MULTIPLE TOLL-LIKE RECEPTOR AGONISTS ACT AS POTENT ADJUVANTS IN THE INDUCTION OF AUTOIMMUNITY

APPROVED BY SUPERVISORY COMMITTEE

Michael K. Racke, M.D.

Nancy E. Street, Ph.D.

James A. Thomas, M.D.

DEDICATION

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MULTIPLE TOLL-LIKE RECEPTOR AGONISTS ACT AS POTENT ADJUVANTS IN THE INDUCTION OF AUTOIMMUNITY

by

BARANDA SANTERI HANSEN

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BARANDA SANTERI HANSEN, M.S.

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Supervising Professor: MICHAEL K. RACKE, M.D.

Multiple sclerosis (MS) is an inflammatory disease of the CNS. Infections can trigger or exacerbate the course of MS, and many agents, both bacterial and viral, have been implicated. These agents are recognized by the innate immune system via pathogen-associated molecular patterns (PAMPs) activating Toll-like receptors (TLRs) on host cells. I investigated the role that PAMPs play in

Experimental Autoimmune Encephalomyelitis (EAE), an animal model of MS. Both genetic and environmental influences contribute to the pathogenesis of MS. I used various PAMPs as the environmental input to imitate infection and activate TLRs in an effort to induce EAE in a resistant mouse strain. To further elucidate the impact of infection on EAE, I examined the role of various TLR agonists in the development of autoimmunity using direct immunization and adoptive transfer of encephalitogenic T cells. Mice developed EAE following immunization with myelin antigen emulsified in IFA with various PAMPs, indicating that various PAMPs can participate as the adjuvant necessary to induce EAE. I also examined the effect of PAMPs on APC activation and found that DCs pulsed with antigen and activated with the TLR agonist LPS enhanced the stimulation of antigen-specific immune cells. Conversely, tolerizing mice to a particular PAMP inhibited the ability of those mice to develop EAE following direct immunization with that PAMP and Ag. To determine the role of the Toll/IL-1 pathway in the target organ, Myelin Basic Protein (MBP) Ac1-11-specific T cells were adoptively transferred into both wild type and IRAK1-deficient mice and disease was monitored in both groups, with the results suggesting that signaling through TLR is not required in the target organ to develop disease. These results suggest that PAMPs play an important role in priming of autoreactive T cells in EAE and potentially MS.

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LIST OF ABBREVIATIONS

CFA	Complete Freund's Adjuvant
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme-linked Immunosorbent Assay
i.p.	intraperitoneal
IRAK	IL-1Receptor-Associated Kinase
IRF3	Interferon Regulatory Factor 3
LNC	lymph node cells
MBP	Myelin Basic Protein
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
PAMP	Pathogen-Associated Molecular Pattern
PRR	Pattern Recognition Receptor
PTX	Pertussis Toxin
Tg	transgenic
TLR	Toll-like Receptor
TRAF6	Tumor Necrosis Factor Receptor-Associated Factor 6
s.c.	subcutaneous
wt	wild-type

CHAPTER ONE
Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS. Many believe MS is an autoimmune disease arising from the breakdown of immune tolerance in T cells specific for myelin antigens (1, 2). MS is characterized by the destruction of myelin in the brain and spinal cord, which is associated with the formation of sclerotic plaques. This sclerosing process leads to increasing disability due to irreparable axonal damage (3, 4). MS is one of most common causes of disability, second only to trauma, in individuals between the ages of 15 and 45, and it affects at least 350,000 people in the United States alone (5). MS usually begins between the ages of 20 and 40, and like many other autoimmune diseases, such as Systemic Lupus Erythematosis and Rheumatoid Arthritis, MS affects women more often than men, with a preponderance of 2:1 (6, 7). Typically the CNS is an immune privileged site, due to the presence of the blood-brain barrier, but in MS, this barrier is breached, allowing the infiltration of inflammatory lymphocytes, which are often seen in MS plaques. CD4+ T cells expressing activation markers such as IL-2 receptors and HLA Class II molecules are a common component of the inflammatory infiltrates found in active MS lesions, and many regard MS to be a CD4+ Th1-mediated autoimmune disease, although debate on this topic is ongoing (2).

There are several clinical forms of MS, each exhibiting a different course of disease. The most common form of MS is relapsing-remitting MS (RRMS), affecting 85-90% of MS patients initially. RRMS is characterized by periods of relatively low disease activity punctuated by exacerbations of disease symptoms. Many patients with RRMS eventually progress to secondary-progressive MS (SPMS). SPMS results from both demyelination and axonal damage, eventually leading to the accumulation of permanent disability. Another form of MS affecting approximately 10% of MS patients is primary-progressive MS (PPMS), in which patients present with slow but steadily progressing disease symptoms from the onset. The most rare clinical form of MS is primary-relapsing MS (PRMS). This form is similar to RRMS in that these patients suffer acute exacerbations of disease symptoms. However, in between relapses there is no remission of symptoms, but rather there is a continual progression of disease, similar to PPMS. This type of MS affects less than 5% of patients.

While the etiology of MS remains unknown, it is widely believed that there is some element of genetic predisposition to MS. MS is usually found in Caucasians, while there are some ethnicities among which MS is almost never found, including black Africans and Asians (8-11). Specific genes have also been found to confer risk, and these include the HLA-DR and HLA-DQ genes. In Caucasians, the HLA-DR15 haplotype appears to be indicative of higher disease risk (12, 13). Familial studies of MS have shown that there is an approximately

2-5% higher risk of developing disease among first-degree relatives of individuals with MS (14). Studies in twins and non-twin siblings have shown a higher concordance rate of disease among monozygotic (MZ) twins, compared with dizygotic twins and non-twin siblings, while siblings in general display substantially increased occurrence of disease compared with non-siblings. This increased prevalence of MS among family members suggests a definite genetic component to the pathogenesis of MS. However, the concordance rate of MZ twins, while higher than that of other siblings, is unexceptional at 25%.

There is also evidence for an environmental component in the pathogenesis of MS. For example, there appears to be an increase in the incidence of MS among higher geographic latitudes (15). This has given rise to the concept that both genetic and environmental factors play a role in the pathogenesis of MS (7), although exact mechanisms of disease pathogenesis remain unknown. While there are several theories regarding the initiation of autoimmunity, two major theories for the initiation of MS are molecular mimicry and bystander activation (16, 17). In molecular mimicry, the host mounts an immune response against a self-antigen that resembles an epitope from a foreign pathogen (18). In bystander activation, an immune response directed against a foreign pathogen activates immune cells that do not cross-react with the original pathogen but do react against self-antigens. For example, infections could

stimulate APCs that activate autoreactive T cells that do not cross-react with the infecting agent to induce autoimmunity.

Clinical and pathologic evidence strongly suggest a link between exposure to an infectious agent and an autoimmune response directed against myelin (19, 20). Epidemiologic studies have suggested that infection can trigger or exacerbate the course of MS, as have case studies that suggest relapses often occur in the setting of infections. Many different agents, both bacterial and viral, such as E. coli, HHV6, and EBV, have been implicated in triggering MS exacerbations (21-29). These bacterial and viral agents express on their surfaces pathogen-association molecular patterns (PAMPs) not found in host cells. Some PAMPs are recognized by the innate immune system via conserved receptors called pattern recognition receptors (PRRs). There are several different PRRs expressed on immune cells, including mannan-binding lectin, the binding of which initiates the mannan-binding pathway of complement activation. Other PRRs include macrophage mannose receptors and scavenger receptors, which can trigger phagocytosis. Another family of membrane-bound PRRs are the TLRs, which are highly conserved signaling receptors and which are important for microbial pathogen recognition and initiation of inflammatory immune responses (30-32). The *toll* gene was first discovered in Drosophila melanogaster, in which it was found to be required for development of the dorsal/ventral axis during embryogenesis (33). Later studies identified an essential role for *toll* in adult

Drosophila in the host defense against fungal infections (34, 35). Mammalian homologs were discovered later and given the name Toll-like receptors. There are 11 known TLRs to date, with similar expression of TLR1-9 in both humans and mice, whereas TLR10 is non-functional in mice, and TLR11 is not expressed in humans (36, 37). The TLR ectodomain is composed of leucine-rich repeats, and it is the organization and distribution of these leucine-rich repeats that distinguishes one TLR from another and confers recognition specificity of each. There is a region of approximately 200 amino acids in the cytoplasmic tail of TLRs that bears similarity to the cytoplasmic tail of the IL-1 receptor, and this region is known as the Toll/IL-1 receptor (TIR) domain (36, 38). This domain is critical for the initiation of TLR signaling (38).

TLRs play an essential role in the initiation of the innate immune response against invading pathogens. PAMPs, such as LPS, bacterial DNA, and dsRNA, are identified as foreign by the host cell following recognition by TLRs, which results in the activation of the Toll/IL-1 pathway, a signaling cascade which can signal through one of at least two pathways (Fig 1). The MyD88-dependent pathway, which is utilized by all TLRs except TLR3, signals via IL-1R-associated kinases (IRAKs). IRAK4 is recruited to the TLR complex following PAMP recognition and mediates phosphorylation of IRAK1. Following phosphorylation, IRAK1 then leaves the TLR complex to associate with TNF receptor-associated factor 6 (TRAF6) and additional kinases. This early signaling leads to the

activation of transcription factors such as early phase NF-κB and AP-1, and culminates in the secretion of inflammatory cytokines, such as TNFα, IL-1, and IL-6 (39-41). The MyD88-independent pathway is utilized by TLR3 and TLR4. This pathway signals through the adaptor molecules TIR-domain containing adaptor protein/ MyD88-adaptor like protein (TIRAP/Mal) and TIR-domain containing adaptor protein inducing IFNβ (TRIF, also known as TIR-domain containing molecule 1 or TICAM-1), to induce the transcription of late phase NFκB, as well as the phosphorylation and translocation of interferon regulatory factor 3 (IRF3), leading to the production of IFNβ and costimulatory molecules.

TLRs are found on many cells, including endothelium, monocytes, and APCs, such as dendritic cells, macrophages, and B cells (42). They are expressed in different locations within the cell. For example, TLR1, 2, and 4 are expressed on the surface of the cell, unlike TLR3, 7, 8, and 9, which can be expressed in intracellular compartments, such as endosomes (36, 43-45).

DCs are unique APCs in that they can initiate primary immune responses (46). Immature DCs reside in tissues and are highly endocytic. When a stimulus is recognized by TLRs on the surface of DCs, it is endocytosed, triggering migration of DCs to local lymph nodes. TLR agonist recognition also triggers maturation, which involves the upregulation of costimulatory molecules and MHC Class II expression, as well as increased antigen presentation (38, 42, 47, 48). Once in the lymph node, the mature DCs present antigen to local T and B

cells. Upon antigen-specific binding to a T cell, DCs release inflammatory cytokines such as IL-12 (48). These cytokines direct T cell differentiation to a Th1 phenotype, which may contribute to the development of autoimmune diseases such as Experimental Autoimmune Encephalomyelitis (EAE) (49).

A popular animal model used to study MS is experimental autoimmune encephalomyelitis (EAE). Murine EAE is used often to study MS because EAE shares many similarities with MS. For example, both disorders exhibit similar clinical courses and CNS pathology. Like the most common form of MS, murine EAE can exhibit a relapsing/remitting course of disease in certain strains. The pathology of EAE is also similar to that of MS patients in that there is inflammation and demyelination in the white matter (2, 50). Other similarities include increased disease incidence among females, and a role for MHC. The role of MHC and genetic background becomes evident when attempting to induce EAE in various mouse strains. For example, the SJL strain is highly susceptible to EAE, while the B10.S strain is naturally resistant.

EAE is known to be a CD4+ T cell-mediated disease in several murine models, although CD8+ T cells can also be encephalitogenic under certain circumstances (51). EAE can be transferred using activated Ag-specific Th1 cells in a dose dependent manner, although it cannot be transferred via antibodies, and few studies have shown that EAE can be transferred using CD8+ T cells (51, 52). One way EAE is induced is through active immunization, in which mice are

immunized subcutaneously (s.c.) with myelin antigens in the presence of an adjuvant. Common proteins and epitopes used include Myelin Oligodendrocyte Glycoprotein (MOG) 35-55, Proteolipid Protein (PLP) 139-151, Myelin Basic Protein (MBP) Ac1-11, and whole MBP. The adjuvant most often used is Complete Freund's Adjuvant (CFA), which contains *Mycobacterium tuberculosis*. Mycobacterial substances have been found to activate the Toll/IL-1 pathway via activation of TLR1, 2 and 4, and it has been shown that the Toll/IL-1 pathway is critical for the expansion of autoreactive T cells and the subsequent development of EAE (53-58). Although CFA is the adjuvant most commonly used to induce EAE, other TLR agonists, such as CpG oligodeoxynucleotide, LPS, and peptidoglycan have been used to induce EAE as well as other autoimmune diseases such as autoimmune myocarditis and autoimmune arthritis (55, 59-61). Since an adjuvant is needed to actively induce EAE, and the commonly used adjuvant CFA contains the TLR agonist mycobacteria, I wanted to investigate the ability of other TLR agonists to participate in the activation of encephalitogenic T cells that results in the initiation of autoimmune processes such as EAE.

CHAPTER TWO
Methodology

Mice

Vα2.3 and Vβ8.2 TCR-transgenic (Tg) mice were kindly provided by Dr. J. Goverman (University of Washington, Seattle, WA) and were crossed to produce Vα2.3/Vβ8.2 TCR Tg mice, which were bred in the animal facility at UT Southwestern. C57BL/6, B10.PL, and B10.S wt mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in the animal facility. IRAK1-deficient mice were originally generated as described (62), and then backcrossed onto a B10.PL background. All mice were housed in a barrier animal facility at University of Texas Southwestern Medical Center (Dallas, TX) in accordance with the Institutional Animal Care and Use Committee. All mice were 7–10 weeks of age when experiments were performed.

