ON THE ROLE OF CD24 IN THE PATHOGENICITY OF MYELIN ANTIGEN SPECIFIC T CELLS

DISSERTATION

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By

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ABSTRACT

CD24 is a glycosylphosphatidylinositol (GPI) anchored cell surface glycoprotein that is expressed in hematopoietic cells and cells of the central nervous system (CNS). Although CD24 is commonly used as a maturation marker for T lymphocytes, its role in thymocyte development is not clear. CD24 has been reported to function as a co-stimulatory molecule independent of CD28 and is required for the induction of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (MS). Bone marrow chimera experiments suggest that CD24 expression on both bone marrow-derived cells and CNS cells are required for EAE development. Studies of single nucleotide polymorphisms (SNPs) suggest that CD24 is related to the risk and progression of MS. We have used a series of genetic models to delineate the role of CD24 in the pathogenesis of EAE.

It has previously shown that CD24 is required for the induction of EAE. In $CD24^{-/-}$ mice, normal levels of myelin oligodendrocyte glycoprotein (MOG) specific T cells were primed, however these T cells were non-pathogenic. To understand this issue, we bred $CD24^{-/-}$ mice with 2D2 TCR transgenic mice, which bear TCR specific to MOG, and generated 2D2 TCR transgenic mice with or without CD24. Here I show that 2D2 TCR transgenic mice with CD24-deficiency ($2D2^{+}CD24^{-/-}$) have remarkably withered thymi. In peripheral lymphoid organs,

transgenic T cells from $2D2^{+}CD24^{-/-}$ mice have an immature phenotype ($CD4^{-}CD8^{-}$), do not respond to MOG peptide stimulation, and fail to cause autoimmune inflammation in the CNS and optical nerves. In contrast, OTII TCR transgenic mice with CD24 deficiency ($OTII^{+}CD24^{-/-}$), which bear TCR specific to chicken ovalbumin (OVA), have normal thymi and their peripheral T cells have a normal response to OVA peptide. These data suggest that CD24 inhibits thymic deletion of myelin antigen, but not foreign antigen-reactive T cells.

To understand the role of CD24 on the resident cells in the CNS during EAE development, I created CD24 bone marrow chimeras and transgenic mice in which CD24 expressions was under the control of a glial fibrillary acidic protein promoter (GFAP) denoted $Astro^{CD24}$. I showed that mice lacking CD24 expression on the CNS resident cells developed a mild form of EAE, but mice with over-expression of CD24 in the CNS developed severe EAE. Compared with nontransgenic mice, the CNS of $Astro^{CD24}$ mice had higher expression of cytokine genes such as IL-17; the CNS of $Astro^{CD24}$ mice accumulated higher numbers of Th17 and total $CD4^{+}$ T cells, whereas $CD4^{+}$ T cells underwent more proliferation during EAE development. Expression of CD24 in CD24-deficient astrocytes also enhanced their co-stimulatory activity to myelin oligodendrocyte glycoprotein-specific, TCR-transgenic 2D2 T cells. Thus, CD24 on the resident cells in the CNS enhances EAE development via co-stimulation of encephalitogenic T cells.

The role of CD24 in the thymic generation and pathogenicity of myelin antigen specific T cells provides a novel explanation for its control of genetic susceptibility to autoimmune diseases in mice and humans.

DEDICATION

To my mother and father, Laurie G. and Joseph W. Carl, who through their love and support, help me to be the best man I can be. And to my son, Adam Joseph Carl, who has inspired me to be the best man I can be.

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PUBLICATIONS

1. **Carl JW Jr**, Bai XF (2008) (Review) IL27: Its Roles in the Induction and Inhibition of Inflammation*, Int J Clin Exp Pathol 1(2):117-123.*
2. Liu JQ, **Carl JW Jr**, Joshi PS, RayChaudhury A, Pu XA, Shi FD, Bai XF (2007), CD24 on the Resident Cells of the Central Nervous System Enhances Experimental Autoimmune Encephalomyelitis, *The Journal of Immunology 178(10)*:6227-35.
3. **Carl JW Jr**, Liu JQ, Joshi P, Yin L, Zhen X, Whitacre CC, Liu Y, Bai XF, Autoreactive T Cells Escape Clonal Deletion in the Thymus by a CD24-Dependent Pathway, *The Journal of Immunology (in Press)*.

FIELDS OF STUDY

Major Fields: Immunology

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ABBREVIATIONS

2D2 – Name of transgenic mice with TCR specific for MOG_{35-55}

aa – Amino Acid

APC – Antigen Presenting Cell

$Astro^{CD24}$ – Name of transgenic mice with CD24 expression under control of glial fibrillary acidic protein promoter

BBB – Blood Brain Barrier

CFA – Complete Freund's Adjuvant

CNS – Central Nervous System

DMEM – Delbecco's Modified Eagle's Medium

DN – Double Negative

DP – Double Positive

EAE – Experimental Autoimmune Encephalomyelitis

ER – Endoplasmic Reticulum

FCS – Fetal Calf Serum

GFAP – Glial Fibrillary Acidic Protein

GPI – glycosylphosphatidylinositol

H&E – Hematoxylin and Eosin

HPRT – hypoxanthine-guanine phosphoribosyltransferase

HSA – Heat Stable Antigen

i.p. – Interaperitonal

i.v. – Intravenous

mTEC – Medulary Thymic Epithelial Cells

MBP – Myelin Basic Protein

MHC – Major Histocompatibility Complex

MOG – Myelin Oligodendrocyte Glycoprotien

MS – Multiple Sclerosis

OTII – Name of transgenic mice with TCR specific for $OVA_{323-333}$

OVA – Ovalbumin

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PLP – Proteolipid Protein

RT-PCR – Real Time PCR

s.c. – Subcutaneous

SNP – Single Nucleotide Polymorphism

SP – Single Positive

TEC – Thymic Epithelial Cells

TCR – T Cell Receptor

UTR – Untranslated Region

VSA – Viral Super Antigen

WT – Wild Type

INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) affecting 1/1000 Caucasians (1). Although the etiology of MS is not known, genetic, gender, and environmental triggers are factors that affect the risk of contracting the disease as well as of its progression. Most people are diagnosed with MS between the ages of 20 and 40 with women being affected twice as often as men. According to the National MS Society, about 400,000 Americans are known to have MS, with 200 new diagnoses each week. Currently there is no cure for MS, although there are therapies that target the immunological checkpoints of MS development.

