differential expression of adhesion or co-activating signals, as molecules known to be involved in T cell-APC interactions such as CD2 and LFA-1 are expressed highly in all T cell subsets (Dustin et al. 1997). This preference was determined to be due to the presence of Nrp1 on the Treg cell surface, where it was nearly undetectable on other T cell subtypes. Further, blocking Nrp1 on either DCs or Treg removed this advantage (Sarris et al. 2008). As microglia are the resident antigen-presenting, Nrp1 expressing cells of the CNS, we hypothesized that Nrp1 would also mediate this preference for Treg interaction and activation (Agudo et al. 2005; Majed et al. 2006; Olson et al. 2001).

Although interactions between DCs and Treg have been observed in real-time (Sarris et al. 2008), this has never been investigated with microglial cells. Using time-lapse video microscopy, we determined the differences in contact times between microglia and Treg in the presence or absence of Nrp1. All studies were performed using microglia isolated from the neonatal cortex, as these cells readily return to a resting state, which is representative of the healthy CNS. As microglia were adherent to the plate, all contacts between the two cell types were due to movement among Tregs which remain in suspension. To obtain microglia that lack Nrp1 expression, the cells were isolated from macrophage/microglial specific Nrp1 knockout mice, which were generated from crossing Csf1R-cre (Deng et al. 2010) to $Nrp1^{fl/fl}$ (Gu et al. 2003) mice, henceforth referred to as Nrp1 KO microglia; littermates without Cre are Nrp1 WT. On the other hand, to ablate Nrp1 from Tregs, cells were isolated from $Nrp1^{fl/fl}$ mice and then polarized to the Treg subset. During this process, they were also transfected with a retroviral-Cre expressing construct (HR-MMPCreGFP), generated as previously described (Silver and Livingston 2001), which will be referred to as Nrp1 KO Treg with cells not exposed to retrovirus noted as Nrp1 WT (Figure V-1).

The interactions between microglia and Treg were monitored for 20 minutes of their co-culture, where we observed both short and long contacts. Wild-type microglial cells and Treg had longer average interaction times than if Nrp1 was knocked out in either cell type, or both (Figure V-2A,C). Transient contacts are indicative of T cells scanning for antigen, while longer interactions are required for rearrangement of proximal signaling molecules and activation (Dustin et al. 1997; Irvine et al. 2002). Based on previous literature reports, we set a threshold of 400 seconds as the

reference duration (Sarris et al. 2008); if contacts lasted longer than 400 seconds they were considered long/stable, but if they were less than 400 seconds the interactions were considered short. Overall, we determined the percentage of contacts that lasted for longer than 400 seconds, as previously described (Sarris et al. 2008). There was a significant reduction in long contacts when homotypic Nrp1 signaling was disrupted (Figure V-2B). These observations follow a similar pattern to those seen in the study of interactions between iDCs and Treg in the periphery (Sarris et al. 2008).

Nrp1 expression on microglia and Treg promotes TGFβ release.

TGFβ is an anti-inflammatory cytokine that is integral to Treg development and function (Huber et al. 2004; Peng et al. 2004; Sakaguchi 2005). Binding of TGFβ directly to Nrp1 on Treg has been shown to promote their immunosuppressive capabilities, either through activation of TGFβ from its latent form (Glinka and Prud'homme 2008), or potentially by interacting with both Nrp1 and its co-receptor TβR1 and signaling via the canonical TGFβ pathway (Nissen et al. 2013). Further, activated Treg become a mobile source of TGFβ, as they are recruited to areas of inflammation and release this cytokine (Chen and Wahl 2003). Through a distinctive approach requiring cell-cell contact, Tregs deliver inhibitory signals to target cells (Chen et al. 2001; Sakaguchi 2005; van Maurik et al. 2002). Long, functional contacts resulting in TCR stimulation promote Treg activation and TGFβ release (Chen and Wahl 2003; Sarris et al. 2008). Further, as microglia interact with Treg on an antigen-specific, TCR mediated basis (Ebner et al. 2013), we observed TGFβ levels as a measure of Treg activation by microglia.

Following the real-time video experiments described above, we kept the microglia and Treg in co-culture for four additional days. At the end of the co-culture period, media were collected and an ELISA assay probing for TGFβ levels was performed. We found that wild-type Tregs, when exposed to wild-type microglia, released significantly more TGFβ than when homotypic Nrp1 interactions were presumably disrupted through loss of Nrp1 expression on either Treg, microglia, or both (Figure V-3). These data indicate that effective activation of Treg, as well as their function in releasing its primary immunosuppressive cytokine TGFβ is mediated by its ability to interact with antigen-presenting immune cells through Nrp1. This also could potentially be through the TGFβ acting upon the Treg themselves through binding to Nrp1, which would explain the slightly elevated TGFβ levels where wild-type Treg were exposed to Nrp1 KO microglia, as these Treg are still capable of responding to this cytokine via Nrp1.

DISCUSSION

Removal of Treg cells (Kim et al. 2007a; Sakaguchi et al. 1995), or interference with their recruitment or retention (Bystry et al. 2001) results in a rapid induction of an autoimmune response, even in unprimed hosts. This suggests that self-reactive T cells are constitutively present, but are held in check by circulating Treg populations. Thus, in the healthy state, it seems that Treg cells have the upper hand over naïve T cell populations in the decision to launch an autoimmune response. Here, we present evidence that indicates that Nrp1 is critically important for this preference, as it is responsible for mediating long contacts between microglia and Treg. Further, this interaction results in the activation of Treg, and promotes their release of TGFβ. These data have many implications in processes ranging from maintenance of tolerance to autoimmunity, particularly in the central nervous system.

Short contacts between T cells and APCs are characteristic of antigen scanning by T cells (Irvine et al. 2002). However, long interactions seem to occur only in the presence of cognate MHC-peptide complexes, which are required for the stabilization and promotion of immunological synapse formation (Grakoui et al. 1999). Contrary to this, it has been shown that Treg make three times as many long interactions with iDCs that naïve T cells do in the absence of exogenous antigen (Sarris et al. 2008). This is possibly due to the bias of the TCR repertoire of Treg cells towards endogenous antigens (Hsieh et al. 2004).

The interaction between Tregs and microglia plays a key role in many CNS disorders. Microglia do not typically interact with Treg in the healthy state, as T cells are

normally excluded from the “immune-privileged” CNS due to the presence of the blood brain barrier (Dhib-Jalbut 2007; Fletcher et al. 2010). However, upon T cell infiltration, Treg can mediate the ability to withstand neurodegenerative conditions, as adoptive transfer of these cells is beneficial during optic nerve injury (Kipnis et al. 2004) and Parkinson’s disease (Appel et al. 2010). Most importantly, Treg play a key role in the recovery process of EAE. While infusion of Treg attenuates EAE symptoms (Kohm et al. 2002), their depletion increases EAE susceptibility in resistant mouse strains (Reddy et al. 2004). Further, mice that do not express Nrp1 in their T cell populations have severe EAE, with symptoms far worse than in control mice. Additionally, they have reduced numbers of Treg, which implies that Nrp1 is necessary for Treg differentiation and function *in vivo* (Solomon et al. 2011). Taken in whole, these data indicate that Nrp1 could be an essential mediator of both microglial and Treg function in EAE.

FIGURES

Figure V-1: T cell differentiation into Treg upregulates Nrp1, which is reduced following retroviral Cre transfection. T cells from $Nrp1^{fl/fl}$ mice were polarized to Tregs in the presence or absence of retroviral Cre. On the final day of polarization, when cells would be used for video imaging, RNA was isolated and PCR was performed for Nrp1. This shows a comparison between naïve T cells, Treg that have been fully polarized in the absence of retrovirus, and Treg that have been fully polarized in the presence of retrovirus.

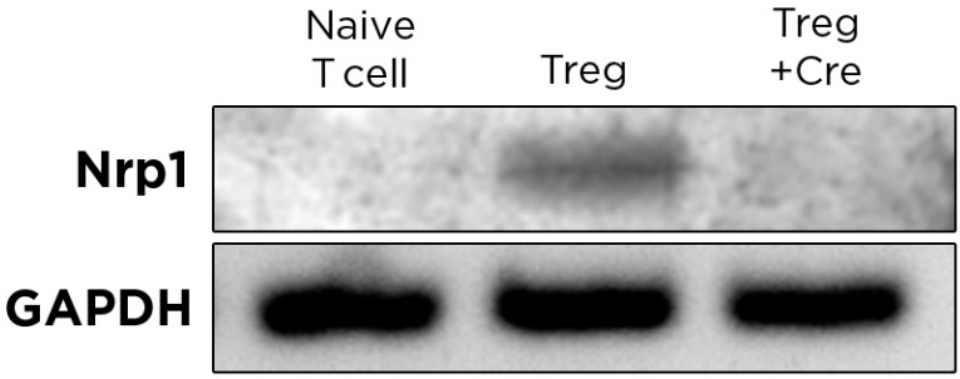

Naive
T cell
Treg
Treg
+Cre
Nrp1
GAPDH

Figure V-2: Nrp1 mediates long contacts between Treg and microglia. Microglia isolated from Nrp1 KO or Nrp1 WT pups were plated in chamber slides, and allowed to return to a resting state. The next day, Nrp1 KO or Nrp1 WT Treg were added, and visualized using real-time time lapse video microscopy. Images were taken every 10 seconds for 20 minutes, and duration of contacts was determined by the number of frames Treg and microglia remained in steady contact **(A)**. The percentage of interactions longer than 400 seconds **(B)**, and average contact length **(C)** were also observed. Significance is relative to WT microglia/WT Treg, n=3, **, $p<0.01$; ***, $p<0.001$