Reagents

LPS from *Escherichia coli* serotype 0111:B4, Poly (I:C), and Zymosan were purchased from Sigma-Aldrich (St. Louis, MO). *Mycobacterium tuberculosis* was purchased from Difco (Detroit, MI). All TLR agonists were tested for LPS contamination using a Limulus Amoebocyte Lysate (LAL) assay (Cambrex Bio Science; Walkersville, MD). MOG 35-55, PLP 139-151, and MBP Ac1-11

peptides were purchased from Biosource International (Camarillo, CA). Whole MBP was prepared by homogenizing whole guinea pig spinal cords. Pertussis toxin, (PTX), isolated from *Bordetella pertussis*, was purchased from List Biological Laboratories, Inc. (Campbell, CA).

EAE induction and evaluation of disease

B6 studies

For active induction of EAE, naïve male and female C57BL/6 wt mice were immunized s.c. in each flank with MOG 35-55 (200 μg/mouse) in an emulsion with CFA (Difco; Detroit, MI) or IFA supplemented with one of various PAMPs: LPS, Mycobacteria, Poly (I:C), or Zymosan (concentrations noted in text or figure legends). PTX was dissolved in PBS and injected intraperitoneally (i.p.) at 200 ng/mouse at the time of immunization and 48 hours later. EAE scoring was performed as previously described (63): 0, no abnormality; 1, a limp tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5, quadriplegia or premoribund state; and 6, death.

For short-term studies, female C57BL/6 wt mice were immunized s.c. with MOG 35-55 (200 μg/mouse) in an emulsion with CFA or IFA plus PAMPs (30 μg/mouse). On Day 10, the draining lymph nodes and/or spleens were harvested for various assays. For PTX experiments, splenocytes were harvested from naïve female C57BL/6 mice, processed, and resuspended at 2×10^6 cells/mL. Cells were

plated at 2 mL/well in 24-well plates with 200 ng/mL PTX or media alone, and supernatants were collected at 24-hour intervals for cytokine assays.

IRAK1$^{-/-}$ studies

For adoptive transfer of EAE, naïve splenocytes were harvested from Vα2.3/Vβ8.2 TCR transgenic (Tg) mice and resuspended at 4x10^6 cells/mL. Cells were cultured at 2 mL/well in 24-well plates with 2 μg/mL MBP Ac1-11. 48 hours later, the cells were collected, washed, and resuspended in PBS at a concentration of 30x10^6 cells/200 μL. 30x10^6 cells were injected i.p. into B10.PL wt or B10.PL IRAK1$^{-/-}$ mice. Mice were monitored daily for disease.

Transgenic studies

For tolerance studies, female Vβ8.2 TCR Tg mice on a C57BL/6 background were tolerized by an i.p. injection of 40 μg LPS emulsified in IFA. 24 hours later, mice were given an i.v. injection of 100 μg LPS dissolved in PBS. 10 days later, mice were either euthanized and splenocytes harvested for proliferation and cytokine assays, or mice were immunized with MOG 35-55 in IFA with an adjuvant (specific adjuvant noted in text or legends), and monitored daily for disease.

B10.S studies

In an experiment using PAMPs to break tolerance in an EAE resistant strain, male B10.S wt mice were immunized i.p. with 200 μg PLP 139-151. 10 days later, the

mice were immunized with 200 μg PLP 139-151 i.p. 10 days after immunization, the mice were immunized s.c. with PLP 139-151 in CFA. 14 days after the final immunization, spleens and LNC were harvested and cultured with Ag, LPS, and Ag + LPS. Supernatants were collected at 24, 48, and 72 hours for cytokine assays. Following a 96-hour culture, a fraction of the cells were used for proliferation assays. The remaining cells were collected, washed, and resuspended at $6x10^6$ cells/200 μL, and naïve male B10.S mice were injected i.p. with $6x10^6$ cells/mouse.

Cell Culture

Draining lymph node cells (LNCs) and/or spleens from different mice were harvested, and single-cell suspensions were obtained by pressing the tissue through a wire mesh screen. The cells were cultured at $4x10^6$ cells/ml in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, HEPES buffer, $5x10^{-5}$ M 2-Mercaptoethanol, and nonessential amino acids for the length of time specified in the text or figure legends. MOG 35-55, PLP 139-151, MPB Ac1-11, whole MBP, and LPS were used as stimulating Ags.

Proliferation Assay

$1x10^4$ mouse splenocytes or LNCs were placed in each well of a 96-well plate. Ag was added (concentrations noted in text or legends) in quadruplicate. The

cells were cultured for 96 hours, and the cells were pulsed with 0.5 μCi/well [*methyl*-^{3}H]thymidine for the final 16 hours of culture. Cells were harvested onto glass fiber filters using a Tomtec harvester (Tomtec, Hamden, CT), and incorporated [*methyl*-^{3}H]thymidine was measured with a Betaplate counter (PerkinElmer Wallac).

Bone marrow-derived DC culture

Bone marrow-derived dendritic cells (BMDCs) were cultured as described in (64), with modifications. Briefly, femurs and tibias were harvested from B10.PL wt mice, and bone marrow was flushed using HBSS. Cells were washed, counted, and resuspended at $1x10^{6}$ cells/mL in RPMI 1640 supplemented with 10% FBS and 1% Pen-Strep. Cells were plated in 6-well plates at 3 mL/well, and rmGM-CSF and rmIL-4 (Peprotech, Frankfurt, Germany) were added at 10 ng/mL each. On Day 2, the media was aspirated off and fresh media and cytokines were added as on Day 0. On Day 4, loosely adherent cells were collected, counted, and replated at $1x10^{6}$ cells/mL in 3 mL/well of fresh media with 10 ng/mL of cytokines. On Day 6, loosely adherent cells were collected and counted, and MBP Ac1-11 was added at 100 μg/mL for 24 hours. During the last 6 hours of incubation, LPS was added at 1 μg/mL to activate the DCs.

DC proliferation assay

For proliferation assay, DCs were collected, washed, and resuspended at $1x10^6$ cells/mL and plated at various concentrations in quadruplicate in a 96-well plate. $1x10^5$ Vα2.3/Vβ8.2 TCR Tg T cells were added to each well. For cytokine assay, DCs were plated at various concentrations in a 24-well plate with $8x10^6$ Vα2.3/Vβ8.2 TCR Tg T cells (Concentrations noted in text or figure legends).

Cytokine detection

IFNγ and IL-12 were measured by ELISA. ELISA plates (Immunol 2; Dynatech Laboratories, Chantilly, VA) were coated with 2 μg/ml (50 μL/well) IFNγ or IL-12 mAb (BD PharMingen, San Diego, CA) in 0.1 M $NaHCO_3$ buffer (pH 8.2) overnight at 4°C. The plates were blocked with 200 μL of 1% BSA in PBS per well for 2 hours. The plates were then washed twice with PBS/Tween 20, and a total of 100 μl of supernatant was added in duplicate. The plates were incubated overnight at 4°C and washed four times with PBS/Tween 20. 100 μL of biotinylated secondary antibody diluted to 1μg/mL in 1% BSA/PBS was added, and plates were incubated at room temperature. The plates were washed six times with PBS/Tween 20, and 100 μL of avidin-peroxidase (2.5 μg/mL) was added and incubated for 30 minutes. The plates were washed eight times with PBS/Tween 20, and 100 μL ABTS substrate containing 0.03% H_2O_2 was added to each well.

The substrate color was allowed to develop, and the absorbance was measured at OD_{405}. A standard curve was generated from the protein standards, and the concentration of cytokine in the samples was calculated.

CHAPTER THREE
Results

LPS increases proliferation and cytokine secretion *in vitro*

While the etiology of MS is unknown, it is believed that both genetic and environmental factors play a role. As such, some individuals could be more susceptible to developing MS than others, due to their genetic background. Likewise, in EAE there are mouse strains that are more susceptible to developing disease, due to their genetic background. However, genes alone do not determine who develops MS; there is also an environmental role. It has been suggested that exposure to infectious agents may play a role in the pathogenesis of autoimmune disease in humans as well as in animal models. Infectious pathogens may play a role by activating APCs, which can then stimulate autoreactive T cells.

B10.S is a mouse strain that is naturally resistant to EAE. I began these studies by attempting to determine if exposure to PAMPs could overcome this resistance. Naïve male wt B10.S mice were immunized with whole MBP in CFA to prime MBP-specific cells. 10 days later, LNC were harvested and cultured with Ag with various doses of the PAMP LPS. Proliferation and cytokine secretion were measured, and it was found that culturing primed cells in the presence of LPS greatly increased proliferation compared to cells that were cultured with Ag alone. Proliferation with LPS was more than five times that of cells cultured without LPS, even at a dose of only 1 μg/mL (Fig. 2A). IFNγ was

also increased in the presence of LPS, with up to five times more IFNγ secreted by cells cultured in the presence of LPS at a concentration of 25 μg/mL (Fig. 2B). I also collected cells that had been cultured with 25 μg/mL of MBP and 25 μg/mL of LPS, as well as cells that had been cultured with 25 μg/mL of MBP only. I then adoptively transferred these cells into naïve B10.S mice. None of the mice developed EAE, suggesting that there are mechanisms at play in the B10.S strain to make it quite resistant to EAE.

As previously stated, B10.S mice are naturally resistant to induction of EAE. In another experiment, I sought to overcome this resistance in B10.S mice using a previously published protocol with modifications (59). In this experiment, naïve male wt B10.S mice were immunized i.p. with PLP 139-151 in an emulsion with IFA. 10 days later, these mice were re-immunized s.c. with PLP 139-151 emulsified in CFA. 14 days later, LNCs were harvested from these mice and cultured with Ag, LPS, or Ag with LPS. When cells were cultured with either LPS or LPS plus a consistent dose of 2 μg/mL PLP139-151, I could not detect antigen-specific proliferation over the proliferation in response to LPS (Fig. 3A). However, when cells were cultured with either PLP 139-151 or PLP 139-151 with 10 μg/mL of LPS, even at a dose of 0.01 μg/mL of Ag, there was increased proliferation in the presence of LPS, while there was no proliferation without LPS, even at a dose of 100 μg/mL of Ag (Fig. 3B, $p>0.0001$). I also measured

IL-12 secretion by cells that were cultured with Ag, LPS, or both. When cells were cultured with Ag alone, there was a significantly decreased cytokine response. Cells cultured with or without Ag in the presence of LPS secreted more than twice as much IL-12 as cells cultured without LPS (Fig. 3C). This highlights the importance of LPS in the stimulation of immune cells.

To determine the ability of these activated cells to transfer disease to an EAE resistant strain, after 96 hours in culture, 6×10^6 cells/mouse were injected i.p. into naïve male wt B10.S mice. The cells that were cultured with PLP 139-151 alone were unable to induce disease at all, while cells that were cultured with LPS or Ag with LPS transferred moderate EAE in at least 80% of the mice (Fig. 3D). This indicates that the presence of a PAMP increases the Ag-specific response of autoreactive immune cells and enhances their encephalitogenic potential.

PAMPs act as adjuvants to induce T cell proliferation.

Infection may play a role in the pathogenesis of autoimmune disease by activating APCs, which then may activate autoreactive T cells. To examine the effect of PAMPs on the *in vivo* activation of APCs, and the subsequent ability of APCs to stimulate proliferation, C57BL/6 wt mice were immunized s.c with MOG 35-55 in IFA alone or in addition to 30 μg of one of four TLR agonists. LNC were harvested 10 days after immunization, and single-cell suspensions

were made. These LNCs were then cultured in the presence of MOG 35-55. When Mycobacteria (activates multiple TLR, including 1, 2, and 4), LPS (activates TLR4), and Zymosan (activates TLR2), were used as adjuvant, they induced a robust dose-dependent proliferative response, compared to Ag with IFA, whereas Poly I:C, an activator for TLR3, did not induce proliferation (Fig. 4). There was a 2-fold increase in proliferation over the IFA control when Zymosan was used ($p<0.0001$), while LPS induced a 3-fold increase ($p<0.0001$), and Mycobacteria induced 5-fold greater proliferation ($p<0.0001$), at both 10 and 100 μg/mL of antigen. This demonstrates that multiple TLR activators function as adjuvants for the *in vivo* activation of APCs, which can then stimulate a proliferative response to MOG 35-55 by encephalitogenic T cells. However, the MyD88-independent pathway used by the TLR3 agonist was very poor in stimulating a proliferative response to MOG 35-55.

Exposure of MBP Ac1-11-specific cells to PAMP activated WT bone marrow-derived dendritic cells results in an enhanced response.

The previous experiments suggest that multiple PAMPs are able to activate APCs *in vivo*, so I next examined the *in vitro* effects of PAMP activation on APCs and subsequent activation of Ag-specific immune cells. To do this, I examined T cell proliferation and cytokine production of Vα2.3/Vβ8.2 TCR Tg T cells in response to MBP Ac1-11 loaded DCs that were activated with LPS. I used

LPS to activate DCs because LPS is a well known trigger for maturation of DCs (47, 48). Bone marrow-derived dendritic cells (BMDCs) from C57BL/6 wt mice were cultured as previously described in Chapter Two: Methodology. On day 7 of culture, BMDCs were pulsed with MBP Ac1-11 for 24h. For the last 6h of culture, 1 μg/mL of LPS was added to the media, and activation was measured by CD86 and MHC Class II upregulation as determined by flow cytometry (data not shown). The cells were then collected, washed, and co-cultured with MBP Ac1-11-specific Tg T cells for 96h. BMDCs pulsed with antigen and activated with LPS stimulated increased T cell proliferation compared to T cells cultured with DCs that were pulsed with antigen in the absence of activation by LPS (Fig. 5A, $p>0.0001$). However, in the absence of antigen, LPS alone was not sufficient to induce robust proliferation. These experiments suggest that a PAMP, such as LPS, can enhance the ability of BMDCs to present Ag and stimulate proliferation by Ag-specific T cells.