MS is a complex disease associated with an inflammatory process in the CNS comprised of infiltrating lymphocytes and activated macrophages/microglia resulting in the demyelination of axons, as well as axonal damage and destruction (2-4). While the sequence of events that initiates MS is unknown, experimental autoimmune encephalitomyelitis (EAE), the best animal model for MS (5, 6), has been instrumental in characterizing the pathological progression.

The EAE model was first developed in monkeys by Rivers, *et al*. (7), and shares many features of MS pathology (2, 8). EAE can be induced in mice by active

immunization with CNS antigens such as myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG). EAE can also be induced by passively injecting purified activated auto-reactive T cells into healthy syngeneic mice. In both methods of EAE induction, *B. Pertussis* toxin is also utilized as another adjuvant to initiate T cell accumulation at the blood brain barrier (BBB) (9, 10), allowing activated T cells access to the CNS. Like MS, EAE is a $CD4^+$ T cell mediated disease (5, 6), and activated $CD4^+$ T cells pass through the permeable BBB secreting Th1 cytokines, such as IFNγ and TNFα (11). These cytokines subsequently induce the creation of lesions that destroy the myelin sheath that normally insulates and protects nerve fibers. Lesions are commonly found in the optic nerve, periventricular white matter, brain stem, cerebellum, and spinal cord white matter (12).

It has been reported (13-15) that targeted mutation of CD24 abrogates the development of EAE induced with MOG-peptide immunization and adoptive transfer. $CD24^{-/-}$ mice had levels of MOG-peptide specific T cell responses similar to those found in WT mice that displayed EAE. However, CD24-deficient MOG-specific T cells were not encephalitogenic upon adoptive transfer. This observation suggests that the MOG-responsive T cells generated in CD24-deficient mice are functionally defective. Adoptive transfer studies also demonstrate that MOG-peptide specific T cells generated from WT mice can only transfer EAE to $CD24^{+/+}$ but not to $CD24^{-/-}$ recipients. These data suggest that encephalitogenic T cells need help from the host cells in order for them to

expand and persist in the CNS. Indeed, bone marrow chimera experiments suggest that both bone marrow-derived non-T and non-B cells or radiation-resistant cells of $CD24^{+/+}$ genotype can provide such help (15). Recently using a series of genetic models to study the role of CD24, I have found that CD24 contributes to both thymic generation and effector function of myelin antigen specific T cells.

CHAPTER 1:

PATHOGENESIS OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS AND MULTIPLE SCLEROSIS

EAE serves as a model for the pathogenesis and progression of MS, and has enabled the development of therapies based on the checkpoints identified by this model. Understanding the checkpoint model of EAE pathogenesis will be useful in following the experimental design discussed in this study. I will describe a 4 checkpoint model (**Figure 1**) of EAE/MS pathogenesis consisting of 1) generation of naïve autoreactive T cells, 2) activation of naïve T cells in the peripheral lymphoid organs and differentiation of activated T cells into pathogenic effector subsets, 3) migration of activated T cells into the CNS, and 4) reactivation and survival of T cells in the CNS.

The thymus is a primary lymphoid organ responsible for the selection and development of T cells (16). It is a bi-lobed structure located on the anterior side of the heart in the pericardial medistinum (17). A normal thymus is comprised of a cortex and a medulla enclosed by thin capsular tissue. Bone marrow-derived precursors enter the thymus via an artery at the cortico-medullary junction. The artery ramifies into fenestrated capillaries in the medulla and rarely fenestrated capillaries in the cortex (18). The lack of fenestration on the capillaries in the cortex forms a blood-thymus barrier

prohibiting entry of perivascular lymphocytes and circulating antigenic molecules. The cortex contains small immature lymphocytes, sparse epithelial cells, and phagocytic macrophages (19). The medulla contains more mature T cells, prominent epithelial cells, Hassall's corpuscles, phagocytic macrophages, non-phagocytic dendritic cells and B lymphocytes(20). Stroma in the thymus play an important role in central tolerance by expressing tissue specific antigens (21). Expression of tissue specific isoforms and doses of myelin components in the thymus determine the efficacy of central tolerance. Several studies suggest that in some strains of mice, thymic expression of myelin components, such as PLP and MBP, contribute to the resistance to EAE induction (22, 23). For example, the SJL strain of mice predominantly express an alternatively spliced form of PLP, which may contribute to the increased frequency of autoreactive T cells in the periphery (22, 23). Central tolerance shapes the repertoire of TCRs, and the existence of autoreactive T cells is the first checkpoint in the EAE model of pathogenesis (**Figure 1 – Checkpoint A**).

The developing lymphocytes are categorized by the expression of CD4 and CD8. The bone marrow-derived precursors express no CD4 or CD8 and are called double negative (DN) cells. The next stage of development is a double positive (DP) (24) cell expressing both CD4 and CD8, followed by maturation to a single positive (SP) cell expressing CD4 or CD8. The TCR genes are formed in the DN stage of development. The stepwise stages of somatic recombination events in

DN cells can be correlated by the expression of the cell surface markers CD44 and CD25.