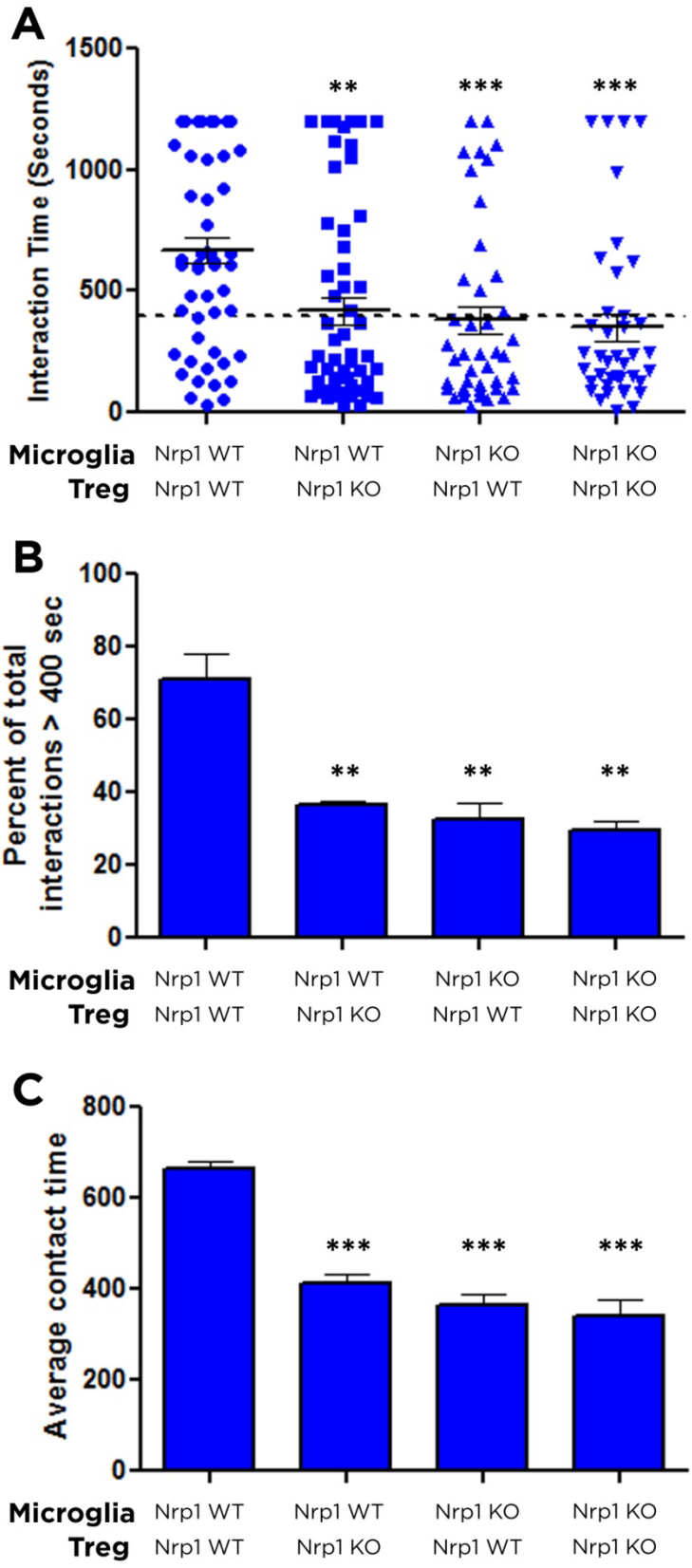
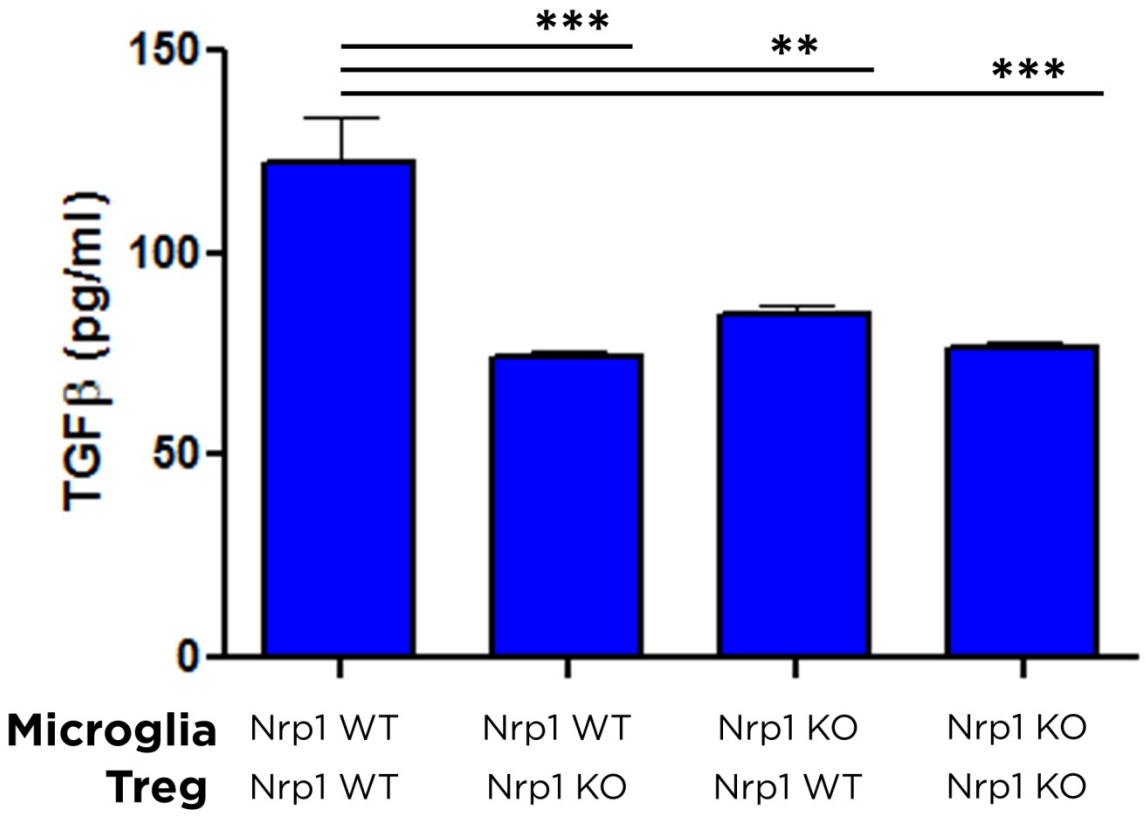

A
Interaction Time (Seconds)
1500
1000
500
0
**
***
***
Microglia Nrp1 WT Nrp1 WT Nrp1 KO Nrp1 KO
Treg Nrp1 WT Nrp1 KO Nrp1 WT Nrp1 KO
B
Percent of total interactions > 400 sec
100
80
60
40
20
0
**
**
**
Microglia Nrp1 WT Nrp1 WT Nrp1 KO Nrp1 KO
Treg Nrp1 WT Nrp1 KO Nrp1 WT Nrp1 KO
C
Average contact time
800
600
400
200
0
***
***
***
Microglia Nrp1 WT Nrp1 WT Nrp1 KO Nrp1 KO
Treg Nrp1 WT Nrp1 KO Nrp1 WT Nrp1 KO

Figure V-3: Loss of Nrp1 expression on microglia and Treg reduces TGFβ release. Microglia isolated from Nrp1 WT or KO pups were co-cultured with Nrp1 WT or KO Treg for four days, after which an ELISA probing for TGFβ cytokine levels was performed. n= 9, **, $p<0.01$; ***, $p<0.001$


***
**
***
150
100
50
0
TGFβ (pg/ml)
Microglia Nrp1 WT Nrp1 WT Nrp1 KO Nrp1 KO
Treg Nrp1 WT Nrp1 KO Nrp1 WT Nrp1 KO

Chapter VI

Microglial Nrp1 is necessary for tuftsin's beneficial effects *in vivo*

INTRODUCTION

MS and its animal model EAE are characterized by the progressive demyelination and degeneration of neurons in the CNS. (Swanborg 1995). The course of MS/EAE is strongly affected by microglia (Kreutzberg 1996; Raivich and Banati 2004; Rott et al. 1994; Stevens et al. 1994), with classically-activated M1 microglia that release pro-inflammatory cytokines associated with increasing disease severity and worsening symptoms. Alternatively, anti-inflammatory M2 microglia have been shown to mediate recovery (Kigerl et al. 2009), and administration of M2 monocytes can suppress ongoing severe symptoms in EAE (Mikita et al. 2010).

Previous work in our laboratory revealed that modulation of the activation state of microglia affected severity and duration of EAE (Bhasin et al. 2007). In particular, the microglial activator tuftsin, which readily crosses into the CNS (Paul et al. 2000), decreased the severity of EAE symptoms and drastically improved recovery in wild-type mice. Recently, we determined that tuftsin functions by promoting an M2 shift among microglial populations, which are then capable of polarizing T cell populations to their anti-inflammatory subsets (Wu et al. 2012). Further we have shown that tuftsin acts specifically through its receptor Nrp1 on microglial cells, which results in signaling through the canonical TGFβ signaling pathway (Nissen et al. 2013). These data strongly indicate that microglial modulation of tuftsin through its receptor Nrp1 is responsible for the beneficial effects seen *in vivo* following tuftsin administration during EAE.

To investigate this hypothesis, we induced EAE in mice that have a macrophage/microglial specific knockout of Nrp1, which would allow us to examine whether it is tuftsin's action on microglia alone that results in the attenuation of

symptoms. Here, we show that following the ablation of Nrp1 expression on macrophages and microglia, tuftsin treatment is completely ineffective in improving symptoms during EAE. Further, we observe that while tuftsin can attenuate pathological hallmarks of this disease such as demyelination and activation of inflammatory microglia in wild-type mice, animals that lack Nrp1 on their macrophages and microglia have no response to tuftsin treatment.

RESULTS

Implantation of drug delivery pumps does not affect EAE disease scores.

Consistent delivery of tuftsin during the month-long EAE course was achieved by implanting osmotic drug delivery pumps subcutaneously in the back of the mice. To determine if the presence of this pump alone affected disease scores, EAE was induced in Nrp1 WT and Nrp1 KO mice with or without a saline-filled 28-day osmotic pump. We observed no difference in disease score between control and saline infused mice at all time points, and in both genotypes (Figure VI-1). Thus, we can attribute any changes that occur following treatment to be a result of tuftsin infusion, rather than an off-target effect due to the presence of a drug delivery pump.

Loss of Nrp1 on microglial/macrophage populations prevents tuftsin's reduction of disease score in EAE.

To evaluate the role of Nrp1 on macrophage and microglial populations in mediating tuftsin's beneficial effects, the clinical course of MOG-induced EAE was assessed in Nrp1 WT and Nrp1 KO mice, using littermate controls for all experiments. In the groups that received tuftsin, drug delivery started at day 0 and lasted for 28 days through implantation of a single osmotic pump containing 500 μM tuftsin. During this period, clinical scores were recorded blinded to genotype. Among Nrp1 WT mice, clinical symptoms appeared around day 7, increased gradually to peak at day 21, and subsequently recovered. However, Nrp1 WT mice treated with tuftsin exhibited a similar disease course, albeit with significantly attenuated symptoms. From day 9 to day 23

post-EAE induction, tuftsin infused Nrp1 WT mice had a significantly reduced disease score (Figure VI-2A). In Nrp1 KO groups, both control and tuftsin infused mice showed no difference in scores for any of the 28 days, with similar severity to Nrp1 WT mice (Figure VI-2B).