I also measured IL-12 production in these cultures, and when Tg cells were mixed with $1.5x10^4$ BMDCs that had been pulsed with antigen and activated by LPS, there was increased IL-12 production compared to BMDCs that had not been activated ($p<0.0007$). In the absence of antigen, LPS alone was able to induce IL-12 by BMDCs (Fig. 5B), indicating that these DCs were activated. In the absence of LPS, there was very little IL-12 produced (48). There was also very little IL-12 secreted in response to LPS if $3x10^4$ BMDCs were used, and no

IL-12 was detected if only $3x10^3$ BMDCs were used (data not shown). IFNγ was measured as well; only BMDCs that were pulsed with antigen and activated by LPS were capable of inducing IFNγ production by Ag-specific T cells (data not shown).

WT mice develop EAE after active immunization using PAMPs as adjuvants.

As shown above, multiple PAMPs, such as LPS, Mycobacteria, and Zymosan, act as adjuvants to enhance T cell proliferation in wt T cells. Since these PAMPs enhance proliferation of immune cells *in vitro*, I examined the ability of these PAMPs, along with Poly I:C, to act as adjuvants to actively induce EAE in wt C57BL/6 mice. Four mice in each group were immunized s.c. with MOG 35-55 emulsified in IFA supplemented with the four previously mentioned PAMPs as adjuvant. PTX was given on Days 0 & 2, and onset of disease was observed beginning 12 days after immunization. In Figure 6A, 30 μg of adjuvant/mouse was added to the emulsion. Mycobacteria and LPS worked best as adjuvants, with 100% of the mice developing moderate to severe EAE. Poly I:C and Zymosan were able to induce mild disease at this dose. However, when the dose of adjuvant was increased to 100 μg/mouse, Zymosan did induce moderate disease, as did LPS and Mycobacteria (Fig. 6B). Even at this higher dose, Poly I:C only induced mild EAE, consistent with the proliferation data showing Poly I:C is inefficient at stimulating APCs for T cell proliferation. These

experiments demonstrate that the TLR activators that utilize the MyD88-dependent pathway, such as LPS, Mycobacteria, and Zymosan, are capable of functioning as adjuvants for the induction of EAE in C57BL/6 mice. Poly I:C, which utilizes the MyD88-independent pathway, is much less efficient in inducing EAE.

Pertussis toxin signals via Toll-like receptors to stimulate T cells and APCs to secrete inflammatory cytokines.

PTX is often used to imitate environmental conditions in the induction of EAE. It is often necessary to inject animals with PTX following active immunization to break down the blood-brain barrier, although the mechanism by which PTX helps induce disease is not known. A recently published paper (65) described the mechanism by which PTX increases permeability of the blood-brain barrier. The study also demonstrated that PTX signals via TLR4 to recruit cells to the blood-brain barrier. I cultured naïve C57BL/6 splenocytes with 200 ng/mL PTX or in media alone for 72 hours. Supernatants were collected at 24-hour intervals to measure cytokine secretion. As shown in Figure 7A, exposure of naïve cells to the TLR4 agonist PTX significantly increased the secretion of inflammatory cytokine IL-12 by APCs. PTX also greatly increased the production of IFNγ by T cells, with almost no cytokine released by cells that were not exposed to PTX (Fig.7B). This supports a recent report showing that PTX is

an agonist for TLR4 and suggests that PTX itself can be used as a TLR agonist to induce EAE.

Tolerance to a PAMP results in reduced proliferation and IL-12 production upon stimulation with that PAMP.

To further explore the effect of TLR signaling on the activation of APCs, I examined the effect of LPS tolerance on the ability of T cells to proliferate and secrete cytokines. C57BL/6 wt mice were injected i.p. with 40μg LPS diluted in PBS in an emulsion with IFA. Twenty-four hours later, mice were given i.v. injections of 100μg LPS dissolved in PBS. Control mice were given PBS at both time points. One week later, mice were given a booster i.v injection of 250 μg LPS; again, control mice received PBS. 10 days later, splenocytes were collected and single-cell suspensions were made. Cells were cultured with various doses of LPS for proliferation and cytokine assays to determine if tolerance did occur. The cells were indeed tolerized, as shown by the inhibited proliferative response of immune cells as compared to control mice, with control cells exhibiting proliferation more than three times that of tolerized cells (Fig. 8A, $p<0.0001$). The response to ConA and anti-CD3 was comparable between the two groups, demonstrating that T cells were still functional and able to proliferate in response to alternate stimuli (Fig. 8B).

IL-12 is an important inflammatory cytokine released by DCs upon TLR activation. If tolerance did occur, IL-12 secretion should be decreased. As expected, IL-12 production was inhibited by tolerance to LPS, further indicating that immune cells were indeed tolerized to LPS. Cells from control mice produced more than twice as much IL-12 than their tolerized littermates (average of 160 pg/mL secreted by control cells compared to 70 pg/mL secreted by tolerized cells at 72h) (Fig. 8C). These same effects also were seen if the mice did not receive the second booster dose of 250 μg LPS (data not shown). Thus, tolerance to LPS appears to result in reduced activation of APCs as shown by proliferative capacity and cytokine secretion. This further supports the view that TLR signaling is important for activation of APCs that play a role in the proliferation of autoreactive immune cells and secretion of inflammatory cytokines.

EAE is inhibited in mice tolerized to LPS when LPS is used as the adjuvant.

Since immune cells that are tolerized in vitro to LPS have decreased proliferation and IL-12 production after stimulation by LPS, I next examined if tolerance would affect development of EAE when LPS is used as an adjuvant. Since active immunization for EAE requires the addition of an adjuvant, it follows that tolerance to that adjuvant would inhibit the development of EAE. Vβ8.2 TCR Tg mice were utilized for this experiment because they have a higher

precursor frequency of MBPAc1-11-specific T cells and do not require PTX for disease induction. Vβ8.2 TCR Tg mice were tolerized to LPS as described above, while control mice were given PBS for all injections. Ten days later, one LPS tolerized group was immunized s.c. with MBP Ac1-11/IFA/30 μg LPS. A second tolerized group was immunized with MBP Ac1-11/CFA. Lastly, the control group was also immunized with MBP Ac-11/IFA/30 μg LPS. As expected, LPS was not able to act as an adjuvant to induce EAE when mice were LPS tolerized. The first tolerized group (Fig. 9A, group 1) had significantly delayed onset of EAE when LPS was used as an adjuvant, with disease developing on day 45 compared to day 27 for control mice (Fig. 9A, $p<0.0002$). It is possible that LPS tolerance acts by downregulating all immune function in these mice. To examine this, a second tolerized group was immunized using CFA as an adjuvant instead of LPS. The second tolerized group (Fig. 9A, group 2) developed moderate to severe EAE when CFA containing Mycobacteria was used as an adjuvant, indicating that immune function was not globally impaired in these mice, as the mice were still capable of developing EAE when an adjuvant other than LPS was used.

In a similar experiment, mice were again immunized, with the first LPS tolerized group receiving MBP Ac1-11 in IFA with 30 μg LPS as the adjuvant, and the second LPS tolerized group receiving MBP Ac1-11 in IFA with 30 μg Zymosan as the adjuvant. The control group was immunized with MBP Ac1-11

in IFA with 30 μg LPS. Four of the five control mice developed moderate to severe EAE, while both tolerized groups were significantly protected from EAE (Fig. 9B, $p<0.0001$). Onset of disease was delayed by 15 days when Zymosan was used as adjuvant, and onset was delayed by more than 20 days when LPS was used in the emulsion (Onset at day 41 compared with day 16 for the control group), indicating that there is some cross-tolerance between TLR2 and 4, in accordance with previously published reports (66, 67). Thus, LPS tolerance *in vivo* inhibits induction of EAE in a disease-prone mouse strain when LPS is used as the adjuvant and to some degree when Zymosan is used as the adjuvant.

Toll/IL-1 pathway is essential for T cell priming.

To examine the role that the Toll/IL-1 pathway may play in the activation of autoimmune cells, Rehana Hussain, a technician in the Racke lab, immunized B10.PL wt and IRAK1$^{-/-}$ mice with 200 μg MPB Ac1-11 in CFA. The mice were then monitored for disease, and it was found that IRAK1-deficient mice were impaired in their ability to develop EAE, as there was no incidence of disease in the absence of IRAK1 (Fig. 10A). The ability of MBPAc1-11 specific T cells to transfer disease to IRAK1-deficient mice was also examined. Rehana harvested cells from naïve Vα2.3/Vβ8.2 TCR Tg mice and cultured them with 2 μg/mL of MBP Ac1-11 for 48h. These cells were then injected i.p. into naïve B10.PL wt or IRAK1$^{-/-}$ mice. These mice were then monitored for signs of disease. As shown

in Figure 10B, IRAK1$^{-/-}$ mice developed EAE with a course similar to that of wt mice, indicating that while IRAK1 and the Toll/IL-1 pathway are necessary for the priming of autoreactive T cells, signaling does not appear to be necessary in the target organ for the expression of disease. I also performed this experiment using $5x10^6$ cells and $10x10^6$ cells. However, the mice did not develop EAE following these adoptive transfers.

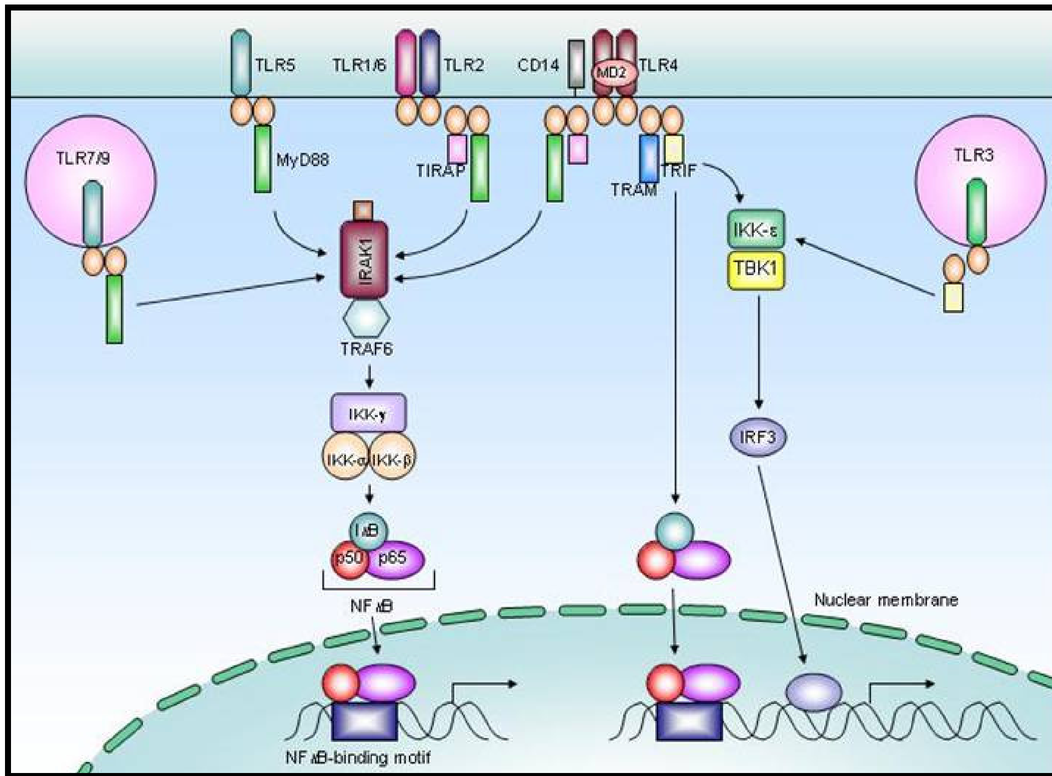

Figure 1. Toll/IL-1 pathway. The Toll/IL-1 pathway is comprised of the MyD88-dependent and MyD88-independent pathways. The MyD88-dependent pathway is utilized by TLRs 1, 2, 4-7, and 9, and results in the transcription of early phase NFκB and the release of inflammatory cytokines. The MyD88-dependent pathway is used by TLR3 and can be used by TLR4. This pathway results in the release of IFNβ and IFN inducible genes. Figure adapted from (38)

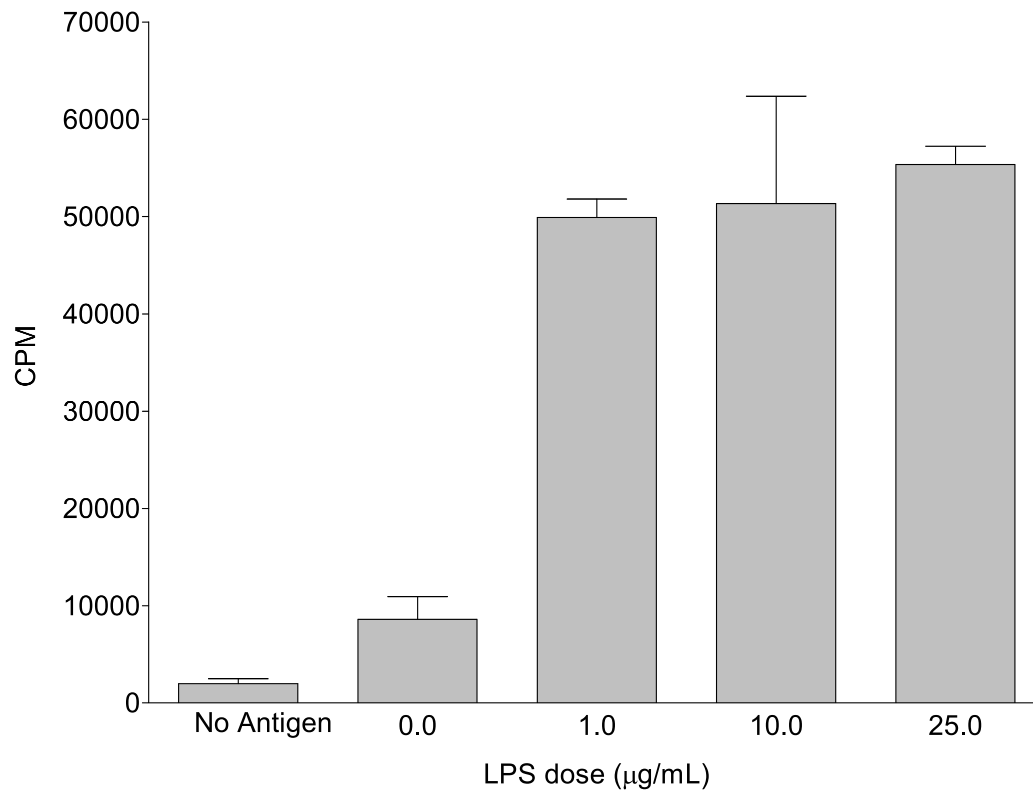

Figure 2A. LPS increases response of MBP-specific immune cells. Male B10.S mice were immunized s.c with 200 µg MBP in CFA on Day 0. On Day 10 LNCs were harvested and cultured with MBP, LPS, or both for 96 hours. [^{3}H]-thymidine was added for the last 16 hours of culture, and proliferation was measured.