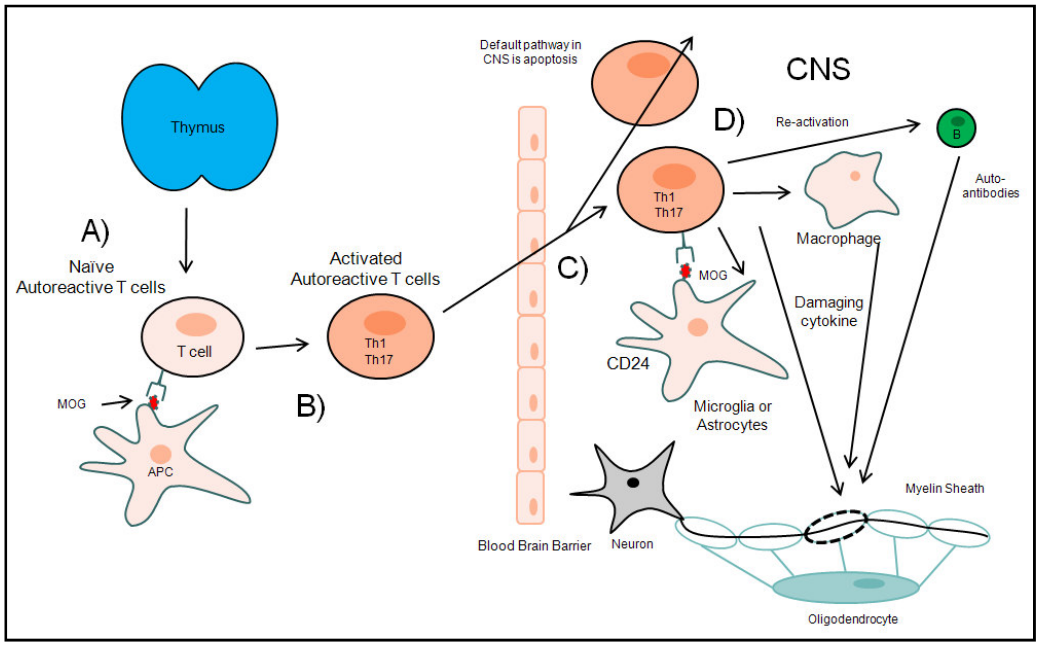

Figure 1: Four checkpoints model of EAE pathogenesis

A) Generation of naïve autoreactive T cells in the thymus is the first checkpoint. B) Activation of naïve T cells by antigen presenting cells and differentiation into pathogenic effector subsets such as Th1 or Th17 is the second checkpoint. C) Migration of activated T cells to the CNS and crossing the BBB is the third checkpoint. D) The fourth checkpoint is reactivation and survival of T cells in the CNS

The earliest cells to arrive from the bone marrow are CD44 positive and CD25 negative denoted DN1 and are found primarily at the cortico-medullary junction. Lymphocytes that will become γδ cells begin to rearrange the δ D-D and V-D gene segments in the DN1 stage. DN1 cells develop into CD44 positive and CD25 positive cells called DN2 and are found throughout the cortex. DN2 cells rearrange δ D-J segments and γ segments (25). Lymphocytes that are destined to become αβ cells begin to rearrange the β D-J segment in the DN3 stage. DN3

cells no longer express CD44 and are concentrated in the outermost part of the thymus just below the capsule (26). DN3 cells then develop into DN4 cells and β VDJ recombination completes and is expressed on the cell surface with a preαTCR. DN4 cells express neither CD25 nor CD44 and will become DP cells. Autoreactive T cells specific for CNS antigens are normally controlled by peripheral tolerance mechanisms such as anergy (27) or functional regulatory cells (28).

The activation and clonal expansion of autoreactive T cells is the second checkpoint in the EAE model of pathogenesis. Active induction of EAE utilizes CNS antigens suspended in an adjuvant such as complete Freund's adjuvant (CFA) and injected subcutaneously (s.c.) in the hind quarters, followed by an intravenous (i.v.) tail vein injection of *B. pertussis*. The activation mechanism of naïve CD4+ T cells follows the "two-signal model" in which the interaction of the TCR with a peptide/MHC complex must be accompanied by a second co-stimulatory signal. Normally the co-stimulatory signal is CD28 on the T cell with B7 family molecules on APCs (14, 29, 30). The activation of CD4+ T cells specific for CNS antigens is the second checkpoint in EAE. Concurrent with the activation of autoreactive T cells is their differentiation into effector cells. CD4+ T cells differentiate into two major subtypes of effector cells defined by the cytokines they release. The two effector subtypes are denoted Th1 and Th2 (31). Th1 is associated with cell mediated immunity and release the cytokines IL2, TNFα and IFNγ (31), while Th2 is associated with humoral mediated immunity and release

IL4, IL5, IL6, IL10 and IL13 (31). A recently identified subset of T cells (Th17) cells has also been shown to be correlated with EAE susceptibility (32, 33). Thus, the development of EAE can be regulated by factors that control the differentiation of Th subsets (5, 34). MS as well as EAE is generally accepted to be mediated by Th1 helper cells. In the CNS Th2 type cytokines play a role in down regulating Th1 responses and macrophage activation (35). The differentiation into Th1/Th17 helper cells is a second checkpoint in EAE (**Figure 1 – Checkpoint B**).

Once activated, the T cells must cross the BBB. The BBB is formed by highly specialized endothelial cells, which inhibit transcellular molecular trafficking (36), and astrocyte foot processes (37-39). The blood vessels on the CNS side of the BBB are ensheathed by astrocytes (40) forming a barrier. During inflammation, the BBB endothelium upregulate traffic signals that aid T cell arrest and transmigration (41-43). Infiltrating activated leukocytes accumulate in the perivacular spaces (44), while non-activated leukocytes do not, because T cells are able to cross the BBB based on their activation state, not TCR specificity (45). Additionally T lymphocytes vary in degree in their ability to enter different sites of the CNS, with higher levels of entry in the spinal cord and lowest levels at the cerebrum (46). The process of migration to the CNS and crossing the BBB is a third checkpoint in the progression of EAE (**Figure 1 – Checkpoint C**).

Once the activated autoreactive T cells cross the BBB, to become reactivated, they must encounter the CNS antigen epitope presented on MHC molecules as expressed by CNS antigen presenting cells (5, 47-49) such as microglia,

astrocytes, or dendritic cells and become reactivated. In addition to the two well-defined cytokine profiles Th1 and Th2, a third profile, associated with autoimmune diseases, has been identified and denoted Th17 (32, 33). Reactivated T cells will induce the production of cell mediated destruction of myelin on axons (50). Activation of microglia, infiltrating macrophages, and B cells will induce these cells to release damaging cytokines or antibodies which result in the destruction of myelin on axons (51-53). Myelin is necessary for the proper conduction of electrical signals along axons.