To further determine the role of Nrp1 in mediating tuftsin's effects, peak and cumulative scores were compared between all groups. Our results show that the peak score of Nrp1 WT mice was 2.29±0.09, which indicates that their most severe symptoms were partial paralysis of the hindlimbs. In contrast, Nrp1 WT mice that were treated with tuftsin had a peak score of 1.23±0.14, which only correlates to a limp tail. However, Nrp1 KO mice attained comparable peak scores whether infused with tuftsin (2.28±0.13) or not (2.16±0.97) (Figure VI-2C). A similar pattern was seen in the cumulative score, where tuftsin-treated Nrp1 WT had a significantly reduced cumulative score (18.46±2.34) compared to Nrp1 WT controls (29.67±1.71). However, this difference was again abolished between Nrp1 KO control (28.25±2.14) and Nrp1 KO tuftsin-treated mice (28.38±1.55) (Figure VI-2D). Overall, tuftsin administration significantly reduced EAE severity, but not when its receptor Nrp1 was removed only from macrophage and microglial cells.

Reduction of weight loss during EAE as a result of tuftsin treatment is disrupted by Nrp1 knockout on macrophages/microglia.

Another classical hallmark of MS and EAE is weight loss, in which as symptoms become worse, body weight steadily decreases. As scores are a subjective value

determined by the observer, recording weight as an indicator of disease severity is not a measure that relies on interpretation of behavior. We weighed mice upon EAE induction and every seven days afterward, with timepoints at day 0, 7, 14, 21, and 28. As shown in Figure VI-3A, Nrp1 WT control mice steadily drop in weight up to day 21, after which they begin to re-gain weight during the recovery phase, paralleling their symptoms. However, Nrp1 WT tuftsin-treated mice gain weight continually throughout the EAE disease course, despite the fact that they do exhibit mild loss of tail tone. Conversely, Nrp1 KO mice in both control and tuftsin treated groups lose weight similarly to that seen in Nrp1 WT control mice, with tuftsin exerting no reduction in weight loss (Figure VI-3B). Thus, loss of Nrp1 specifically on macrophage and microglial populations disrupts tuftsin's prevention of weight loss during EAE.

Macrophage/microglial Nrp1 knockout mice do not exhibit reduced demyelination following tuftsin infusion.

Demyelination is a well-defined characteristic of both MS and EAE. Fluoromyelin dye was used to detect the presence of myelin in the white matter of the mouse spinal cord. Intact myelin was distinguished by green fluorescent staining, with reduced fluorescence indicative of demyelinated areas. Significant reduction in demyelination in Nrp1 WT tuftsin-infused groups was evident at day 14, for the white matter in control mice was 6.18±0.50% demyelinated, with tuftsin-treated mice exhibiting only 3.63±0.23% demyelination. This difference was further exacerbated at day 21, where extensive demyelination (25.51±3.69%) was observed in control mice, but only mild

demyelination (5.75±1.33%) was evident in the white matter of tuftsin-treated mice (Figure VI-4A,C). However, no significant change in demyelination was observed at any timepoint between Nrp1 KO WT and tuftsin-treated mice (Figure VI-4B,C). In all groups, recovery (remyelination) was apparently by day 28, although there was still reduced demyelination in tuftsin-treated Nrp1 WT mice compared to control that was not evident in Nrp1 KO groups. As Nrp1 expression on microglia thus appears to be necessary for tuftsin to exert its beneficial effects on three physical and pathological hallmarks of MS- paralysis, weight loss, and demyelination- its role in mediating tuftsin's effects on a cellular level had yet to be determined.

Microglial activation is diminished by tuftsin treatment, which is reversed by Nrp1 loss on microglia/macrophages.

During EAE, infiltrating T cells attack the engodenous myelin, which results in cellular debris in injured areas. Activated microglia are then recruited, and through phagocytosis function to clear these regions (Benveniste 1997). As the timing and extent of microglial activation contributes significantly to the ultimate outcome of EAE (Bhasin et al. 2007), we stained coronal spinal cord sections from all groups for Iba1 to observe the activation state of microglia.

Iba1 staining on both Nrp1 WT and KO mice on day 0 revealed resting microglia/macrophages, as defined by their ramified morphology and long, thin processes (Figure VI-5A,B). By day 14, Nrp1 WT control and both Nrp1 KO control and tuftsin treated mice began to show clustering of microglia with enlarged cell bodies and

retracting cell processes in the white matter, presumably in demyelinating lesions, which was not seen in Nrp1 WT tuftsin-treated mice. As the disease progressed to the peak at day 21, microglia with an amoeboid morphology characteristic of full activation as well as extensive microglial/macrophage infiltration was observed in both groups of Nrp1 KO mice and Nrp1 WT control mice, while Nrp1 WT tuftsin-treated mice not only had reduced overall microglial/macrophage infiltration, but individual cells displayed a more ramified, resting morphology as well (Figure VI-5A,B). By day 28, during the recovery phase, microglia in Nrp1 WT tuftsin-treated mice exhibited a far more resting phenotype compared to any other group.

When the Iba1 signal during EAE was quantified, there was a significant increase in microglial/macrophage staining in both Nrp1 KO groups as well as Nrp1 WT control mice as early as day 14, while NrpWT tuftsin-treated mice did not have a significant Iba1 increase until day 21. Compared to Nrp1 WT control mice, Nrp1 WT tuftsin-treated mice had significantly reduced Iba1 expression at all timepoints during EAE, which was not the case between Nrp1 KO control and tuftsin-treated mice (Figure VI-5C). These results indicate that Nrp1 WT tuftsin-treated mice experience both delayed and attenuated microglial activation.

Tuftsin infusion increases M2 microglial populations, which is abolished in macrophage/microglial specific knockout mice.

Microglia can play both a detrimental and beneficial role in MS/EAE due to their ability to polarize to pro-inflammatory M1 or anti-inflammatory M2 subsets. M1 microglia

have been observed in the CNS in the early stages of EAE, which shifts to a prevalence of M2 microglia during recovery (Mikita et al. 2010; Zhang et al. 2011b). As the balance between these two populations is an essential mediator of the MS/EAE disease course, it is important to determine tuftsin's effect on M2 polarization.

To distinguish the microglial activation state, those that expressed low levels of the M1 marker iNOS or the M2 marker arginase-1 (Arg1) in conjunction with Iba1 were defined as M0, resting microglia. Cells expressing either iNOS or Arg1 were considered to be M1 or M2 activated microglia, respectively. In line with previous studies, there was a predominance of M1 microglia in Nrp1 WT control mice at day 14 of EAE, with approximately 72.56% of microglia activated at day 14, comprised of 13.74±0.91% Arg1-positive, M2 cells, and 58.82±1.73% iNOS-positive, M1 cells. This predominance continued through day 21, with approximately 87.77% activated cells consisting of 20.27±1.49% Arg1-positive and 67.50±1.79% iNOS-positive (Figure VI-6A,C-E). In contrast, only about 42.97% of microglia were activated in Nrp1 WT tuftsin-treated mice, which had an M2 predominance at day 14 of 27.04±2.13% Arg1-positive microglia with only 15.93±4.56% of cells staining positive for iNOS. This trend continued through day 21, albeit with less of a drastic difference as of the ~56.69% of activated microglia, 29.76±3.69% were Arg1-positive while 26.93±6.98% were iNOS positive (Figure VI-A,C-E). During the recovery phase at day 28, both control and tuftsin-treated Nrp1 WT mice switched to an M2 predominance, although Arg1 levels were still significantly elevated in tuftsin-treated mice over controls. Overall, there was a significant decrease in M1 microglia at all time points in Nrp1 WT tuftsin-treated mice, with a concomitant significant increase in M2 microglia on days 14 and 28 (Figure VI-C,D). However, this

M2 predominance as a result of tuftsin treatment was completely abolished in Nrp1 KO mice, as both control and tuftsin-treated groups exhibited similar proportions among their resting, M1, and M2 microglial populations (Figure VI-B-E).

These results indicate that tuftsin functions through Nrp1 on macrophage/ microglial cells by polarizing them to a protective M2 subset early, which gives an advantage to anti-inflammatory responses later during the EAE disease course.

DISCUSSION

In this chapter, we assessed the effects of tuftsin on EAE, and found that tuftsin significantly ameliorated a variety of hallmarks of EAE, including reduction of clinical score, weight loss, microglial activation, and M1 microglial polarization. However, if tuftsin binding to microglia and macrophages is disrupted through loss of expression of its receptor Nrp1 on only these cell types, tuftsin's beneficial effects are completely abrogated. These results suggest that tuftsin functions exclusively through microglial populations *in vivo* during EAE.

Tuftsin's broad activities on phagocytic cells, especially microglia and macrophages, make the peptide a potential candidate for immunotherapy. As evidence, tuftsin and its analogs have been chemically synthesized and applied in a variety of clinical studies (Fridkin and Najjar 1989). As the normal tuftsin serum level is 250 – 500 ng/ml and the LD_{50} dose of tuftsin is 2.4 mg/ml, a serum level of 60 μg/ml is a suitable physiological concentration for applying tuftsin in therapeutic studies. Here, we delivered tuftsin through mini-osmotic pumps at a concentration of 500 μM, with an infusion rate of 0.25μl/hour. In theory, an infusion of tuftsin at 0.06μg per hour will diffuse along the length of the spinal cord and surrounding tissues, bringing it to a physiological concentration for the therapeutic effects without other untoward effects.

When Nrp1 is abolished from all T cell populations *in vivo*, mice undergo a significantly more severe EAE disease course. Further, they exhibit a preferential Th17 lineage commitment and decreased Treg functionality (Solomon et al. 2011). These results differ from ours shown here, as macrophage/microglial specific Nrp1 KO mice

undergo an EAE disease course that is comparable to wild-type controls. There are several potential explanations for this difference- first, we showed that tuftsin treatment promoted the upregulation of Nrp1 on microglial cells, where Nrp1 is normally expressed at very low levels (Wu et al. 2012)(Chapter III), which would indicate that loss of Nrp1 expression in Nrp1 KO mice would not differ significantly from the endogenous state in the absence of treatment. Further, Tregs in these mice still express Nrp1, which promotes their function through the binding and activation of TGFβ (Glinka and Prud'homme 2008). The disruption of antigen presentation preference described in Chapter V could also potentially be circumvented, as during EAE DCs and iDCs from the periphery are recruited into the CNS (Jain et al. 2010; Sagar et al. 2012), which are capable of interacting with T cells on a Nrp1-mediated basis (Sarris et al. 2008). Thus, while infiltrating Treg from the periphery would not be favored for interactions with Nrp1 KO microglia, they would still make contacts with Nrp1-expressing, antigen-presenting iDCs in a Treg-preferential manner. Endogenously, Nrp1 on microglial cells has been described to be upregulated following CNS injury to the spinal cord or optic nerve (Agudo et al. 2005; Majed et al. 2006), but a role for this protein in the healthy state has not been discussed. So while microglial/macrophage specific Nrp1 knockout mice respond differently to tuftsin treatment, they would still undergo a similar disease course to wild-type mice.