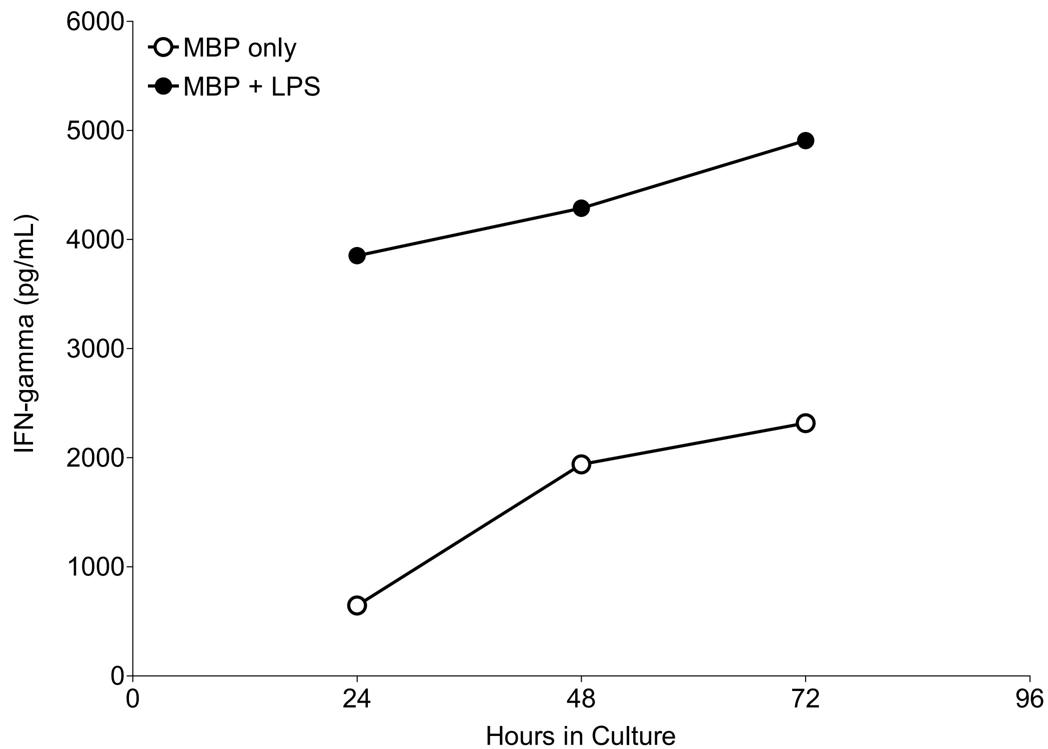

Figure 2B. LPS increases IFNγ secretion. Male B10.S mice were immunized s.c. with 200 μg MBP in CFA on Day 0. On Day 10 LNCs were harvested and cultured with MBP, LPS, or both for 96 hours, and IFNγ secretion was measured by ELISA.

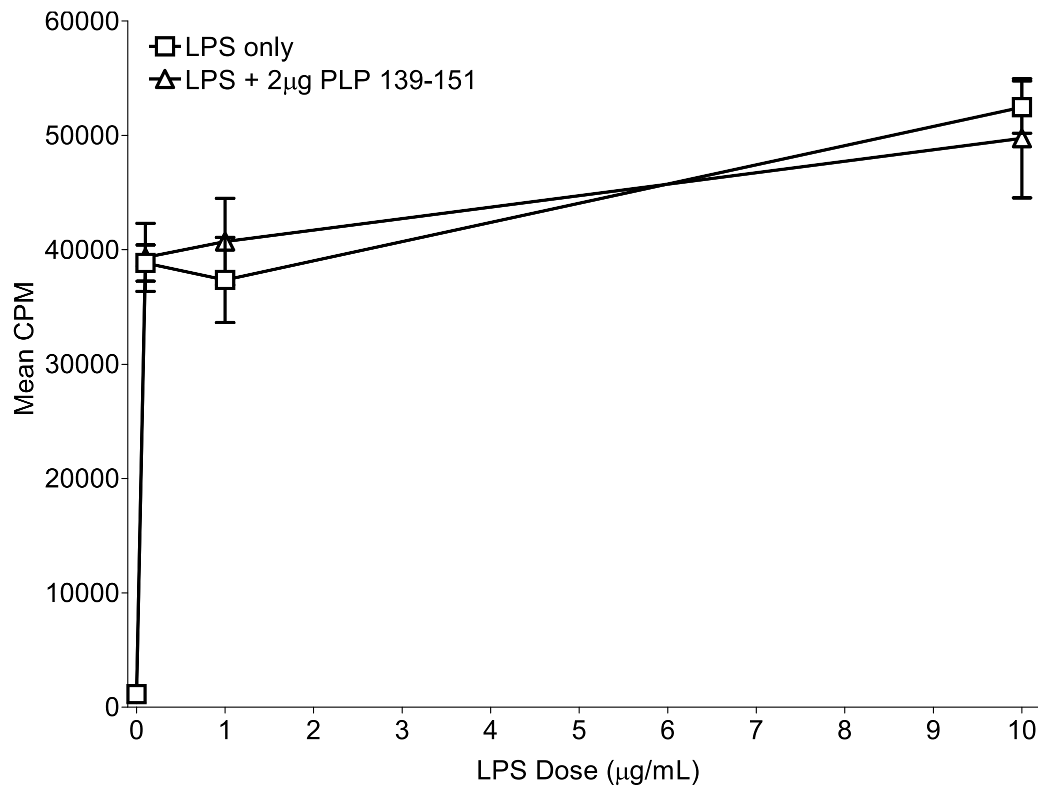

Figure 3A. LPS increases proliferation of PLP 139-151 specific immune cells. Male B10.S mice were immunized i.p. with 200 μg PLP 139-151 on Day 0. On Day 10 mice were immunized s.c. with PLP 139-151/CFA. On Day 24 spleens and LNCs were collected and stimulated with PLP 139-151, LPS, and PLP 130-151 + LPS. Cells were cultured with varying doses of Ag with or without a consistent dose of LPS, and proliferation was measured.

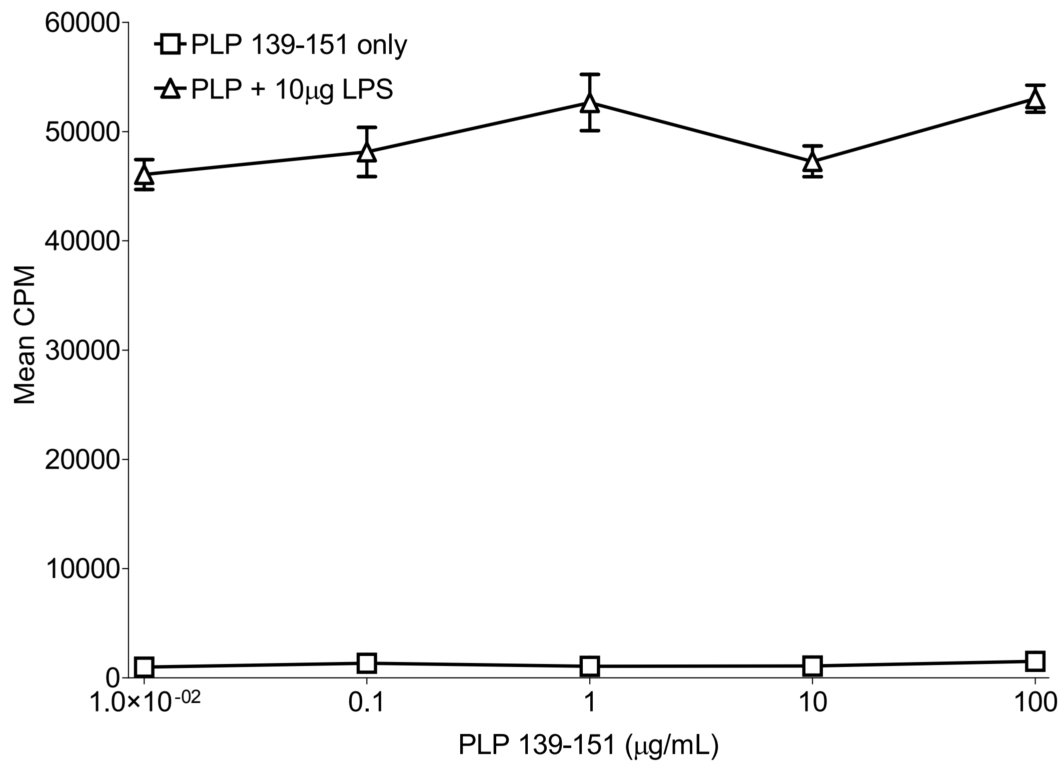

Figure 3B. LPS overcomes resistance *in vitro* and *in vivo*. Male B10.S mice were immunized i.p.with 200 μg PLP 139-151 on Day 0. On Day 10 mice were immunized s.c. with PLP 139-151/CFA On Day 24 spleens and LNCs were collected and stimulated with PLP 139-151, LPS, and PLP 130-151 + LPS. Cells were cultured with varying doses of LPS with or without a consistent dose of Ag, and proliferation was measured.

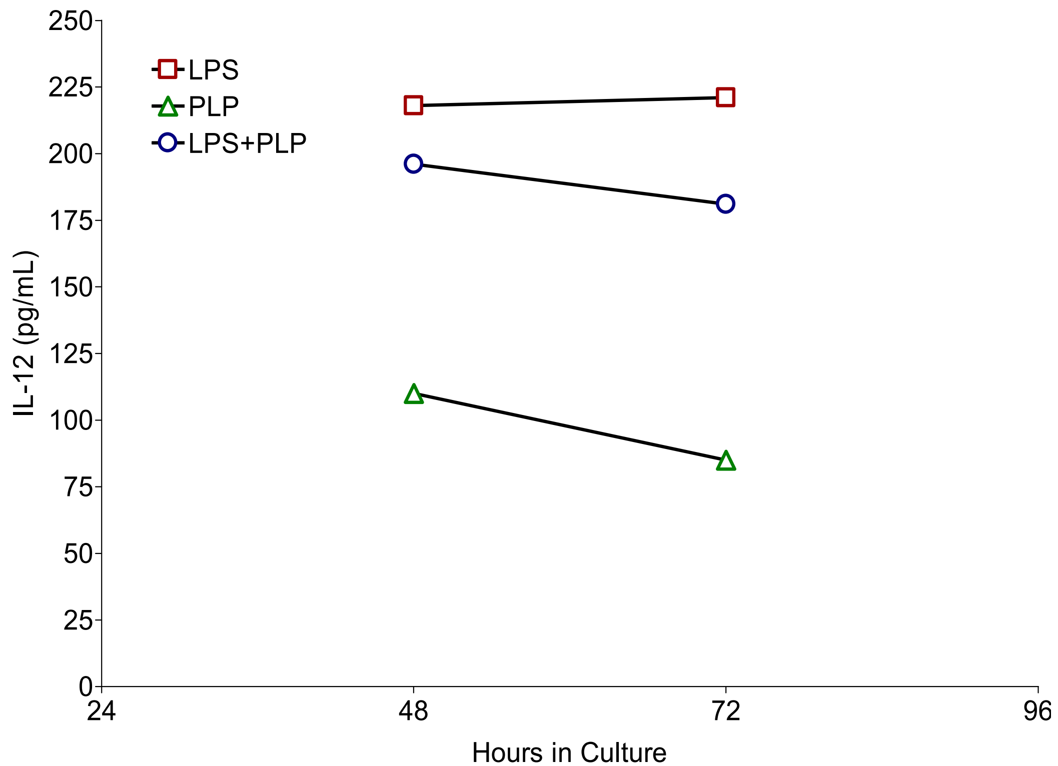

Figure 3C. LPS increases IL-12 secretion. Male B10.S mice were immunized i.p. with 200 µg PLP 139-151 on Day 0. On Day 10 mice were immunized s.c with PLP 139-151/CFA. On Day 24 spleens and LNCs were collected and stimulated with PLP 139-151, LPS, and PLP 130-151 + LPS. IFNγ secretion by cells that were cultured with Ag, LPS, or both was measured using ELISA.