Generally, T cells find the CNS an inhospitable environment for their survival, and will undergo apoptosis (54) unless rescued by supporting cells co-stimulation (55). Microglia constitute the tissue specific macrophage of the CNS (56), regularly engulfing material in their environment and helping to maintain the integrity of the brain (57). Under inflammatory conditions microglia up-regulate MHC class molecules and can promote the reactivation of T cells in the CNS (58). Astrocytes influence the formation and maintenance of the BBB (59). Under inflammatory conditions they will up-regulate adhesion molecules facilitating leukocyte transmigration. The ability of astrocytes to present antigens is a controversial one; *in situ* and *in vivo* evidence supports the expression of MHC-II and co-stimulatory molecules (60), yet other studies indicate that astrocytes do not present antigens to prime T cells (61, 62). It is clear that astrocytes do not normally express MHC-II but its expression can be induced in inflammatory conditions (63). Furthermore human astrocytes do not express B7-1 or B7-2

either constitutively or due to inflammatory stimuli (64), while murine astrocytes do express B7 molecules under inflammatory conditions (60). Under inflammatory conditions, CNS APCs will upregulate MHC-II and co-stimulatory molecules. Activated T cells that have crossed the BBB will encounter CNS APCs and become reactivated. Mechanism of reactivation by CNS APC constitutes a fourth checkpoint in EAE (**Figure 1 – Checkpoint D**).

CHAPTER 2:

THE ROLE OF CD24 IN MULTIPLE SCLEROSIS AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Springer (65) identified CD24 in 1978 while searching for lymphocyte differentiation markers. Because of its resistance to heat, it was called Heat Stable Antigen (HSA). CD24 is a glycosylphosphatidylinositol (GPI) link protein expressed on numerous cells of the immune system, including T cells (66, 67), dendritic cells (68), B cells (69), macrophages (70), neutrophils (71), as well as cells of the CNS (13, 72, 73). CD24 has been shown to be expressed on a broad range of epithelial cells such as hair follicles (74) and intestine (75) but the significance is not clear. Expression kinetics of CD24 are best understood on T cells and B cells, while expression on developing neuronal cells is still a field of ongoing study.

2.1 CD24 Molecular Genetics

First identified in the mouse using two rat antibodies M1/69 and M1/75 (65), CD24 can also be recognized by other antibodies (76-78). Located on the mouse chromosome 10 26.0 cM as two exons and on the human chromosome 6q21 as a single exon, the resulting transcripts share 63% homology (**Figure 2**A). Other intronless pseudogenes at 3p26, 15q12, 15q22, 20q11.2 and Yq11.1 exist and

most likely they do not encode for CD24 proteins (79); however, care needs to be taken when utilizing PCR to analyze gene products.

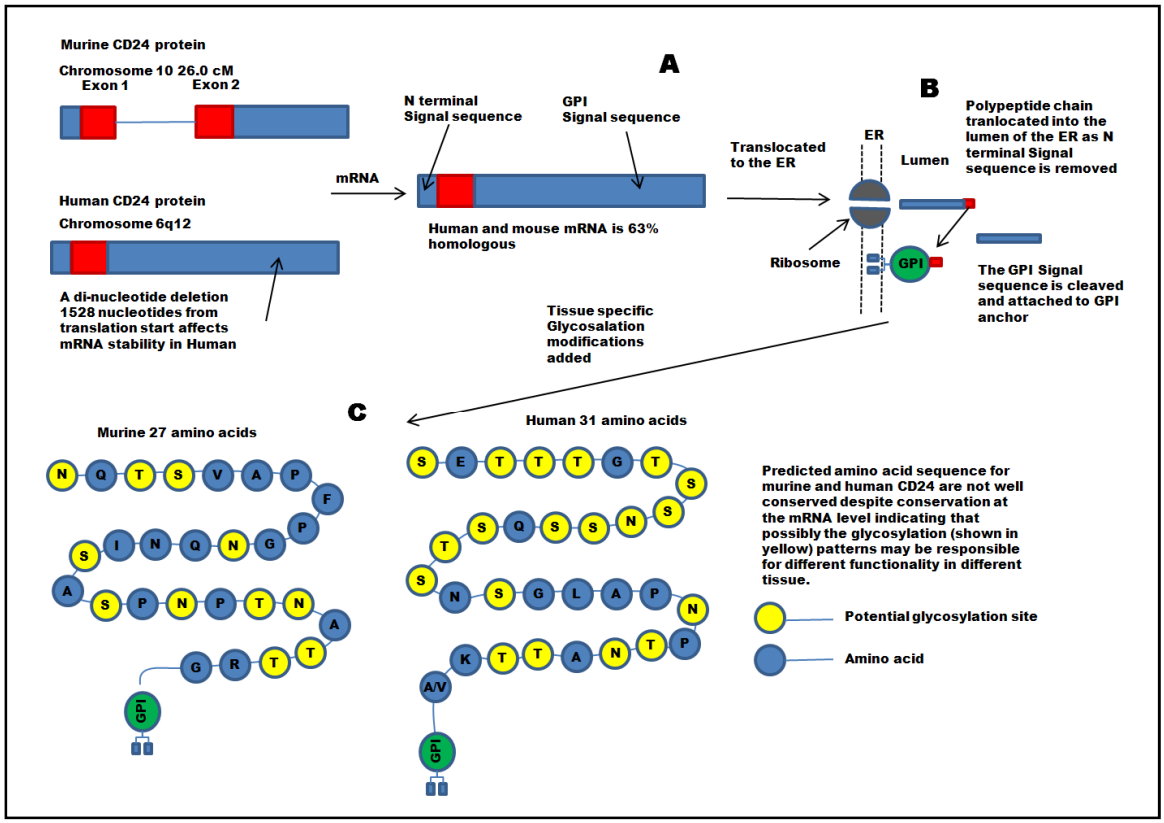

Figure 2: CD24 molecular genetics – DNA to mRNA to protein

A) Murine HSA (CD24) is located on Chromsome 10 26.0 cM, while Human CD24 is located on chromosome 6q12. There is a 63% homologue between the mouse mRNA and human mRNA. B) The mRNA translocates to the ER and the GPI signal sequence is cleaved as the nascent protein is attached to a GPI anchor. C) The resulting protein is 27 amino acids (aa) in length in the mouse and 31 aa in the human. The yellow aa's are predicted glycosylation sites