Microglial polarization to pro- and anti-inflammatory subsets (Mikita et al. 2010) as well as their temporal activation during EAE (Bhasin et al. 2007) is essential in determining whether they will function in a beneficial or harmful manner. As evidenced here, tuftsin not only seems to delay the activation of microglial cells, but gives them an

early, lasting prevalence of M2-polarized populations. The fact that this polarization is more pronounced at day 14 than day 21 (Figure VI-6E) could potentially explain why the disease course in tuftsin-treated Nrp1 WT mice is characterized by a “soft peak” that is very significantly reduced from controls. Regardless, mice in all treatment groups show a steady increase in M2 microglia that only reaches predominance in control and Nrp1 KO mice during the recovery phase. M1 microglia have been reported to promote T cell differentiation toward Th1 and Th17 fates (Becher et al. 2006), which both induce neurodegeneration (Centonze et al. 2009) and impair recovery through remyelination (Li et al. 2005). On the other hand, M2 macrophages are associated with increased Th2 and Treg populations (Frisancho-Kiss et al. 2009), and M2 microglia drive oligodendrocyte differentiation during CNS remyelination (Miron et al. 2013). Taken in whole, tuftsin appears to function by promoting an overall anti-inflammatory environment in the CNS during EAE, which is critically important for its beneficial effects.

FIGURES

Figure VI-1: The presence of a drug delivery pump does not affect the EAE disease course. EAE was induced by injection of MOG_{35-55} in CFA and pertussis toxin in all groups. Saline infused mice had a 28-day osmotic pump implanted at day 0, while control mice did not. Comparisons between saline and control Nrp1 WT **(A)** and Nrp1 KO **(B)** disease scores were recorded. n= 4-12.

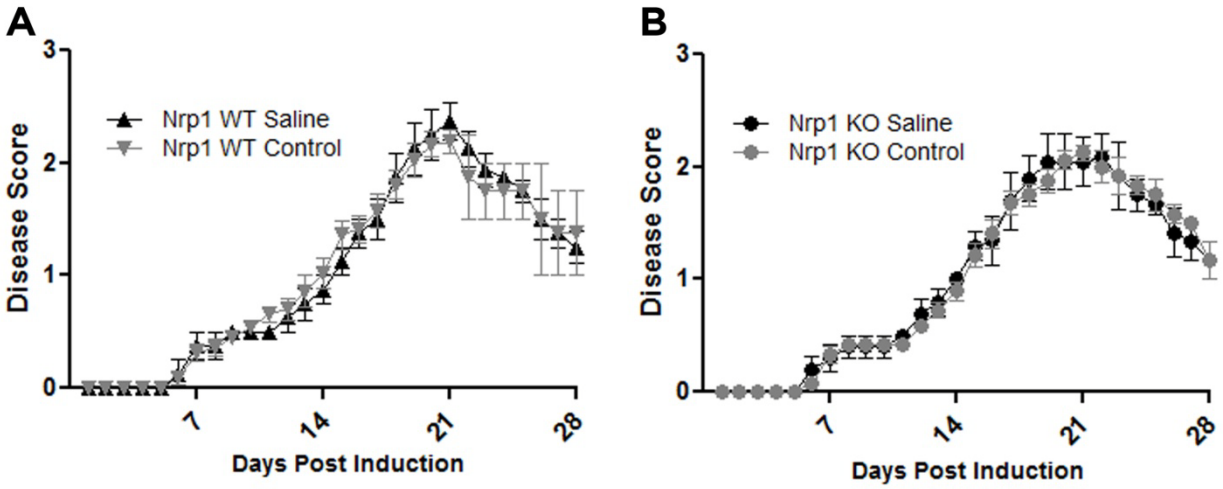

A
Disease Score
Nrp1 WT Saline
Nrp1 WT Control
Days Post Induction
B
Disease Score
Nrp1 KO Saline
Nrp1 KO Control
Days Post Induction

Figure VI-2: Loss of Nrp1 on macrophage/microglial cells reverses the improved disease score observed in the presence of tuftsin. EAE was induced by injection of MOG_{35-55} in CFA and pertussis toxin in all groups. Osmotic pumps filled with 500 μM tuftsin in PBS were implanted subcutaneously on day 0 after MOG immunization, and tuftsin was infused for 28 days. Comparisons between Nrp1 WT control and tuftsin infused mice **(A)** and Nrp1 KO control and tuftsin infused mice **(B)** were made. Cumulative score **(C)** and peak score **(D)** were compared between all groups. n= 13-17, *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$.

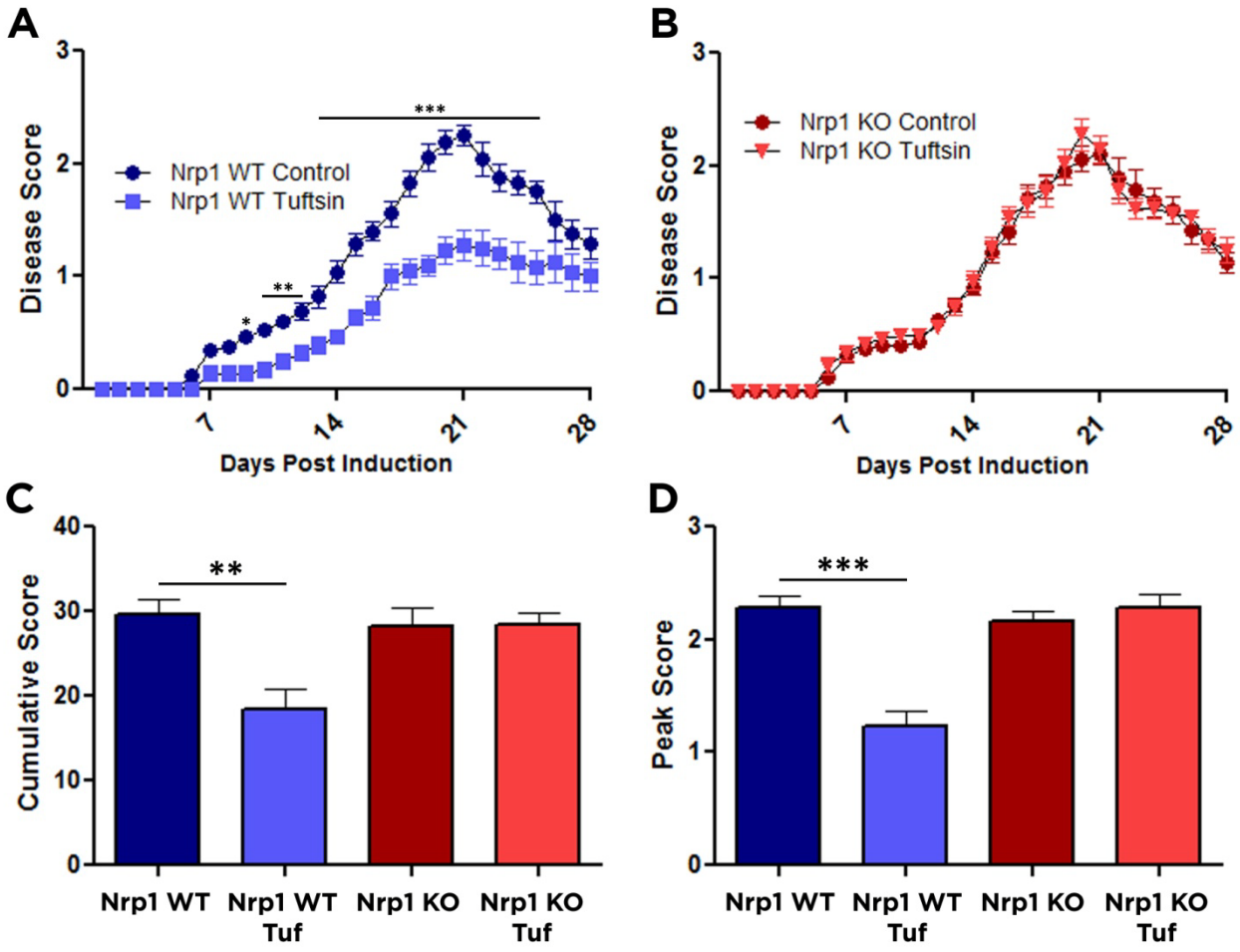

A
Disease Score
Nrp1 WT Control
Nrp1 WT Tuftsin
***
**
*
Days Post Induction
B
Disease Score
Nrp1 KO Control
Nrp1 KO Tuftsin
Days Post Induction
C
Cumulative Score
**
Nrp1 WT
Nrp1 WT Tuf
Nrp1 KO
Nrp1 KO Tuf
D
Peak Score
***
Nrp1 WT
Nrp1 WT Tuf
Nrp1 KO
Nrp1 KO Tuf

Figure VI-3: Macrophage/microglial specific Nrp1 KO mice do not have reduced weight loss with tuftsin treatment during EAE. Weight changes were recorded and compared between Nrp1 WT control and tuftsin infused mice **(A)** and Nrp1 KO control and tuftsin infused mice **(B)**. All weights were plotted as percentages of day 0 weights. n= 13-16, **, $p<0.01$, ***, $p<0.001$.