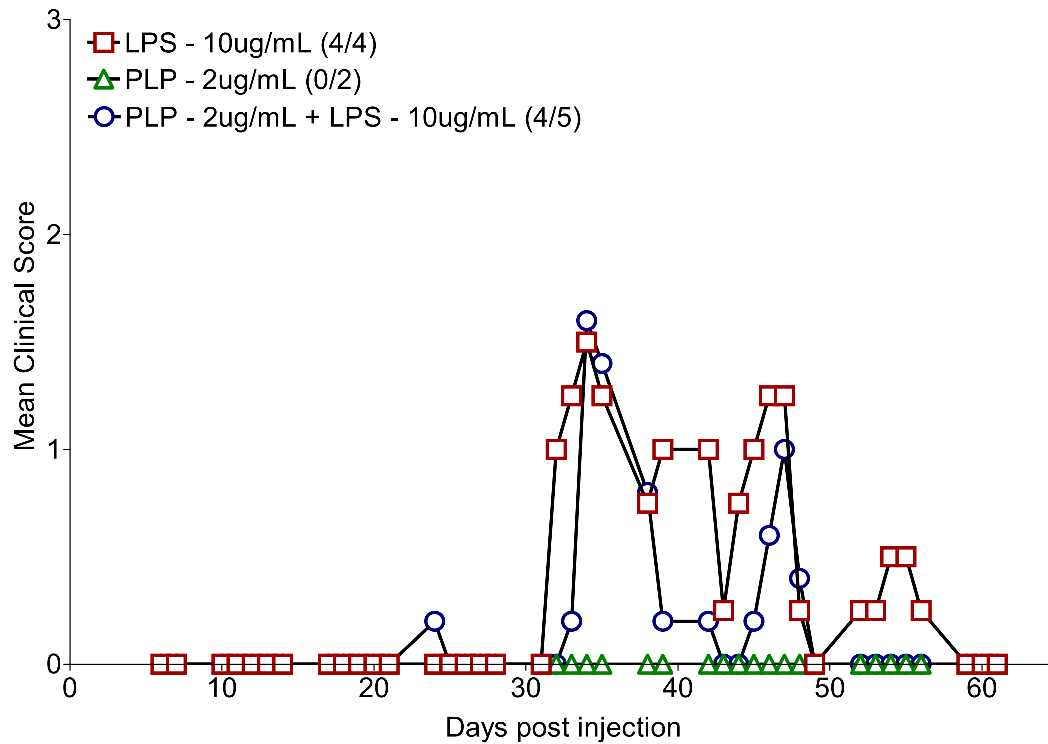

Figure 3D. LPS overcomes resistance *in vitro* and *in vivo*. Male B10.S mice were immunized i.p. with 200 µg PLP 139-151 on Day 0. On Day 10 mice were immunized s.c with PLP 139-151/CFA On Day 24 spleens and LNCs were collected and stimulated with PLP 139-151, LPS, and PLP 130-151 + LPS for 96 hours, and then adoptively transferred into naïve B10.S mice.

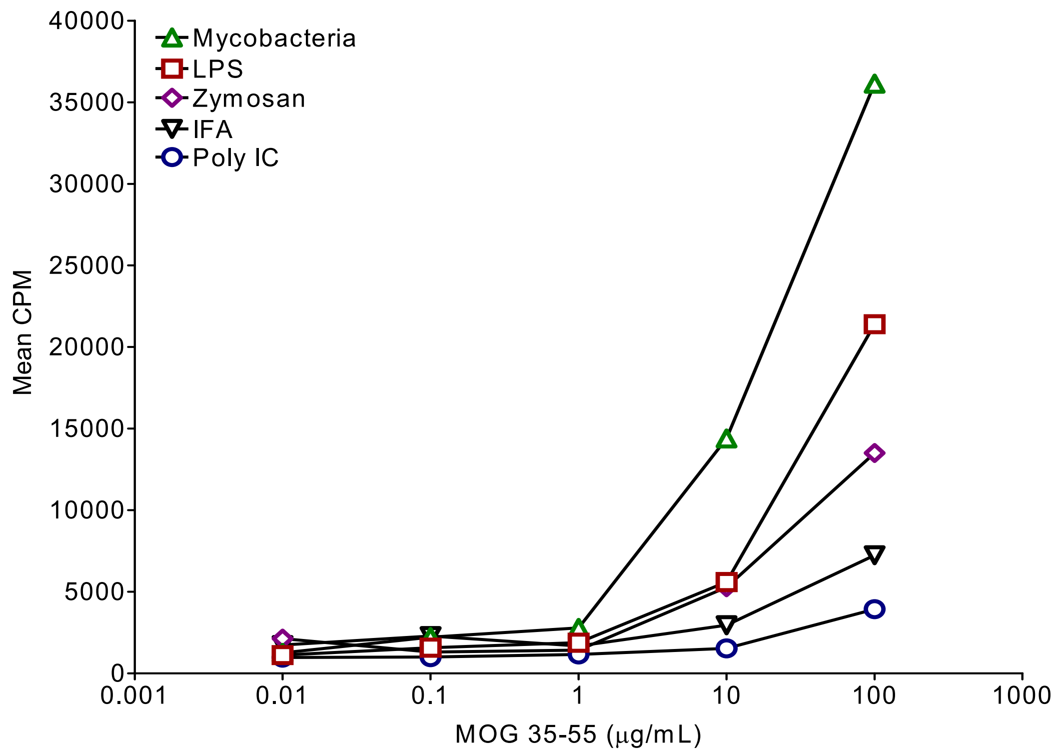

Figure 4. TLR agonists act as adjuvants to induce the proliferation of encephalitogenic T cells. C57BL/6 wt mice (n=4/group) were immunized s.c. with MOG 35-55 in IFA supplemented with 30 μg TLR agonist. On Day 10, LNCs were harvested and cultured with MOG 35-55 in quadruplicate for 96 hours. Cells were pulsed with 0.5 μCi [^{3}H]Thymidine for the final 16 hours of culture. Incorporated radioactivity was measured using a Betaplate scintillation counter.

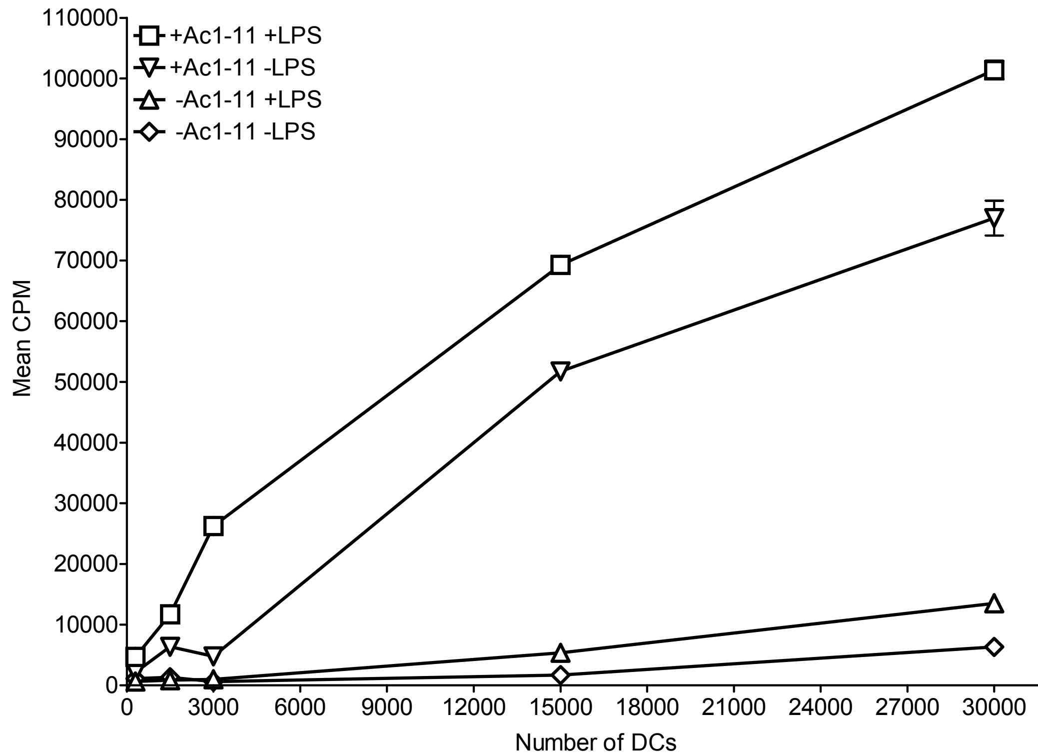

Figure 5A. LPS-activated DCs induce an enhanced proliferative T cell response. Wt DCs were pulsed with MPB Ac1-11 or media for 24 hours, as indicated. LPS was added to the cultures for the last 6 hours of culture where indicated. The cells were then washed and added to Vα2.3/Vβ8.2 TCR Tg cells. DCs were added at various concentrations to $1x10^4$ Tg T cells and cultured for 96 hours. [^{3}H]-thymidine was added for the last 16 hours of culture and proliferation was measured.

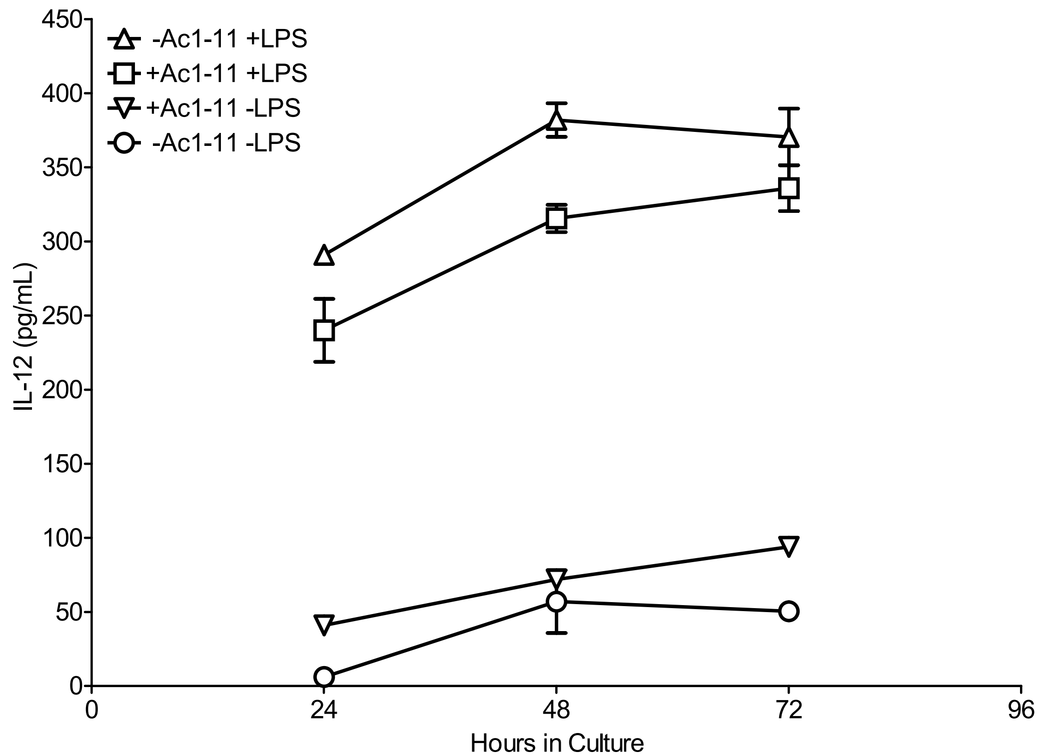

Figure 5B. LPS-activated DCs induce enhanced cytokine secretion. Wt DCs were pulsed with MPB Ac1-11 or media for 24 hours, as indicated. LPS was added to the cultures for the last 6 hours of culture where indicated. The cells were then washed and added to Vα2.3/Vβ8.2 TCR Tg cells. DCs were cultured with $2x10^6$ Tg T cells for 72 hours. Supernatants were collected at 24, 48, and 72 hours, and IL-12 secretion was analyzed by ELISA.

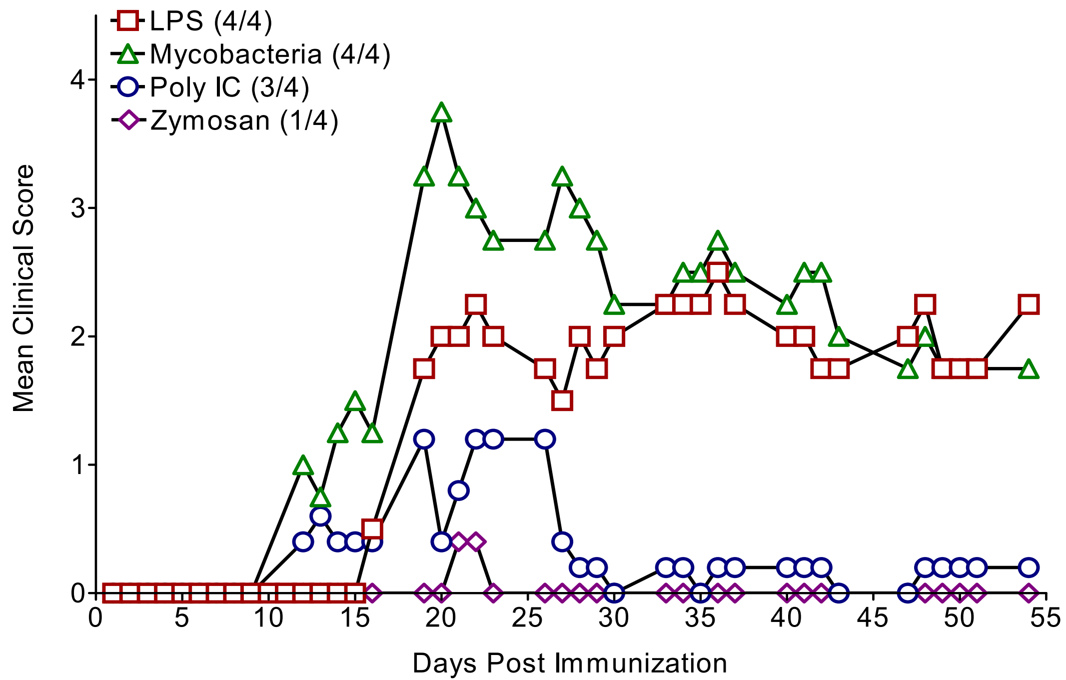

Figure 6A. TLR agonists act as adjuvants to induce EAE.. C57BL/6 wt mice were immunized s.c. with 200 µg MOG35-55 in IFA supplemented with 30 µg TLR agonist. 200 ng PTX was given i.p. on Days 0 & 2. Mice were then monitored daily for disease.