The mRNA contains a 5' terminal signal sequence directing the mRNA to the endoplasmic reticulum (ER) where the signal sequence is removed as it is translated. Because of its GPI anchor, the nascent protein has a GPI signal sequence associated with the long untranslated 3' region. Through mechanisms

still under investigation, the nascent protein is translated fully into the lumen of the ER, where CD24 protein is removed from the GPI anchor signal sequence and subsequently attached to a GPI anchor (68, 80) (**Figure 2**B). Numerous tissue specific N- and O- glycosylations (variations not completely elucidated) are added (81) in the ER and then trans-located to the cell surface (**Figure 2**C).

2.2 CD24 and MS

At least four polymorphisms have been identified in the human CD24 coding regions, one of which results in a SNP associated with an increased risk of MS (82). The other three polymorphisms are found in the 3' untranslated region, one of which is associated with an increased protection of MS (reducing the risk by a factor of 2) and systemic lupus erythermatosus (79). The replacement of alanine with valine at amino acid 31 results in an increased expression of CD24 on the cell surface (82) , possibly by facilitating the insertion of the GPI anchor. The increased expression of CD24 due to this SNP raises the relative risk of MS by two-fold, while at the same time increases the risk of rapid progression of MS. Fifty percent of the patients with a homozygous valine SNP reached an expanded disability score of 6.0 within 5 years compared to 16 years for heterozygous and 13 years for those with homozygous alanine SNP (82). The protective risk modification of CD24 reduction supports the hypothesis that increased CD24 expression increases the risk of MS. The di-nucleotide deletion of an amino acid at position 1527 from the mRNA translation start site affects the stability of the mRNA (79). The reduced stability of the mRNA produced two-fold less CD24

mRNA compared to wild-type alleles. The reduced expression of CD24 due to this di-nucleotide deletion reduced the relative risk of MS by greater than two-fold. Additionally, MS patients who have this di-nucleotide deletion have a slower progression (79).

2.3 CD24 and EAE

It has been reported (13-15) that targeted mutation of CD24 abrogates the development of EAE induced with MOG-peptide immunization and adoptive transfer. $CD24^{-/-}$ mice had levels of MOG-peptide specific T cell responses similar to those found in WT mice that displayed EAE. However, CD24-deficient MOG-specific T cells were not encephalitogenic upon adoptive transfer. This observation suggests that the MOG-responsive T cells generated in CD24-deficient mice are functionally defective.

Adoptive transfer studies also demonstrate that MOG-peptide specific T cells generated from WT mice can only transfer EAE to $CD24^{+/+}$ but not to $CD24^{-/-}$ recipients. It was found (15) that upon adoptive transfer, activated MOG-specific $CD24^{+/+}$ T cells can infiltrate the CNS of both $CD24^{+/+}$ and $CD24^{-/-}$ recipients. However, donor T cells remained in the perivascular area of the $CD24^{-/-}$ recipients and did not cluster in the CNS parenchyma, in contrast to $CD24^{+/+}$ recipients. In addition, proliferation of the donor T cells was lower in $CD24^{-/-}$ recipients and the number of T cells in the CNS rapidly declined within 20 days in the CNS of $CD24^{-/-}$ mice. The number of T cells in the CNS of $CD24^{+/+}$ mice increased for 20 days and then declined, remaining detectable at 40 days after

adoptive transfer. These data suggest that encephalitogenic T cells need help from the host cells in order for them to expand and persist in the CNS. Indeed, bone marrow chimera experiments suggest that both bone marrow-derived non-T and non-B cells or radiation-resistant cells of $CD24^{+/+}$ genotype can provide such help (15). We hypothesize that CD24 on antigen presenting cells (such as bone marrow-derived dendritic cells and CNS local microglia and astrocytes) provide a co-stimulatory signal to CNS infiltrating T cells. Indeed, it was shown that astrocytes and microglia from $CD24^{-/-}$ mice have a 4-fold and 10-fold, respectively, reduced ability to stimulate proliferation of MOG-reactive T cells. Over-expression of CD24 in the CNS significantly enhanced EAE development, via accumulation of high numbers of Th17 cells in the CNS (13). These data suggest that APCs in the CNS contribute to the expansion and persistence of encephalitogenic T cells in the CNS via co-stimulation of CD24.

2.4 Goals of this study

The first goal of this study was to determine the role of CD24 in the pathogenicity of myelin antigen specific T cells. While CD24-deficient mice are resistant to the induction of EAE, CD24 deficient mice can generate MOG specific helper T cell response, but the T cells are not encephalitogenic suggesting that the CD24 deficient, MOG specific T cells are defective in some manner. To investigate this issue, I crossed CD24 deficient mice with myelin specific TCR transgenic mice (2D2) to produce CD24 deficient 2D2 mice. I used this model to determine if CD24 deficiency affects pathogenicity of MOG specific T cells.

The second goal was to assess the role of CD24 in the CNS. The Bai lab created transgenic mice which over-expressed CD24 on astrocytes, driven by the GFAP promoter element. I used this model to determine if CD24 on the resident cells of the CNS can affect T cell pathogenicity in EAE development.