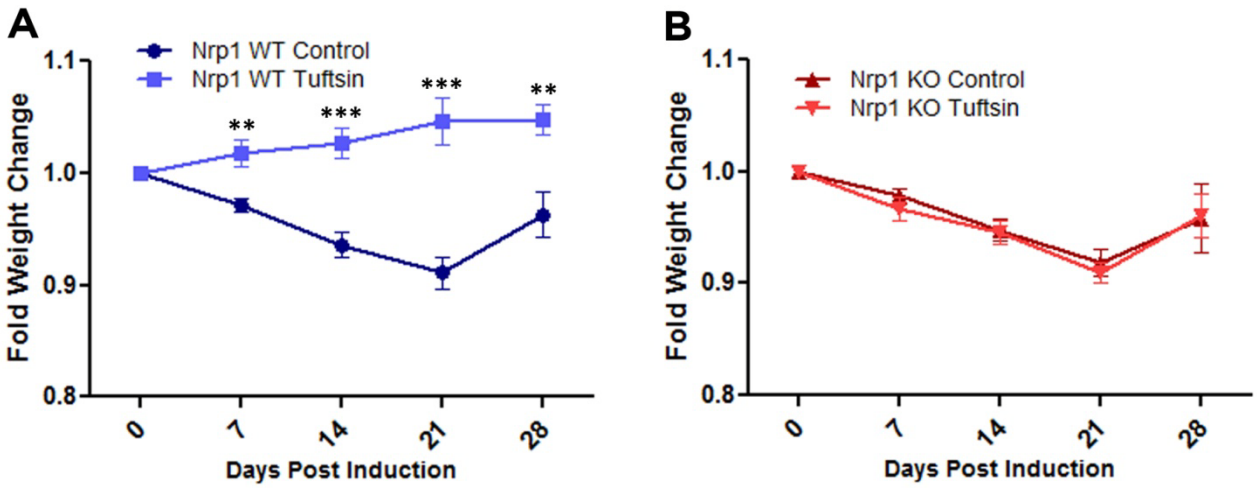
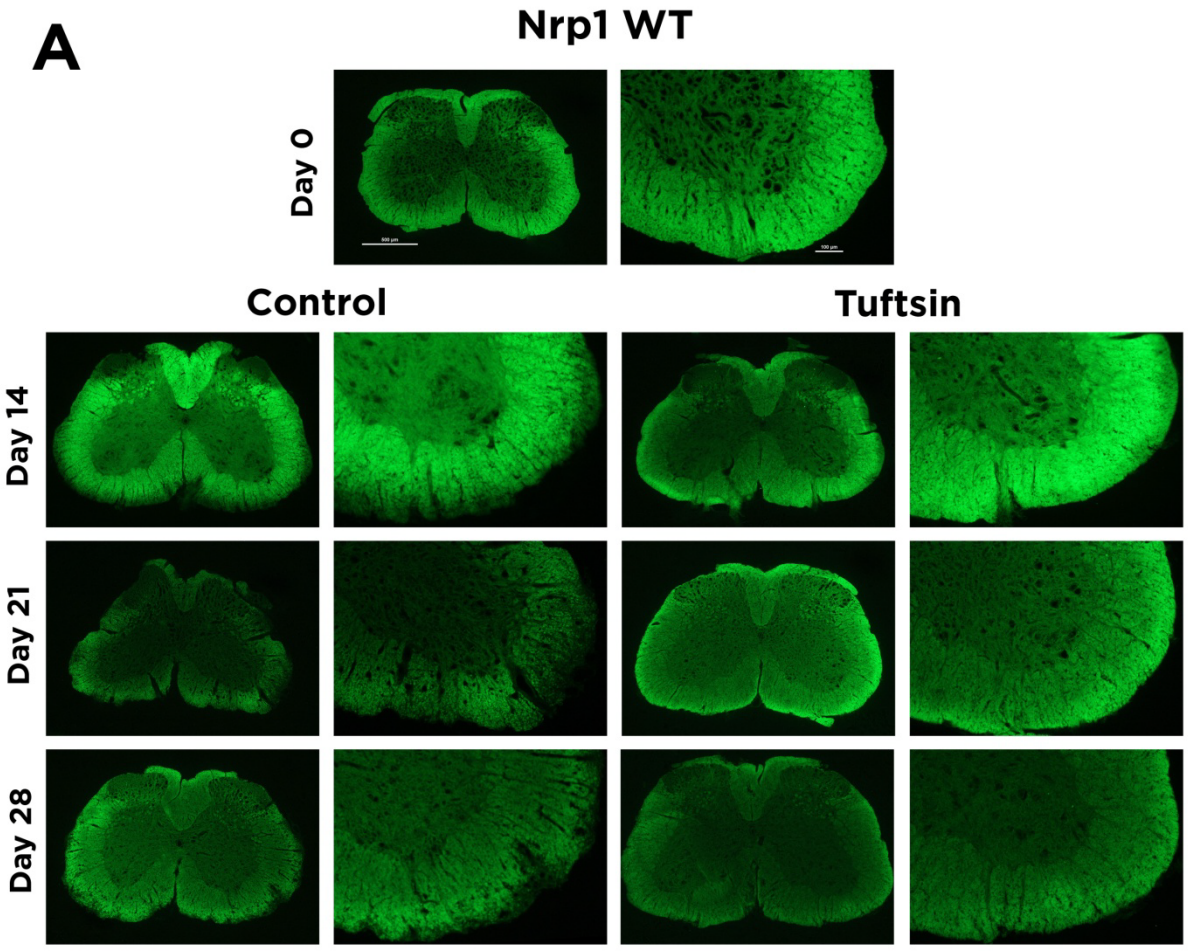
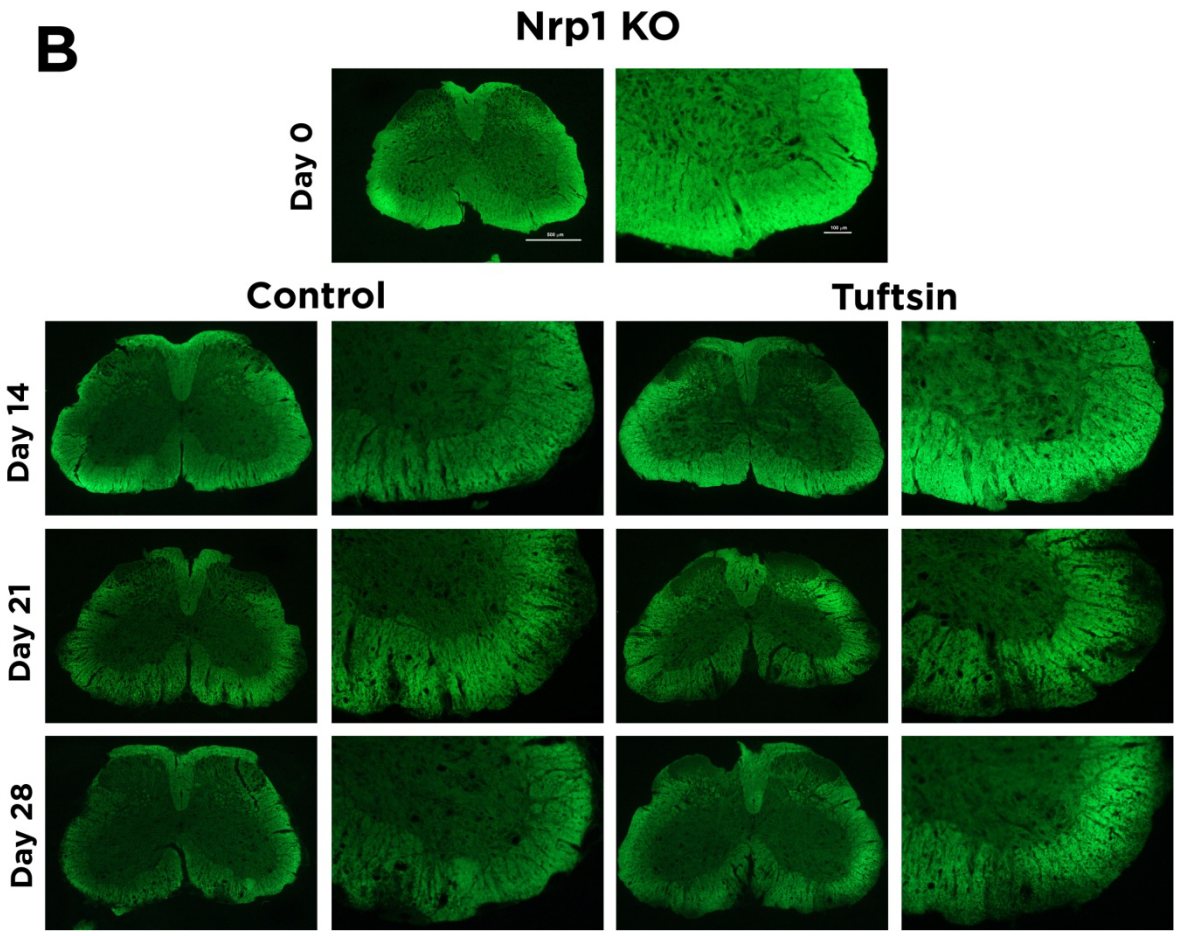

A
Nrp1 WT Control
Nrp1 WT Tuftsin
Fold Weight Change
1.1
1.0
0.9
0.8
**
***
***
**
0
7
14
21
28
Days Post Induction
B
Nrp1 KO Control
Nrp1 KO Tuftsin
Fold Weight Change
1.1
1.0
0.9
0.8
0
7
14
21
28
Days Post Induction

Figure VI-4: Tuftsin's reduction of demyelination is disrupted by the loss of Nrp1 on macrophages/microglia. Frozen spinal cord sections were isolated from control and tuftsin infused Nrp1 WT and KO mice at day 0, 14, 21, and 28 days post induction of EAE. To visualize demyelination, fluoromyelin dye was used. Myelinated regions of the white matter display strong green fluorescence, where demyelinated regions are indicated by diminished color. Images of full coronal sections are shown, with higher magnification images to further visualize demyelinated areas. Comparisons between Nrp1 WT control and tuftsin infused **(A)** and Nrp1 KO control and tuftsin infused **(B)** spinal cord myelination are shown. Demyelinated areas were measured using ImageJ and quantified in **(C)**. n=3, *, $p<0.05$; **, $p<0.01$, Scale bar: 500 μm, 100 μm.