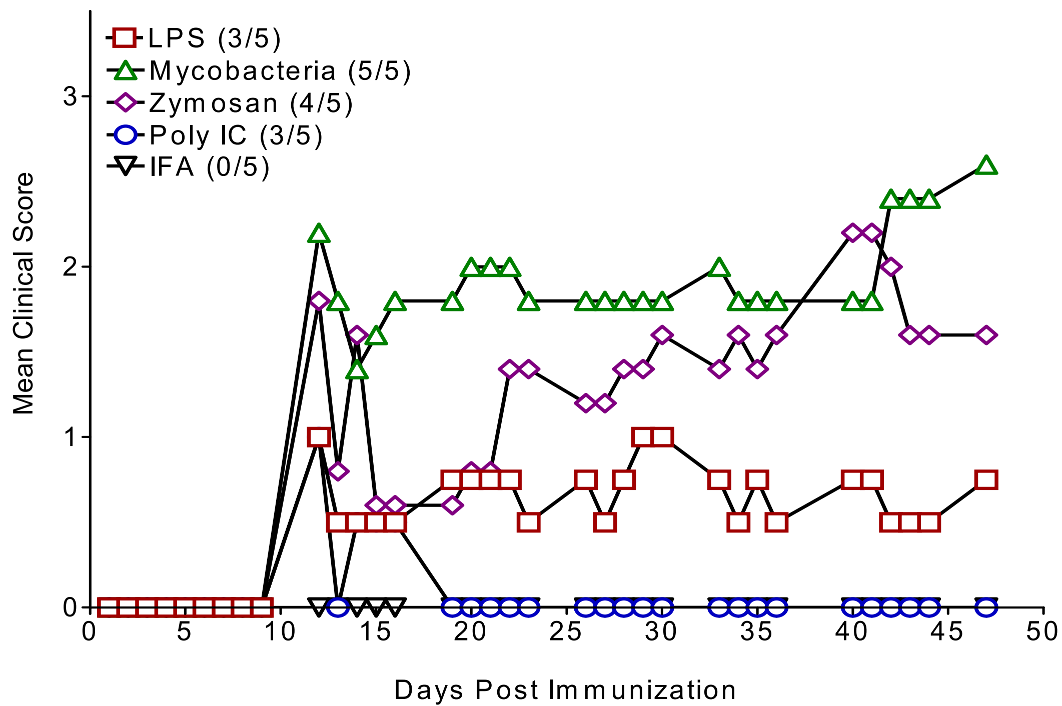

Figure 6B. TLR agonists act as adjuvants to induce EAE. C57BL/6 wt mice were immunized s.c. with 200 µg MOG35-55 in IFA supplemented with 100 µg TLR agonist. 200 ng PTX was given i.p. on Days 0 & 2. The mice were then monitored daily for disease.

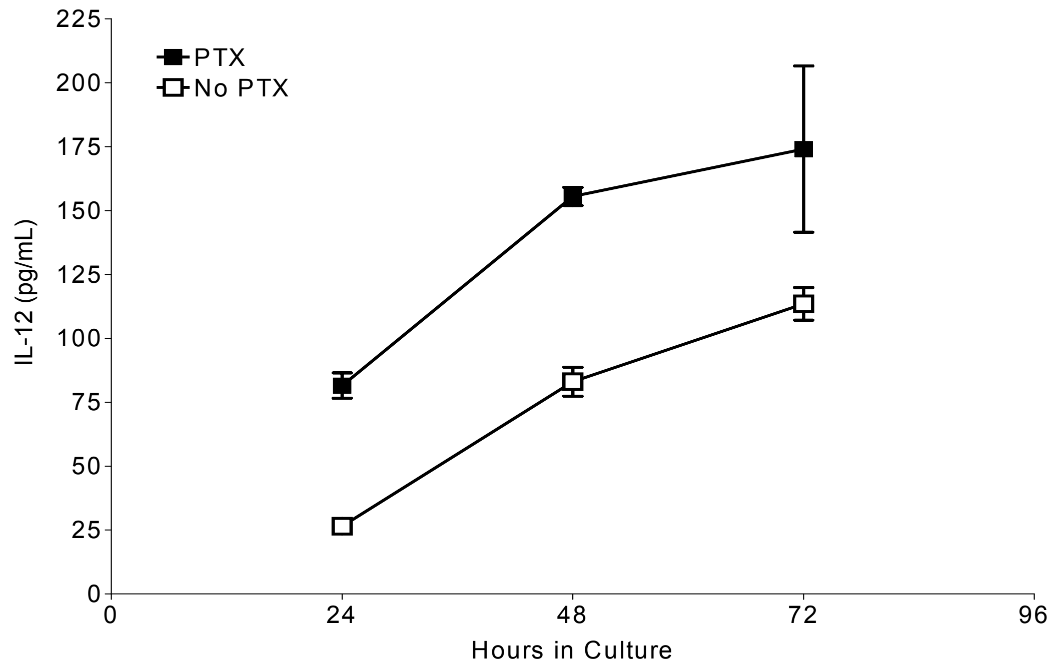

Figure 7A. PTX activates naïve cells to secrete inflammatory cytokines. Splenocytes from wt C57BL/6 mice were cultured with 200 ng/mL PTX or in media alone. Supernatants were collected at 24-hour intervals and IL-12 production was measured by ELISA.

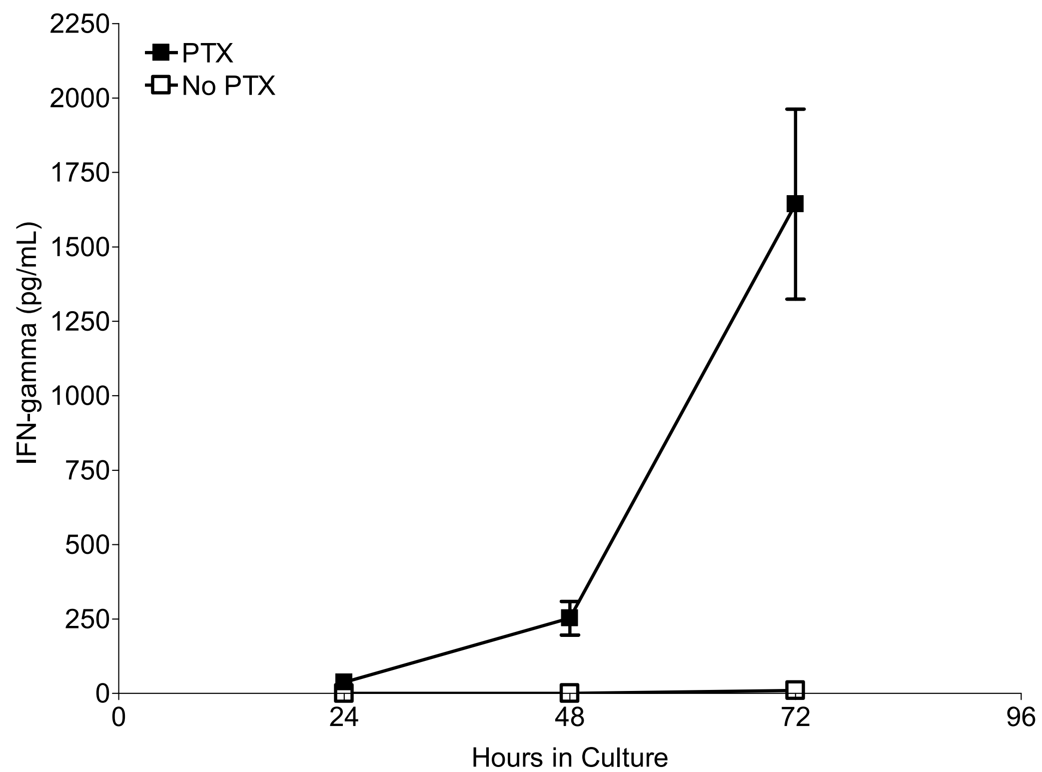

Figure 7B. PTX activates naïve cells to secrete inflammatory cytokines. Splenocytes from wt C57BL/6 mice were cultured in the presence of 200 ng/mL PTX or in media alone. Supernatants were collected at 24-hour intervals and IFNγ production was measured by ELISA.

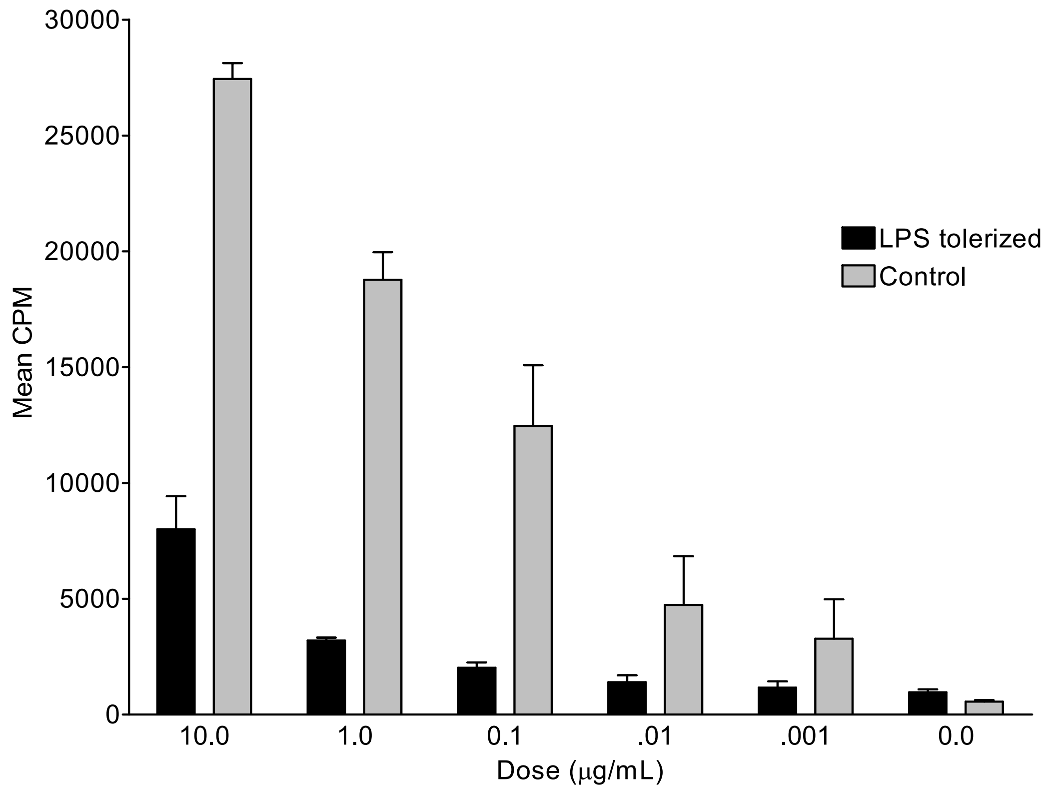

Figure 8A. LPS tolerance. C57BL/6 wt mice (n=2) were immunized i.p. with 40 μg LPS in IFA. Control mice were immunized with PBS in IFA. Mice were injected i.v. with 100 μg LPS in PBS 24 hours later. Control mice received PBS only. Splenocytes from tolerized and control mice were harvested after 10 days and cultured with various doses of LPS for 96 hours. [^{3}H]-thymidine was added for the last 16 hours of culture and proliferation was measured.

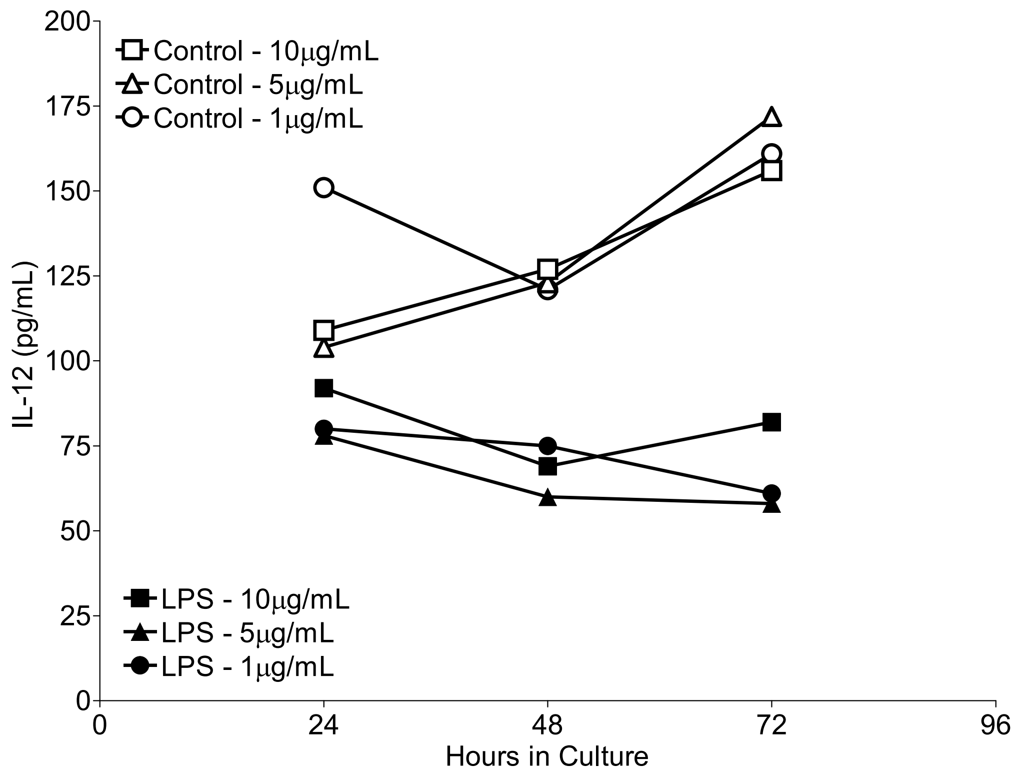

Figure 8B. LPS tolerance. C57BL/6 wt mice (n=2) were immunized i.p. with 40 μg LPS in IFA. Control mice were immunized with PBS in IFA. Mice were injected i.v. with 100 μg LPS in PBS 24 hours later. Control mice received PBS only. Splenocytes from tolerized and control mice were harvested after 10 days and cultured with various doses of LPS for 96 hours. IL-12 production was then measured by ELISA.