CHAPTER 3:

MATERIALS AND METHODS

3.1 Mice

C57BL/6 mice were purchased from the Jackson Laboratory or National Cancer Institute. 2D2 TCR transgenic mice (83) were kindly provided by Dr. V.K. Kuchroo (Harvard Medical School). CD24-deficient mice in the C57BL/6 background have been described (14, 15). OTII TCR transgenic mice were purchased from the Jackson Laboratory. Transgenic mice expressing CD24 exclusively on T cells have been described (67). Mice with CD24 over-expression in the CNS (designated Astro^{CD24}) were generated as I describe below. All mice were bred and maintained in a specific pathogen-free animal facility of The Ohio State University. The animal facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

3.2 Production of Astro^{CD24} mice

The transgenic construct used for microinjection of C57BL/6 oocytes was generated based on the transgenic construct pGfa2-cLac (84) (provided by Dr. M. Brenner, University of Alabama, Birmingham, AL). The construct contains a 2.2kb DNA fragment of the human FGAP promoter sequence (84-86), a fragment of the mouse protamine-1 gene that supplies an intron to stabilize 3' untranslated region (UTR) and polyadenylation signal. We used the full-length

mouse CD24 cDNA (1.8kb) to replace the *LacZ* gene in the original vector. After excising the vector backbone, the full-length DNA fragment used for microinjection was 4.6kb. Oocytes were injected using conventional microinjection technology by the core facilities of The Ohio State University.

3.3 Antibodies and Flow Cytometry

The following antibodies were used in the experiments according to the manufacturer's recommendations: purified, FITC-, PE- PerCP-, or biotin-labeled anti- CD4 (GK1.4), CD8α (53-6.7), CD24 (M1/69), CD25 (7D4), CD44 (IM7), CD45 (30-F11), CD62L (Mel-14), CD69 (H1.2F3), Vα2 (B20.1) Vα3.2 (RR3-16), Vβ3 (KJ25), Vβ5.1/5.2 (MR9-4), Vβ8 (F23.1), Vβ11 (RR3-15), Vβ12 (MR11-1) and anti-rat IgG2a (RG7/1.30) These antibodies were purchased from either BD Pharmingen (San Diego, CA) or eBioscience (West San Diego, CA). For flow cytometry analysis, cells were incubated with antibodies on ice for 30 min followed by extensive washing; Cells were analyzed on a FACSCalibur cytometer (Becton Dickinson, Mountain View, CA, USA).

3.4 Bone Marrow Chimeras

Bone marrow cells were prepared by flushing donor mice femur and tibia bones with Phosphate Buffered Saline (PBS). Recipient mice were lethally irradiated (1000 rads) and reconstituted with $10x10^6$ bone marrow cells by intravenous injection. Engraftment took place over a six to eight week period. For the thymic experiments I used 2D2 TCR transgenic mice as a basic model and generated three types of bone marrow chimeras. $2D2^{+}CD24^{-/-}$ > $CD24^{-/-}$: bone

marrow cells from 2D2^{+}CD24$^{-/-}$ mice were injected into irradiated CD24$^{-/-}$ mice resulting in bone marrow-derived (macrophages, dendritic cells, and T cells) and thymic stroma cells deficient in CD24 expression. 2D2^{+}CD24$^{-/-}$ > CD24$^{+/+}$: bone marrow cells from 2D2^{+}CD24$^{-/-}$ mice were injected into irradiated CD24$^{+/+}$ resulting in bone marrow-derived cells devoid of CD24 while thymic stroma expressed CD24. 2D2^{+}CD24$^{+/+}$ > CD24$^{-/-}$: bone marrow cells from 2D2^{+}CD24$^{+/+}$ mice were injected into irradiated CD24$^{-/-}$ resulting in bone marrow-derived cells expressing CD24 while thymic stroma do not. For the CNS experiments we used CD24$^{-/-}$ mice as our basic model and generated two types of bone marrow chimeras WT> CD24$^{-/-}$: bone marrow-derived cells from WT mice were injected into irradiated CD24$^{-/-}$ mice resulting in bone marrow-derived cells devoid of CD24 while CNS expressed CD24. WT > WT: bone marrow-derived cells from WT mice were injected into irradiated WT mice resulting in CD24 on both bone marrow-derived cells and the CNS.

3.5 Real Time RT-PCR

Total RNA was isolated from thymi or stroma enriched thymi using the Trizol method (Invitrogen). The first strand cDNA of each sample was synthesized using a reverse transcription kit (Invitrogen). Quantitative real time PCR was performed using an ABI Prism 7900-HT sequence system (PE Applied Biosystems) with the QunatiTect SYBR Green PCR kit (Qiagen) in accordance with the manufacturer's instructions. The following primers were used: mMOG.F: 5'-GCAGCACAGACTGAGAGGAA-3'; mMOG.R: 5'-CAGATGATCAAGGCAACCAG-3';

IL4.F: 5'-TGT ACCAGGAGCCATATCCA-3'; IL4.R: 5'-TTCTTCGTTGCTGTGAGGAC-3'; IL10.F: 5'-ACAGCCGGGAAGACAATAAC-3'; IL10.R: 5'-CAGCTGGTCCTTTGTTTGAA-3'; IL12b.F: 5'-TTGCTGGTGTCTCCACTCAT-3'; IL12b.R: 5'-GGGAGTCCAGTCCACCTCTA-3'; IL17.F: 5'-CCTCCAGAATGTGAAGGTCA-3'; IL17.R:5'-CTATCAGGGTCTTCATTGCG-3'; IFNγ.F: 5'-AGCTCTTCCTCATGGCTGTT-3'; IFNγ.R, 5-TTTGCCAGTTCCTCCAGATA-3'; TNFα.F: 5'-ATGAGAAGTTCCCAAATGGC-3'; and TNFα.R: 5'-CTCCACTTGGTGGTTTGCTA-3'; NG2.F: 5'-GCTGAGGTAAATGCTGGGA-3'; NG2.R: 5'-GCATCGAAAGACACCATCAC-3'; DCX.F: 5'-CCTTGGATGAGAATGAATGC-3', DCX.R: 5'-TGAGTCAGCTGGAGACTTGC-3'; P8.F: 5'-AGACGGAGCTGGAGATAAGG-3', P8.R: 5'-AGGCCTAGGTCCTGCTTACA-3'; HPRT.F: 5'-AGCCTAAGATGAGCGCAAGT-3'; and HPRT.R: 5'-TTACTAGGCAGATGGCCACA-3'. The hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was amplified and served as endogenous control. PCR was performed using an optimal condition. 1 μl of first strand cDNA product was amplified with platinum Taq polymerase (Invitrogen) and gene-specific primer pairs. Each sample was assayed in triplicate and experiments were repeated twice. The relative amounts of mRNA were calculated by plotting the Ct (cycle number), and average relative expression was determined by the comparative method ($2^{-\Delta\Delta Ct}$).