A
Nrp1 WT
Day 0
Control
Tuftsin
Day 14
Day 21
Day 28


B
Nrp1 KO
Day 0
Control
Tuftsin
Day 14
Day 21
Day 28

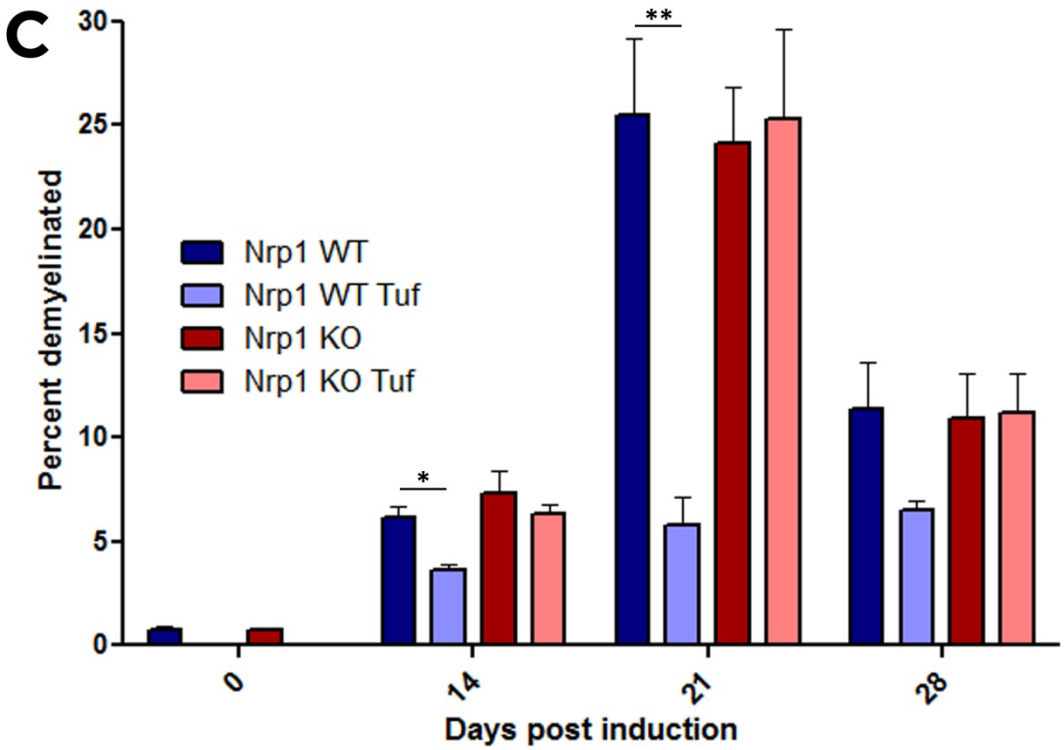
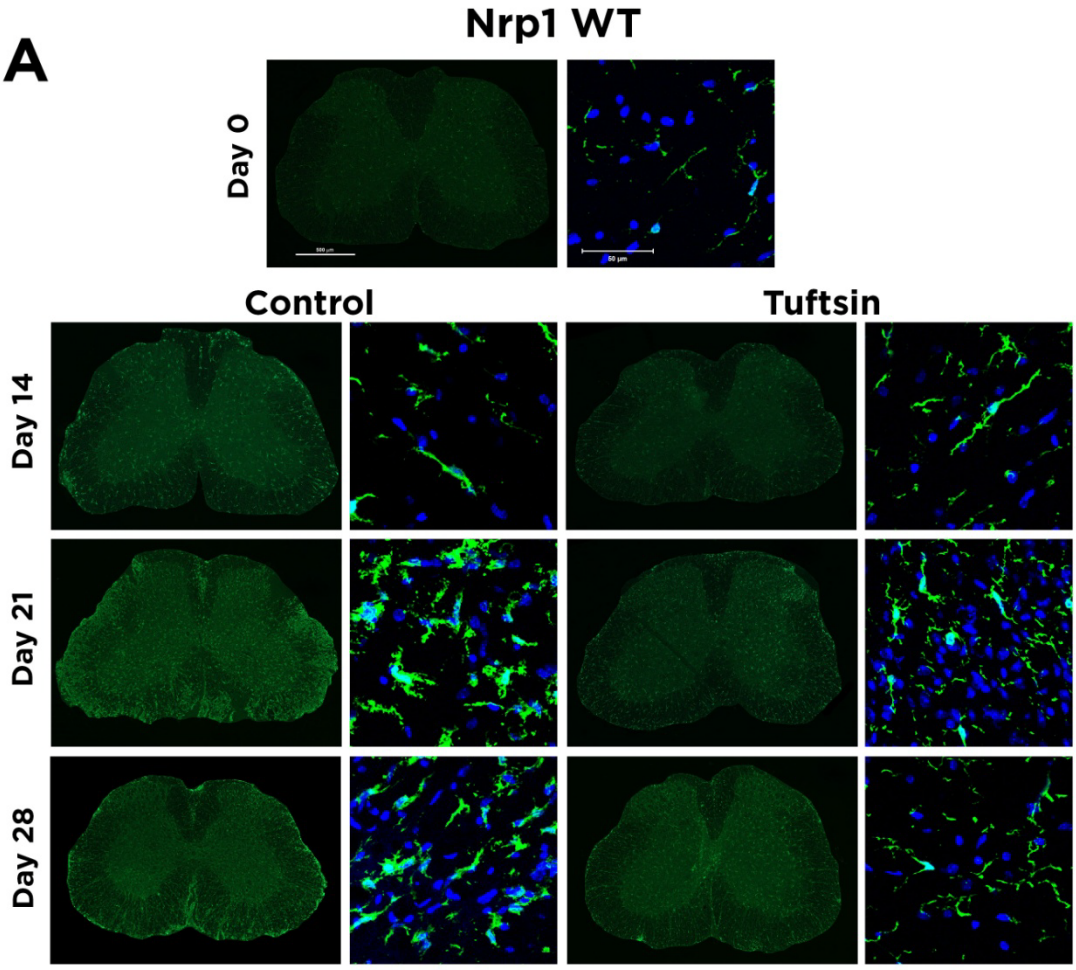
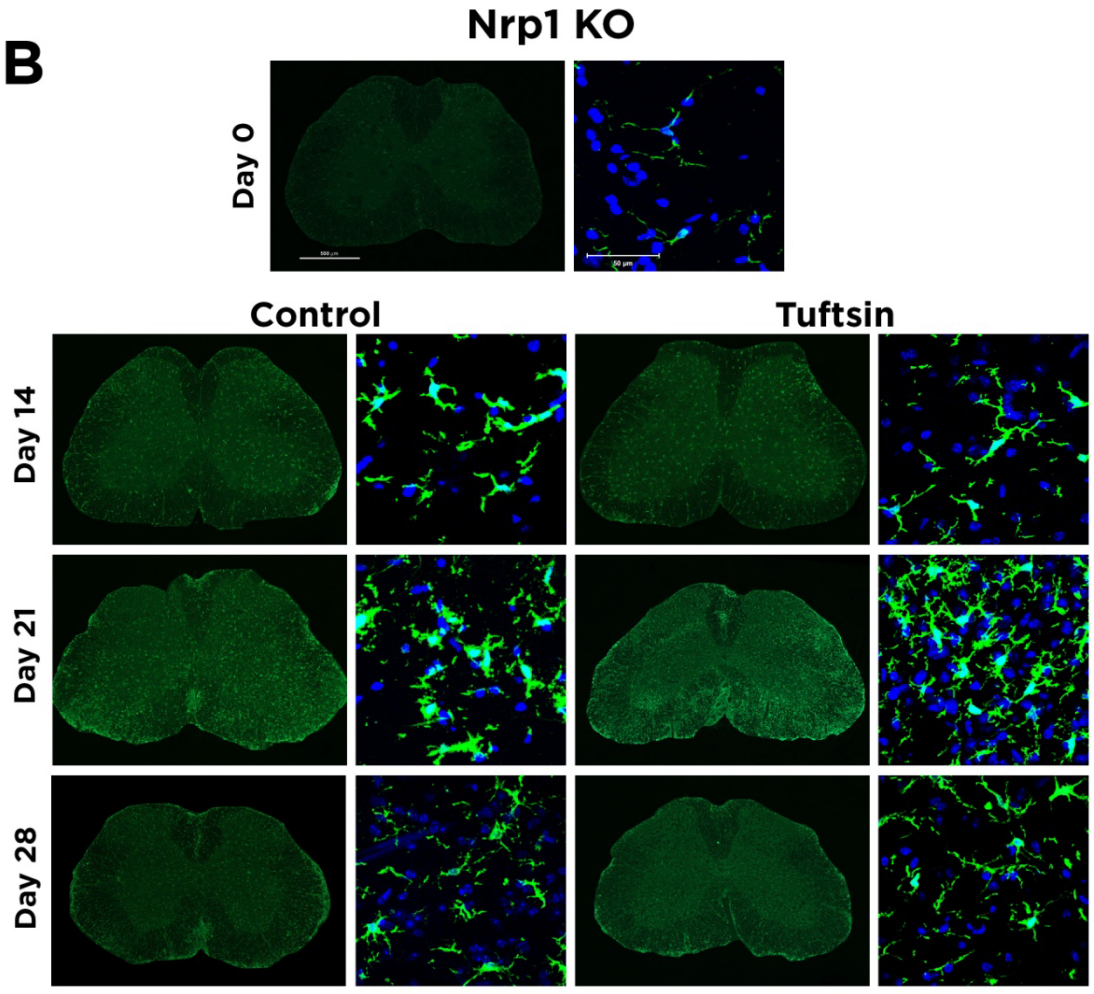

C
Percent demyelinated
30
25
20
15
10
5
0
Nrp1 WT
Nrp1 WT Tuf
Nrp1 KO
Nrp1 KO Tuf
*
**
0
14
21
28
Days post induction

Figure VI-5: Reduction of microglial activation by tuftsin is disrupted in macrophage/microglial specific Nrp1 KO mice. Iba1 (green) and DAPI (blue) staining was utilized to detect microglia in control and tuftsin infused Nrp1 WT **(A)** and KO **(B)** mice at day 0, 14, 21, and 28 days post induction of EAE. Higher magnification images show the morphology of microglia in the white matter. While resting microglia are characterized by small cell bodies with long processes, activated microglia obtain a more amoeboid shape with short, multi-branched cellular protrusions. Quantification of Iba1 signal intensity is shown in **(C)**. n=3, *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$. Scale bar: 500 μm, 50 μm.