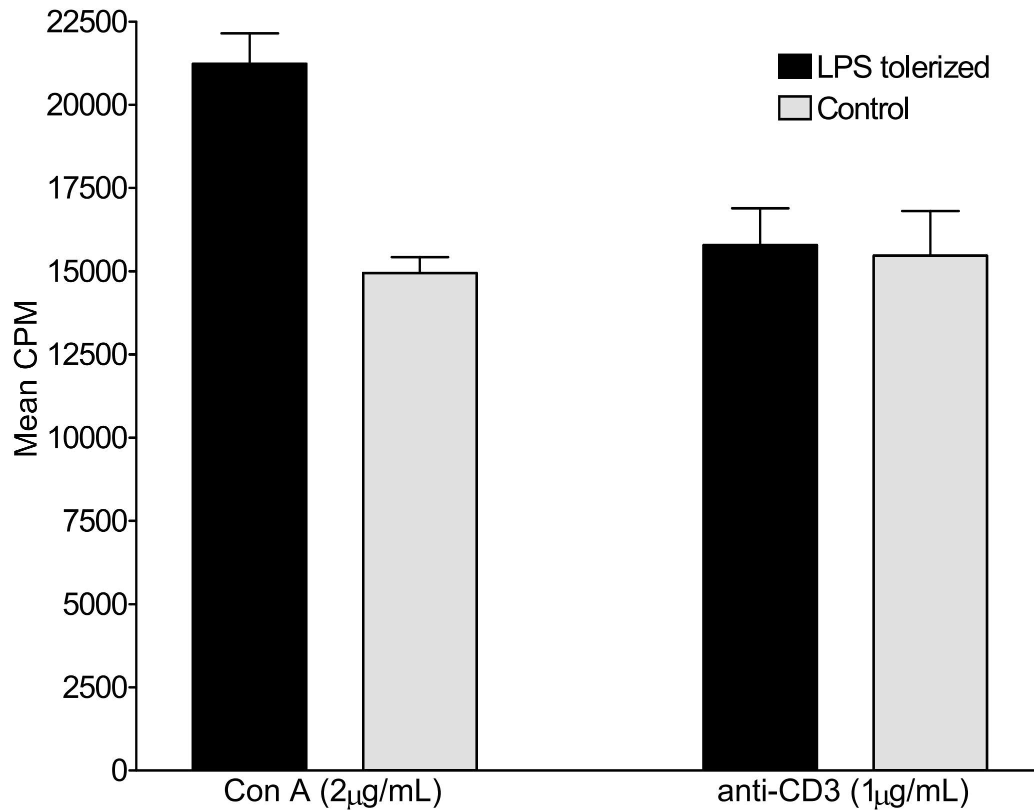

Figure 8C. LPS tolerance does not result in global immune cell impairment.. C57BL/6 wt mice (n=2) were immunized i.p. with 40 μg LPS in IFA. Control mice were immunized with PBS in IFA. Mice were injected i.v. with 100 μg LPS in PBS 24 hours later. Control mice received PBS only. Splenocytes from tolerized and control mice were harvested after 10 days and cultured with ConA or αCD3 for 96 hours for proliferation assay.

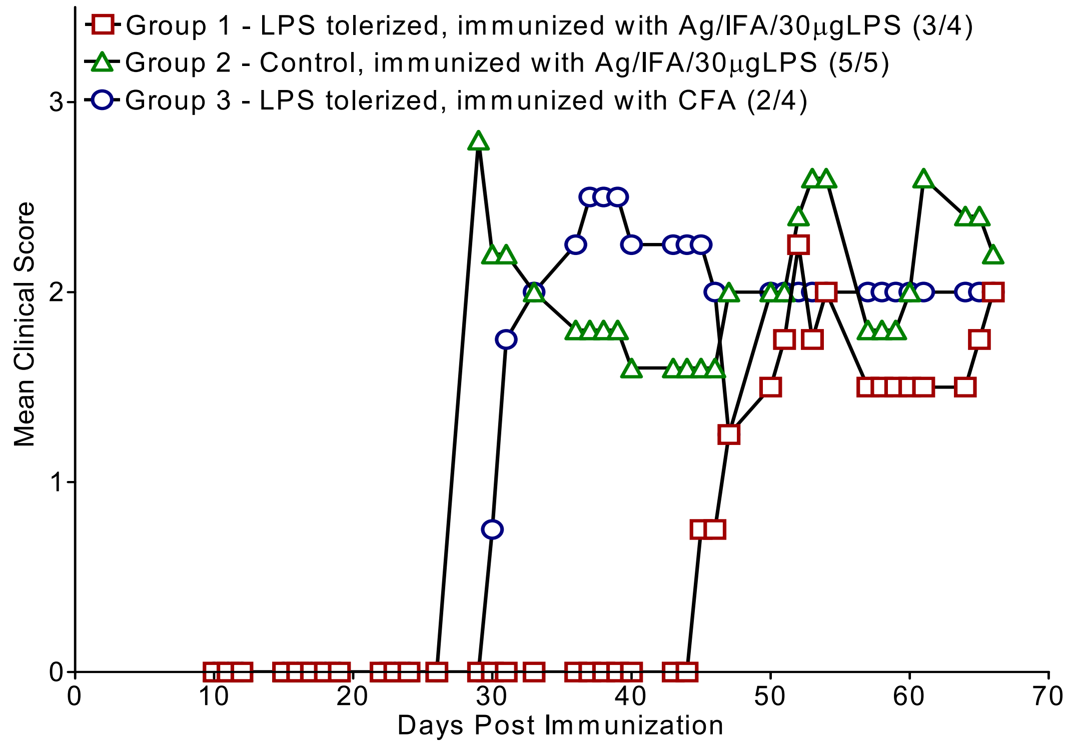

Figure 9A. Tolerance to LPS inhibits EAE induction when LPS is used as adjuvant. Tolerized and control Vβ8.2 Tg mice were immunized s.c. with 200 μg MOG 35-55 in IFA supplemented with 30 μg LPS or with Ag in CFA. Mice were then monitored daily for disease.

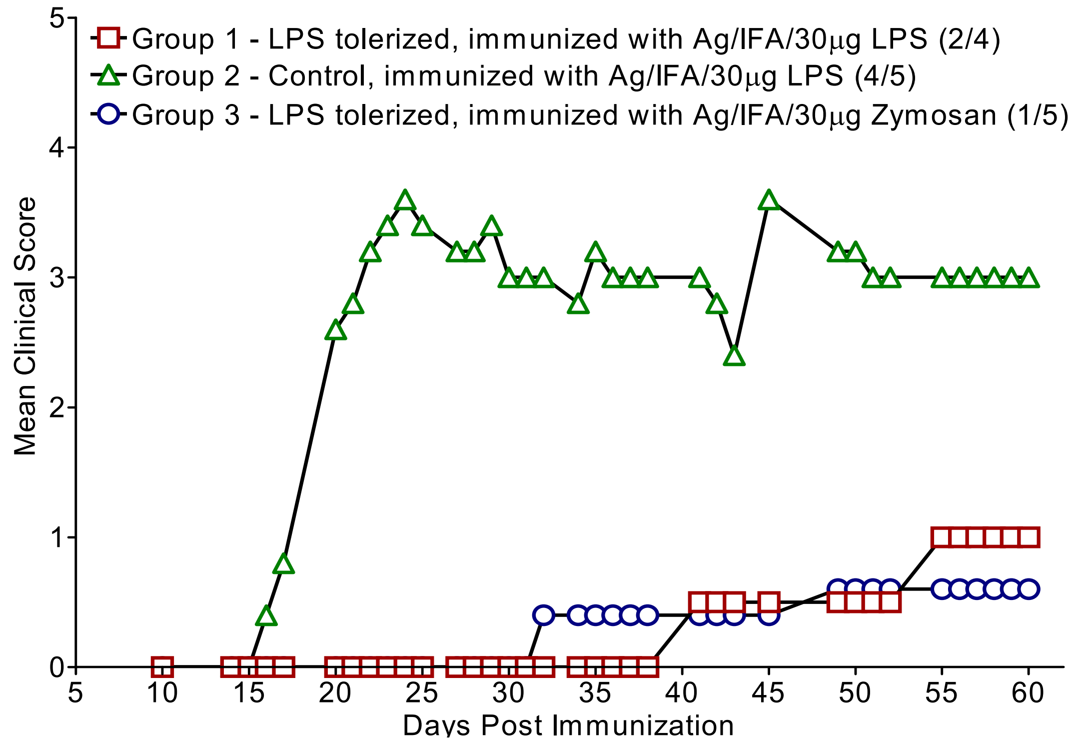

Figure 9B. Tolerance to LPS inhibits EAE induction when LPS is used as adjuvant. A, Tolerized and control Vβ8.2 Tg mice were immunized s.c. with 200 μg MOG 35-55 in IFA with 30 μg LPS or Zymosan. Mice were then monitored daily for disease.

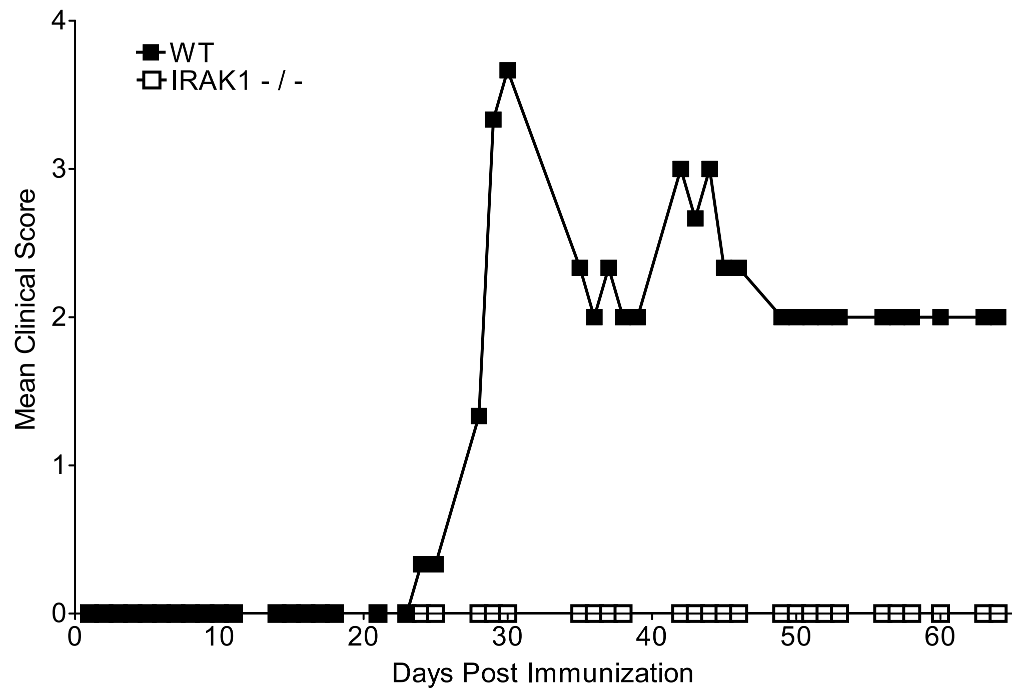

Figure 10A. Toll/IL-1 pathway is essential for T cell priming in EAE. B10.PL wt and IRAK1-/- mice were immunized s.c. with 200 µg MPBAc1-11 in CFA and monitored daily for disease. (This experiment was performed by Rehana Hussain.)