3.6 BrdU Incorporation Assay

At day 19 after EAE induction, mice were given an interaperitonal i.p. injection of 1mg of BrdU (BD Pharmingen) . Brains and spinal cords of recipient mice were

harvested 12 hrs later. The CNS-infiltrating monoculear cells were isolated and stained for cell surface makers, followed by intracellular stained with anti-BrdU ab (BD Pharmingen).

3.7 Proliferation Assay

Splenocytes from each strain of TCR transgenic mice (2D2, OTII) with or without CD24 were stimulated with titrated peptide antigens in 96-well U-bottomed plates. In some experiments, we purified CD4 cells from spleen and lymph nodes and stimulated them with peptide antigens and irradiated (2000 rad) syngenic APCs. ^{3}H-Thymidine was added into the culture at 48 h and harvested 12 h later. ^{3}H-incorporation was measured with a scintillation counter. The OVA peptide antigen used in the assay (OVA $_{323-33}$) was purchased from Sigma (St. Louis, MO). MOG peptide (MOG_{35-55}) (MEVGWYRSPFSRVVHLYRNGK) was purchased from Genemed Synthesis, Inc (South San Francisco, CA).

3.8 Purification of 2D2 T cells from $2D2^{+}CD24^{+/+}$ or $2D2^{+}CD24^{-/-}$ mice

2D2 T cells were purified by negative selection. Briefly, spleen and lymph node cells from 2D2 TCR transgenic mice were incubated with a cocktail of mAbs (anti-CD8, mAb TIB210, anti-FcR mAb 2.4G2 and anti-CD11c mAb N418). After removing the unbound antibodies, the cells were incubated with anti-Ig coated magnetic beads (Dynal Biotech). A magnet was used to remove the Ab-coated cells. The remaining cells were $CD4^{+}$ or $CD4^{-}CD8^{-}$ T cells. The purified 2D2 T cells were used for the proliferation assay.

3.9 Induction and Assessment of EAE

The immunogen, myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (MEVGWYRSPFSRVVHLYRNGK), was purchased from Genemed Synthesis. C57BL/6 Mice of 8-12 wks of age were immunized s.c. with 200 μg of MOG peptide in CFA (containing 400 mg of *Mycobacterium tuberculosis*) in a total volume of 100μl. They received 200 ng of pertussis toxin (List Biological Laboratories) in 200 μl of PBS in the tail vein immediately after the immunization and again 48 hr later. 2D2 TCR transgenic mice (8 – 12 weeks of age) of different CD24 genotypes received 200 ng of Pertussis toxin (List Biological, Campbell, CA, USA) in 200 μL PBS in the tail vein at day 0 and again 48 hours later. Adoptive transfer induction of EAE was accomplished by immunizing s.c. 8 – 12 wk C57BL/6j mice with 100 μg of MOG peptide in CFA in a total volume of 100 μl. Ten days after immunization, draining lymph nodes were harvested and stimulated at a density of $2x10^6$ml in Click's Eagle's Hanks' amino acid medium supplemented with 15% Fetal Calf Serum (FCS), 20 ng /ml rIL-12 and 50 μg/ml MOG peptide for 4 days. MOG-activated lymphoid cells measuring $30x10^6$ were injected i.p. into each recipient mouse that had been irradiated (350 rad) 1 hr earlier. The mice were observed every day and were scored on a scale from 0 – 5 with graduations of 0.5 for intermediate scores: 0 – no clinical signs; 1 – loss of tail tone; 2 partial paralysis of hind leg; 3 – hind limb paralysis; 4 – moribund; and 5 – death.

3.10 Histology

Mice were sacrificed by inhaling $C0_2$. Spinal cords, cerebellum and optical nerves were removed and fixed in 10% formalin/PBS. Paraffin sections were prepared and stained by the histology core facilities of the Department of Pathology (The Ohio State University) for hematoxylin and eosin (H&E) and luxol fast blue (myelin staining). To calculate the extent of spinal cord pathology in mice, an interactive digital analysis system and camera lucida attached to a photomicroscope (Zeiss) were used. The percentages of spinal cord inflammation and demyelination per mouse were calculated by first determining the total white matter area for all spinal cord sections by manually tracing the regions. Next the areas of spinal cord inflammation were determined by manually tracing each of the sections. Pathological changes of each spinal cord were scored as follows: 0 – no changes; 1 – focal area involvement; 2 – less than 5% total myelin area involvement; 3 – 5% to 10% of total myelin area involvement; 4 – 10% to 20% of total myelin area involvement; and 5 – greater than 20% of total myelin area involvement.

3.11 Immunofluorescence and Confocal Laser Scanning Microscopy

The Abs used for immunofluorescence staining of spinal cord sections were the following: biotinylated CD24 (M1/69: BD Pharmingen); Alexa-633-streptavidin (Molecular Probes); FITC-rat anti-mouse CD24 (M1/69: BD Pharmingen); Alexa-488-GFAP (Molecular Probes); Alexa-488-Mouse-anti-neuron Nuclei (Molecular Probes); Alexa-488-IB4 (Molecular Probes); and biotinylated anti-CNPase

(Chemicon International). Spinal cord sections were harvested and frozen in Tissue-Tek OCT medium (Sakura Finetek), and 10 μm thick slices were cut. Tissue sections were fixed in 1% paraformaldehyde and were blocked with 10% normal mouse serum fro 1 hr at 24°C Sections were then stained with the corresponding fluorescent Abs for another 2 hr at 24°C. After washing with PBS, slides were mounted with Fluoromount G mounting medium (Southern Biotechnology Associates) and were examined on a confocal laser-scanning microscope (Zeiss 510)