A
Nrp1 WT
Day 0
Control
Tuftsin
Day 14
Day 21
Day 28


B
Nrp1 KO
Day 0
Control
Tuftsin
Day 14
Day 21
Day 28

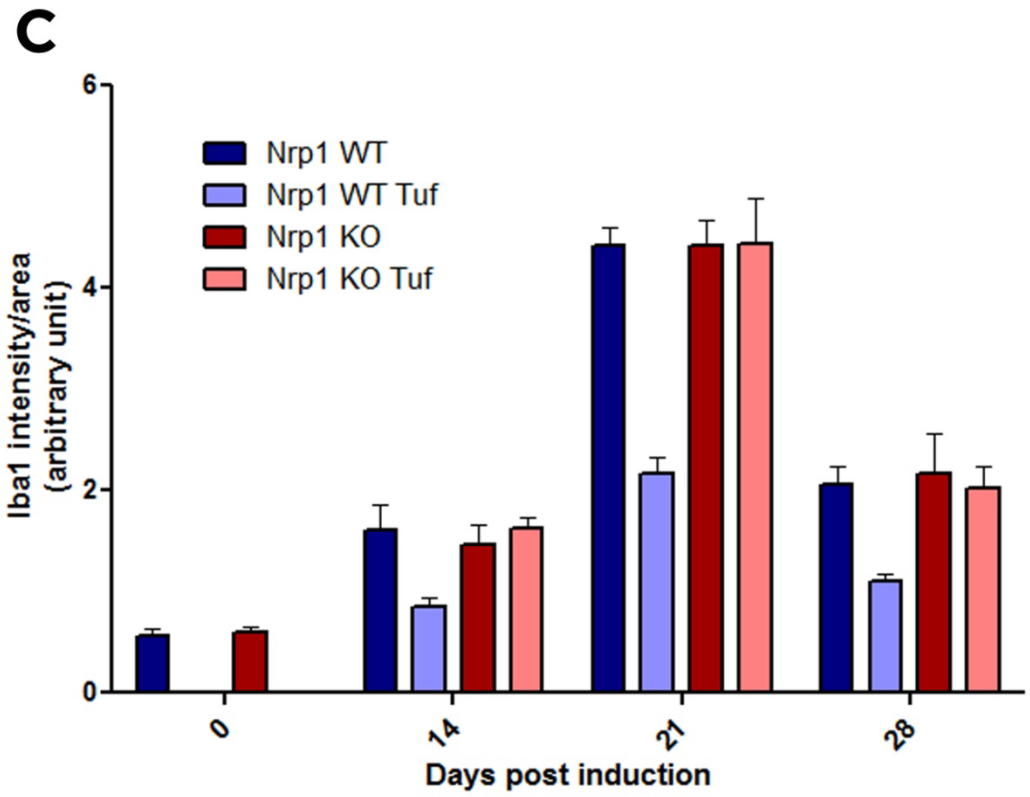
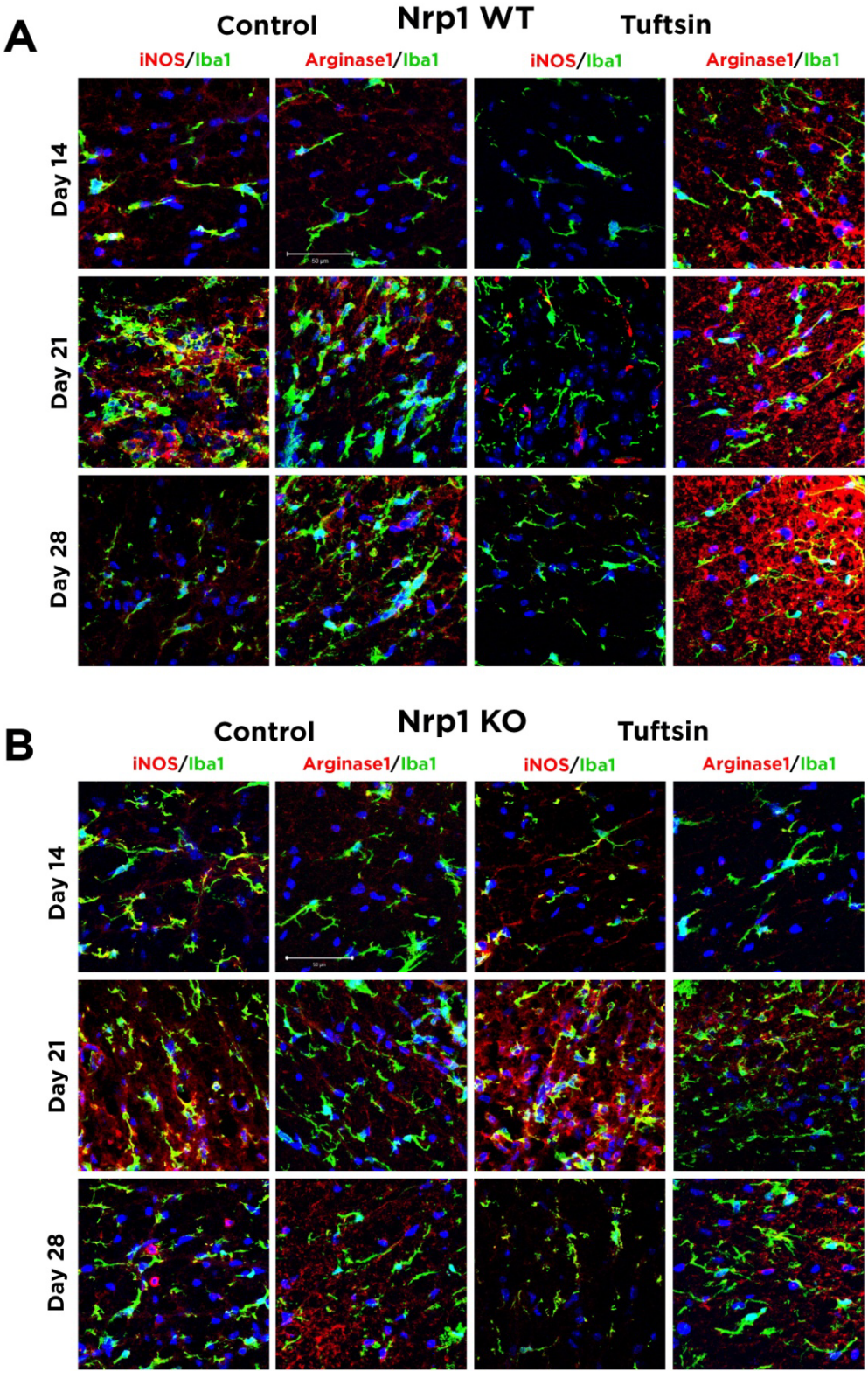
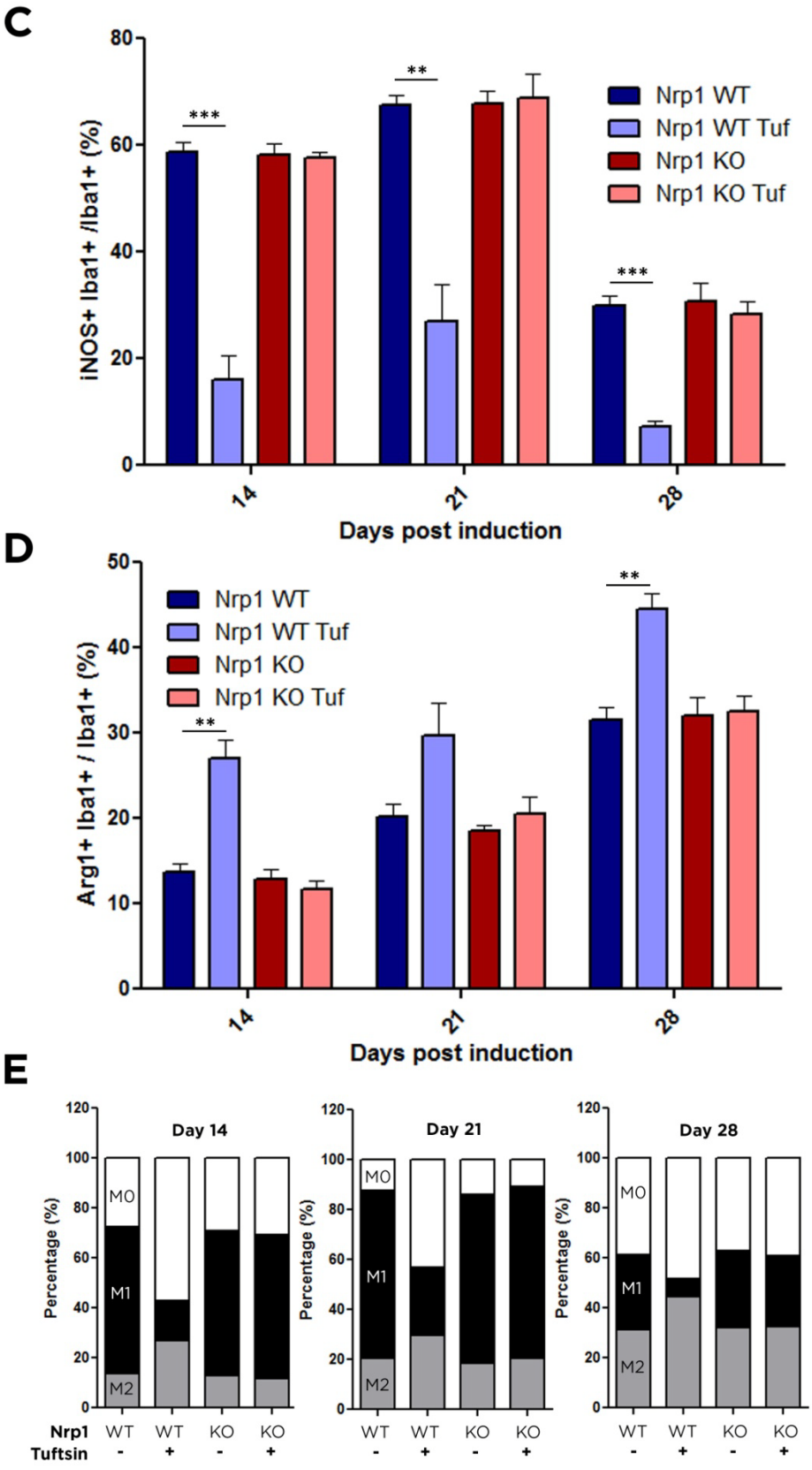

C
Nrp1 WT
Nrp1 WT Tuf
Nrp1 KO
Nrp1 KO Tuf
Iba1 intensity/area (arbitrary unit)
0
2
4
6
0
14
21
28
Days post induction

Figure VI-6: Tuftsin promotes an M2 shift among microglia, which is abolished in macrophage/microglial specific Nrp1 KO mice. Pro-inflammatory (M1) and anti-inflammatory (M2) microglia were identified by colocalization iNOS/Iba1 and Arginase1/Iba1 labeling, respectively, with control and tuftsin infused Nrp1 WT mice in **(A)** and control and tuftsin infused Nrp1 KO mice in **(B).** $iNOS^+/Iba1^+$ **(C)** and $Arg1^+/Iba1^+$ **(D)** were counted and compared between tuftsin treated and control Nrp1 WT and Nrp1 KO mice, at day 14, 21, and 28. Percentages of each population after treatment are indicated in **(E)**. Nuclei are stained with DAPI (blue). n=3, **, $p<0.01$, ***, $p<0.001$. Scale bar: 50 μm.


A
Control
Nrp1 WT
Tuftsin
iNOS/Iba1
Arginase1/Iba1
iNOS/Iba1
Arginase1/Iba1
Day 14
Day 21
Day 28
B
Control
Nrp1 KO
Tuftsin
iNOS/Iba1
Arginase1/Iba1
iNOS/Iba1
Arginase1/Iba1
Day 14
Day 21
Day 28


C
iNOS+ Iba1+ /Iba1+ (%)
Nrp1 WT
Nrp1 WT Tuf
Nrp1 KO
Nrp1 KO Tuf
***
**
***
Days post induction
D
Arg1+ Iba1+ / Iba1+ (%)
Nrp1 WT
Nrp1 WT Tuf
Nrp1 KO
Nrp1 KO Tuf
**
**
Days post induction
E
Day 14
Day 21
Day 28
Percentage (%)
M0
M1
M2
Nrp1 WT WT KO KO
Tuftsin - + - +

Chapter VII

General Conclusions and Discussion

CONCLUSIONS

Tuftsin can effectively promote recovery during EAE, and is a strong potential candidate for immunotherapy in MS. In this project, we focused on determining the mechanism by which tuftsin and its receptor Nrp1 mediate this effect.

How does tuftsin and Nrp1 affect microglia and T cells on a cellular level?