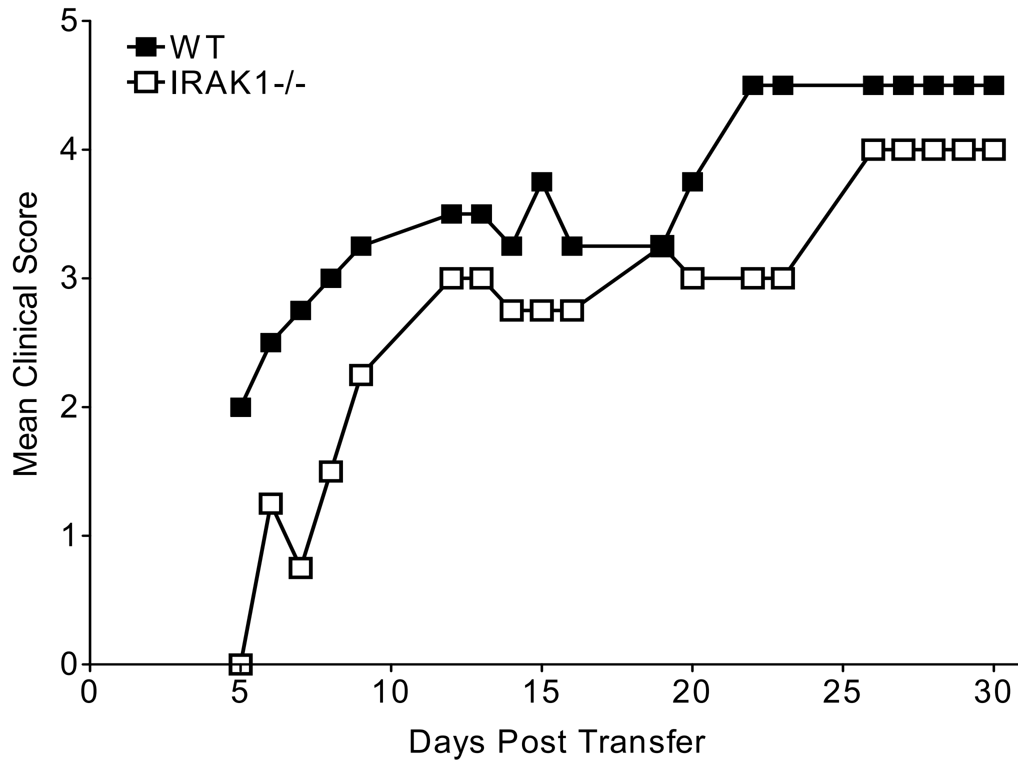

Figure 10B. Toll/IL-1 pathway is not necessary in the CNS for the development of EAE. Splenocytes were harvested from Vα2.3/Vβ8.2 TCR Tg mice and cultured with 2 μg/mL MPBAc1-11 for 48 hours. Cells were washed and injected at 30x10^6 cells/200 μL/mouse into naïve B10.PL wt and IRAK1$^{-/-}$ mice. (This experiment was performed by Rehana Hussain.)

CHAPTER FOUR
Discussion

In this study, I explored the impact of various TLR agonists on the activation of autoreactive immune cells and their subsequent effect on the course of EAE. I found MyD88-dependent TLR agonists to be potent adjuvants, further strengthening the argument that exposure to infection plays a significant role in the induction of autoimmunity.

Studies indicate that there is both a genetic and an environmental component to the pathogenesis of MS. This would suggest that neither genetics nor environment alone would necessarily result in MS, and that a combination of both would increase the likelihood of developing the disease. Indeed, studies have indicated links between susceptibility to MS and ethnicity, geographic location, HLA haplotype, and exposure to childhood infections (8-15). I investigated the contribution of genetics and environment by examining the development of EAE in the resistant B10.S mouse strain. When MBP-specific cells were harvested from mice previously primed with CFA and cultured with Ag alone or Ag in addition to the TLR agonist LPS, proliferation and IFNγ secretion were markedly enhanced by LPS. This suggests that in this genetically resistant strain, the Ag-specificity may be present, but without an environmental input, such as infection, these cells do not induce autoimmunity. However, even though

autoreactive cells were present, and proliferated in response to TLR agonists, when these cells were adoptively transferred, no disease occurred. This is perhaps because there are other mechanisms that factor into the resistance of B10.S mice to EAE, such as a lower precursor frequency of autoreactive T cells or decreased TLR expression, compared with a more EAE susceptible strain. It would be interesting to examine whether higher doses of Ag-specific cells could overcome this resistance.

In another attempt to break tolerance in B10.S mice, I injected mice with Ag alone and then followed this with a priming of Ag in CFA 10 days later. Cells from these mice were harvested and cultured with Ag or LPS, with the expected result being that cells cultured with LPS would proliferate more than cells cultured only with Ag. Indeed, cells that were cultured with LPS only or with Ag and LPS both displayed enhanced proliferation compared with cells cultured with Ag only. There was also increased IL-12 production in response to LPS or Ag with LPS, compared with Ag-only conditions (Fig 3A-C). I also adoptively transferred these cells into B10.S mice and found that, similar to *in vitro* results, mice that were injected with cells stimulated with LPS or Ag plus LPS induced moderate EAE, while Ag-only cultured cells induced no disease. It is not clear why these cells were able to induce EAE, while the previous adoptive transfer failed to work. In this experiment, I used PLP 139-151 as the Ag. In Figure 2, I used whole MBP as the Ag. It could be that some component of PLP 139-151 is

more potent in activating autoreactive T cells. It is possible that repeat experiments will yield more comparable results. This remains to be investigated. Performing *in vitro* proliferation assays and cytokine profiles on cells stimulated simultaneously with either whole MBP or PLP 139-151 could show possible differences in stimulative potential. A parallel EAE adoptive transfer experiment using whole MBP as the priming Ag in one group and PLP 139-151 as the priming Ag in the other group might show which Ag appears to better activate immune cells to adoptively transfer EAE.

I also measured the ability of each of the PAMPs to activate APCs *in vivo*, as demonstrated by the capacity of these APCs to stimulate T cells to proliferate *in vitro*. Wt C57BL/6 mice were immunized with a myelin peptide in an emulsion with one of four TLR agonists. It is important to note that a LAL assay indicated a trace amount of LPS in the stock of Poly (I:C) and Zymosan, so proliferation and induction of EAE were measured using this concentration of LPS. I found that LPS at this concentration was neither able to induce proliferation of T cells nor to induce EAE. Following immunization with myelin peptide a TLR agonist, LNCs from the immunized mice were harvested on Day 10 and cultured with Ag, and proliferation was assessed. I found Mycobacteria, LPS, and Zymosan to be quite effective activators, confirming that exposure to components of infectious agents can activate APCs *in vivo*. It is possible that there may be differences in the percentage of CD4+ T cells in mice that were

primed with different PAMPs, which could account for the different proliferative potential. Future experiments will include CFSE assays using purified CD4+ T cells, to account for these potential differences. These results support studies showing that relapses in MS can be exacerbated in the setting of an infection (20) possibly as a result of activated APCs stimulating autoreactive immune cells.

I suggest that PAMPs activate APCs *in vivo* that subsequently activate autoreactive T cells. To examine this, DCs were cultured with Ag and activated with the TLR agonist LPS. These DCs were then co-cultured with Ag-specific immune cells. In the presence of the TLR agonist, Ag-loaded DCs induced a marked increase in T cell proliferation and inflammatory cytokine secretion over unactivated DCs. In cultures where DCs were not loaded with Ag, but were only activated with LPS, there was increased IL-12 secretion, while IFNγ could not be detected. IL-12 is released by DCs, while IFNγ is released by T cells, suggesting that in the absence of Ag, a TLR agonist can still activate DCs, but these DCs will not stimulate Ag-specific T cells. These results further support a model in which otherwise tolerogenic DCs expressing a self-peptide could become activated, leading to activation of autoreactive immune cells. Further studies will show if other TLR agonists will follow this same pattern. Whether these activated T cells can transfer EAE better than T cells cultured with DCs that have not been exposed to LPS remains to be investigated.

In view of that fact that TLR agonists could activate APCs *in vitro* and *in vivo* to activate autoreactive immune cells, it was necessary to investigate whether these activated APCs were able to stimulate autoreactive cells to induce the development of disease. C57BL/6 wt mice were actively immunized with Ag in IFA, using PAMPs as adjuvants. I found Mycobacteria, LPS, and Zymosan to be efficient adjuvants, although Poly I:C was not able to induce disease. This agrees with my proliferation results (Fig 2) and points to the possibility that some TLR agonists are more potent adjuvants than others. It is interesting to note that Mycobacteria, Zymosan, and LPS, which signal through TLR1, 2, and 4, respectively, all signal through a MyD88-dependent pathway, while Poly I:C, an agonist for TLR3, utilizes a completely MyD88-independent pathway. Signaling through the MyD88-dependent pathway results in recruitment and interaction of IRAK4, IRAK1, and TRAF6, as well as other adapter molecules, culminating in the transcription of NF-κB and secretion of inflammatory cytokines. Signaling via the MyD88-independent pathway involves signaling through IRF-3, ultimately resulting in expression of NF-κB, costimulatory molecules and type-1 interferons. MyD88 is necessary for expression of cytokines such as IL-12, so it would follow that MyD88-independent signaling, utilized by TLR3, would not result in expression of IL-12. Another possibility is that TLR3 may be inefficient as a signal transducer. Studies showing low surface level expression of TLR4 suggest that low expression is sufficient, as TLR4 is very efficient at signaling. It

has also been shown that TLR3 has a high surface expression in immature DCs (68). Perhaps this is because signaling is not as efficient via TLR3. It is also possible that a different dose of each agonist would result in better activating abilities. However, comparing TLR agonists on an equimolar basis is difficult because of the complex nature of these agents. It is difficult to determinine biochemical properties of different TLR agonists, such as binding affinity, since no studies have shown definite binding of TLR agonists to their receptor. Therefore, a standard dose of all agonists was used. Future experiments will be testing different concentrations to look for dose-dependent effects.

To further assess the impact of TLR agonists on the pathogenesis of EAE, I used LPS tolerance to inhibit the ability of LPS to act as an adjuvant. While the mechanisms responsible for endotoxin tolerance remain unknown, several possibilities exist. Downregulation of TLR4 on macrophages occurs, although this alteration alone is insufficient to cause tolerance (69). Particular steps in major pathways have been shown to be downregulated in endotoxin tolerance. For example, IRAK1, which is necessary for TLR signaling via MyD88, is downregulated in endotoxin tolerance. I put forth that TLR agonist activation of APCs is integral to the stimulation of autoreactive T cells and the development of disease. The necessity of TLRs has been shown in other autoimmune models, such as rheumatoid arthritis, autoimmune myocarditis, and autoimmune diabetes mellitus (55, 60, 70). Therefore I presume that impairing the ability of LPS to act

as an adjuvant should impair development of disease following active immunization. This is precisely what occurred, suggesting that TLR activation is required for induction of disease. I did not use PTX in these experiments because of a recent report showing that PTX signals via TLR4 (65). My experiments showing that PTX stimulation alone, in the absence of Ag, could result in secretion of the inflammatory cytokines IL-12 and IFNγ also indicated that PTX could influence the outcome of these experiments. Therefore, the exclusion of PTX eliminates a possible confounding variable. Also, Vβ8.2 TCR Tg mice were used in this experiment due to their high precursor frequency of MBPAc1-11 specific T cells. These conditions were optimal for determining what effect the inhibition of LPS signaling would have on the induction of EAE. Impaired development of disease when LPS was used as the adjuvant was not the result of an overall impairment in immune cell function, as mice immunized with CFA developed classical EAE with a normal time course. In a similar experiment, Zymosan was used as another adjuvant, along with LPS. Published reports have suggested that there is cross-tolerance between TLR2 and 4 (66, 67). My results are in agreement with these published findings, as shown by the delay in onset of disease in mice tolerized to LPS and immunized with Zymosan. And while much research has been done on LPS tolerance, the effect of inhibition of signaling via other TLRs has not yet been examined.

I have shown that TLR agonists activate DCs, which can in turn activate autoreactive T cells to enhance *in vitro* proliferation as well as *in vivo* disease progression. The importance of the Toll/IL-1 pathway was further indicated by an experiment performed by research technician Rehana Hussain, in which B10.PL wt and B10.PL IRAK1$^{-/-}$ mice were immunized with Ag in IFA with a TLR agonist. The IRAK1$^{-/-}$ mice were impaired in their ability to develop EAE following direct immunization, suggesting that the Toll/IL-1 pathway is necessary for the induction of disease. In a study performed by Deng et al. (53), it was shown that IRAK1 is necessary in C57BL/6 mice for the secretion of inflammatory cytokines involved in autoimmunity, such as IL-12 p40. However, when cells were stimulated *in vitro* and adoptively transferred into IRAK1$^{-/-}$ mice, these mice developed EAE comparable to wt mice, indicating that IRAK1 and the Toll/IL-1 pathway are necessary for the priming of autoreactive T cells, although this pathway is not essential in the target organ for the expression of disease. Future experiments will determine potential differences between wt and IRAK1$^{-/-}$ mice if fewer cells are used. Also, reciprocal experiments, in which IRAK1$^{-/-}$ mice are used as donors for adoptive transfer into wild type recipients, will help further elucidate the role of TLRs in priming of encephalitogenic T cells.

In this study, I investigated the impact of TLR agonists on the pathogenesis of autoimmunity. I have shown TLR agonists to be crucial to the induction of autoimmunity. The specific mechanism by which these PAMPs help

induce autoimmunity is not yet clear, although I offer a potential scenario. Under normal circumstances, a DC that endocytoses a self-antigen and expresses a self-peptide on its surface will not activate autoreactive T cells due to a lack of costimulatory molecules on the DC. However, in the setting of an infection, a normally tolerogenic DC could become activated, presenting a self-peptide to an autoreactive T cell, expressing costimulatory molecules and cytokines such as IL-12 needed for the activation of the autoreactive T cell.

It has long been speculated that exposure to infection plays a part in the pathogenesis of autoimmunity. Here I show that several TLR agonists can be utilized as the adjuvant in EAE. This study suggests that the major role of these PAMPs is in activating APCs for T cell priming and that the Toll/IL-1 pathway is not required in the CNS for the development of EAE.

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VITAE

Baranda Santeri Hansen was born in Joplin, Missouri, on December 5, 1978, the daughter of Jayne D. Hansen. After completing her work at Grove High School, Grove, Oklahoma in 1997, graduating as Valedictorian with a 4.2 GPA, she entered the University of Arkansas at Fayetteville, Arkansas. During the summer of 2000, she attended the University of Oklahoma. She received the degree of Bachelor of Science with a major in Biology from the University of Arkansas in May 2002. In August 2002, she entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. She was awarded the degree of Master of Science in December 2005. Since August 2005 she has been employed as a Research Associate at the University of Texas Southwestern Medical Center at Dallas in the Department of Ophthalmology.

Permanent Address: P.O. Box 450915
Grove, OK 74345-0915