When primed microglia were exposed to tuftsin, they readily polarized to their anti-inflammatory, M2 subsets. Further, we observed that tuftsin was capable of upregulating its receptor Nrp1 on microglia, both on the cell surface and shed into the media. Our results indicated that tuftsin binding to its receptor Nrp1 was necessary for this anti-inflammatory shift to occur, which implies that tuftsin did not exert its effects through any other route on microglial cells. Downstream of this interaction, we found that tuftsin signaled via the canonical TGFβ pathway, which had not been determined over the 45-year history of this peptide.

However, Nrp1 itself in the absence of tuftsin was also capable of mediating immunosuppressive effects. Through the use of time-lapse video microscopy, we observed interactions between Nrp1 KO or WT microglia and Nrp1 KO or WT Treg. Our results showed that loss of Nrp1 expression on either cell type, or both, significantly reduced the incidence and duration of long, functional contacts between microglia and Treg. Additionally, when this interaction was disrupted we showed decreased Treg activation through the reduction of TGFβ levels, a characteristic Treg cytokine. Extrapolating from these data, Nrp1 could provide Treg with an advantage for activation

by microglia, which would promote the suppression of autoimmunity in the healthy state (Figure VII-1).

Does tuftsin's receptor Nrp1 mediate its beneficial effects *in vivo*?

Following these observations *in vitro*, we investigated the mechanism of tuftsin action *in vivo*. As infusion of tuftsin into wild-type mice promoted the M2 polarization of microglia during EAE, the role of Nrp1 in this process became our focus. Using macrophage/microglial specific Nrp1 KO mice, we demonstrated that infusion of tuftsin had no effect on reduction of disease score or weight loss, which both saw significant improvements following tuftsin treatment in Nrp1 WT mice. Further, while tuftsin-treated Nrp1 WT mice had significant improvements in demyelination, this effect was abolished in Nrp1 KO mice. As the timing and type of microglial activation is an essential determinant of the MS/EAE disease course, we then sought to define how tuftsin affects these parameters. Our results showed that tuftsin-treated Nrp1 WT mice show microglial activation at day 21 of EAE, with a predominance of M2 microglia for the duration of disease. In contrast, Nrp1 WT control mice as well as both control and tuftsin-treated Nrp1 KO mice had evidence of activated microglia as early as day 14, of which the population was heavily comprised of M1 microglia until the recovery stage at day 28. As the loss of expression of Nrp1 specifically on microglia and macrophages is the only difference between these two genotypes, it is clear that our data show that tuftsin functions exclusively through microglia to exert its beneficial effects during EAE (Figure VII-1).

FUTURE DIRECTIONS

Potential use of Nrp1 as a diagnostic marker for MS

Microglia express Nrp1, which is upregulated under inflammatory conditions (Agudo et al. 2005; Majed et al. 2006). Tregs also express Nrp1 (Bruder et al. 2004; Sarris et al. 2008), and the cytokine TGFβ can bind to this receptor, where it promotes Treg differentiation and activity (Glinka and Prud'homme 2008). Nrp1 is critical for maintaining tolerance and attenuating autoreactivity in EAE (Solomon et al. 2011). These observations lead to the hypothesis that Nrp1 levels are an indicator of the recovery phase of MS/EAE. As Nrp1 is highly expressed on immunosuppressive Tregs, which are an essential contributor to the attenuation of EAE symptoms (Kohm et al. 2002; Liu et al. 2006; McGeachy et al. 2005), and can be shed from the cell surface by ADAM10 (Swendeman et al. 2008), levels of Nrp1 in the cerebrospinal fluid (CSF) of MS patients could be indicative of disease state. Low levels of Nrp1 could suggest exacerbation of MS whereas elevated levels could indicate a remission period. Generating a reliable method to determine Nrp1 levels during EAE/MS would be an indicator of disease progression and state of the immune system in MS patients.

Despite the long history of study into MS, it is difficult to accurately diagnose and predict symptoms in patients (Katz Sand and Lublin 2013; Lublin 2002; Lublin and Reingold 1996). Development of a diagnostic Nrp1 ELISA assay could allow us to not only better predict disease progression in MS patients, but can also be used to gauge the effectiveness of other disease modifying agents. Further, as currently all treatments are in response to symptoms that are observed by physicians, being able to predict

upcoming relapses in patients could allow for proactive rather than reactive interventions.

Combination of tuftsin with additional treatments to improve disease outcomes

In the studies shown above, we administered tuftsin in a prophylactic rather than a therapeutic manner. As treatment in MS patients begins following the presence of visible symptoms such as muscle weakness or cognitive deficits, it is important to determine if tuftsin would work as effectively following the onset of disease. Thus, it would be pertinent to administer tuftsin at day 14, where significant microglial activation and demyelination is evident, in order to observe whether tuftsin is capable of reversing ongoing MS/EAE.

While tuftsin alone clearly exerts a significant improvement in EAE symptoms and pathology, it does not result in returning animals to the normal, uninjured state. Therefore, there is room for improvement upon this treatment model. One potential method for boosting tuftsin's beneficial effects would be to address lingering issues in MS/EAE- the presence of M1 microglia following tuftsin administration, the low levels of Nrp1 expression on microglial cells, and the incidence of demyelinating lesions.

One approach to reduce the numbers of M1 microglial cells would be to combine tuftsin with an additional treatment that would further polarize microglial cells to an M2 phenotype. As we showed in Chapter IV, administration of a c-Met inhibitor to primed microglia in concert with tuftsin resulted in a stronger M2 polarization in target cells than tuftsin alone. This could potentially be by dampening signaling via Nrp1's co-receptor c-

Met, which would promote more effective interactions between Nrp1 and TβR1 and thus improve the efficacy of tuftsin signaling. As this c-Met inhibitor has already been effectively used in animal models (Zou et al. 2012), this approach is entirely plausible.

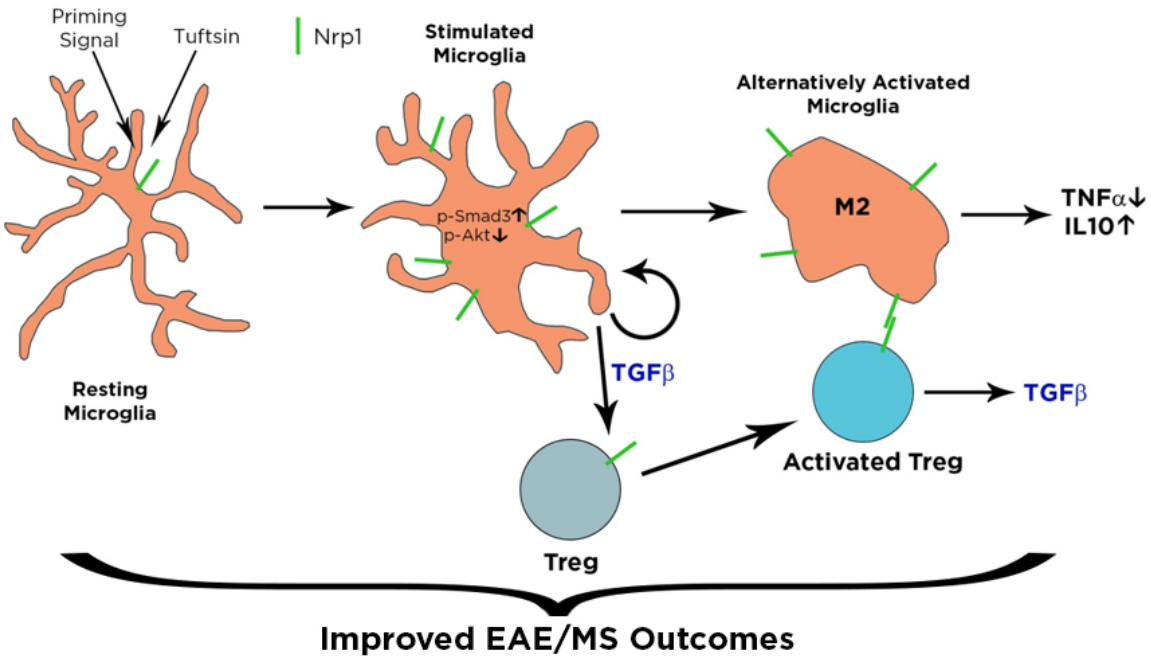
Another avenue by which tuftsin treatment could be improved is through further upregulation of its receptor Nrp1 on microglial cells, which would allow for more efficient binding and activity. Basic fibroblast growth factor, or b-FGF, has been shown to promote the increase of Nrp1 levels on vascular smooth muscle cells (Liu et al. 2005). In a model of chronic tendon tear in rats, b-FGF was infused via osmotic pump for 4 weeks, where it not only improved healing but also resulted in no untoward effects. As Nrp1 upregulation by tuftsin is already a characteristic aspect of its function, further promoting this effect could result in a stronger, more rapid anti-inflammatory response.

Most importantly, significant demyelination is a hallmark of MS/EAE and is responsible for the majority of clinical symptoms seen in patients. While tuftsin promotes the polarization of protective, M2 microglia, its effect on myelinating oligodendrocyte populations and their precursors has not been observed. Loss of the myelin sheath on neurons is a pathology associated with a range of diseases, and thus many varied strategies have been developed to combat this problem. Many psychiatric disorders such as schizophrenia are also associated with myelin abnormalities. Several antipsychotic drugs prescribed for this disease, including haloperidol, quietapine, and olanzapine, have been associated with the promotion of oligodendrocyte progenitor cell (OPC) differentiation, by various mechanisms (Ren et al. 2013). As increased presence of OPCs is an important component of recovery and remyelination, mechanisms by which these cell numbers are increased are extremely beneficial (Zhang et al. 2011a).

In addition, factors secreted from mesenchymal stem cells from the bone marrow are capable of promoting oligodendroglial maturation. Mature oligodendrocytes then function as the primary myelinating cells in the CNS (Jadasz et al. 2013). The drug glatiramer acetate, which is already approved for the treatment of MS in humans, has also been shown to promote oligodendrogenesis and myelin repair (Skihar et al. 2009). Within our own lab, we have shown that microglial inhibitory factor (MIF/TKP) increases OPC differentiation following spinal cord injury (Emmetsberger and Tsirka 2012). Taken together, determining the effect of tuftsin on OPC survival and differentiation is an essential direction of investigation. Combination of tuftsin with any of the above mentioned oligodendrocyte-promoting treatments could potentially mitigate early damage, and allow for rapid, effective remyelination of any lesions that do occur.

FIGURES

Figure VII-1: A model for tuftsin and Nrp1 function during EAE.


Priming Signal
Tuftsin
Nrp1
Stimulated Microglia
Alternatively Activated Microglia
p-Smad3↑
p-Akt↓
M2
TNFα↓
IL10↑
TGFβ
TGFβ
Resting Microglia
Activated Treg
Treg
Improved EAE/MS Outcomes

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