3.12 Preparation of Astrocytes from Newborn Mice and Proliferation Assay

Primary glial cell cultures were prepared from brains of newborn Astro^{CD24} or CD24$^{-/-}$ mice as described previously (15, 87, 88). After removal of the meninges, the brains were dissociated mechanically by nylon sieves. The cells were seeded in Delbecco's Modified Eagle's Medium (DMEM) containing 20% FCS in 75cm^2 tissue culture flasks. On day 4, the medium was replaced with DMEM containing 10% FCS and subsequently changed every 3 days thereafter. The firmly adherent cells were stained with anti-GFAP ab to confirm their identity as astrocytes. To assess the Ag-presenting functions of cultured astrocytes, the purified MOG-specific 2D2 T cells were used as a responder. In brief, irradiated (3000 rad) astrocytes were cultured in round-bottom microtiter plates in 200 μl of DMEM containing 10 U/ml IFNγ. Three days later, the medium was removed, and 5x10^4 T cells in Click's Eagle's Hanks' amino acid medium and the indicated

concentrations of MOG peptide were added into each well. After 48 hr, the cultures were pulsed with 1 μCi/well ^{3}H for another 12 hr and incorporation for ^{3}H was measured in a liquid scintillation beta plate counter.

3.13 Statistical Analysis.

Statistical analysis was conducted using Microsoft Excel 2007 software. Cell quantity comparison was analyzed by the Student's t test. Differences were considered significant at $P \leq 0.05$, and P values are shown were applicable.

CHAPTER 4:

CD24 AND THYMIC GENERATION OF MYELIN ANTIGEN SPECIFIC T CELLS

4.1 Thymic clonal deletion of MOG-specific T cells in CD24-deficient mice

Kuchroo and colleagues(83) have produced 2D2 TCR transgenic mice, whose TCR recognizes MOG_{35-55}, the pathogenic epitope in the C57BL/6 mice. Since MOG specific transgenic T cells developed normally in C57BL/6 mice, and other groups have demonstrated MOG mRNA expression in the thymus (89, 90) or thymic epithelial cells (91), we tested if MOG is ectopically expressed in the thymus of C57BL/6 mice. Significant expression of MOG mRNA was detected in the thymus by real time PCR **(Figure 3**A**)**. Thus, 2D2 T cells have escaped clonal deletion despite expression of MOG in the thymus. Furthermore, the targeted mutation of CD24 caused a massive reduction of thymic cellularity in the 2D2 transgenic mice but not in $CD24^{+/-}$ or $CD24^{-/-}$ mice without 2D2 TCR transgene (**Figure 3**B). To determine whether the thymus reduction was related to age, we examined young mice (18-20 days after birth) and observed similar thymus size reduction in $2D2^{+}CD24^{-/-}$ mice (data not shown). Correspondingly, numbers of $\alpha\beta$-positive 2D2 T cells and the $CD4^{+}CD8^{+}$ and $CD4^{+}CD8^{-}$ populations were dramatically reduced in the thymi of the $2D2^{+}CD24^{-/-}$ mice compared with that of the $2D2^{+}CD24^{+/+}$ mice (**Figure 3**C).

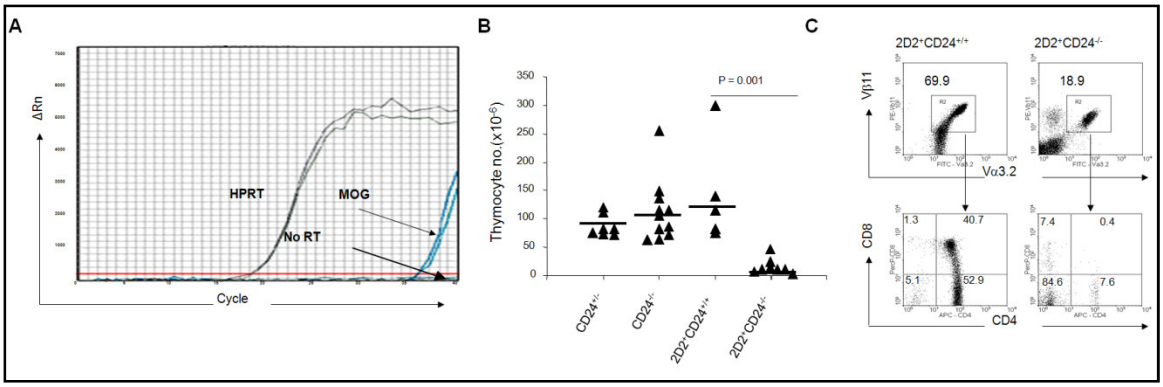

Figure 3: CD24 inhibits thymic deletion of MOG-specific T cells.
A) MOG antigen is expressed in the thymi of C57BL6 mice. A standard 40-cycle realtime PCR was used to detect MOG mRNA expression in the thymus. Data shown represent three experiments with similar results. B) Thymocyte cellularity. Each triangle represents the value from a single mouse. Thick lines represent median numbers of each group of mice. Student's t test was used for the comparison. C) Impact of CD24-deficiency on the development of 2D2 TCR transgenic mice. Thymocytes from $2D2^{+}CD24^{-/-}$ and $2D2^{+}CD24^{+/+}$ mice were stained for Vα3.2, Vβ11, CD4 and CD8. The thymi from $2D2^{+}CD24^{-/-}$ mice had dramatically reduced $V\alpha3.2^{+}V\beta11^{+}$ populations and failed to generate $CD4^{+}CD8^{+}$ and $CD4^{+}CD8^{-}$ T cells compared with that of $2D2^{+}CD24^{+/+}$ mice. Data represent at least five experiments with similar results.

4.2 Restoration of CD24 on thymocytes is insufficient to rescue clonal deletion of autoreactive T cells; CD24 on radio-resistant thymic stromal cells is necessary

Since the majority of thymocytes expressed high levels of CD24, we tested the possibility that CD24 expressed on thymocytes may be responsible for the enhanced clonal deletion. We generated $2D2^{+}CD24^{-/-}$ mice that express CD24 under the control of proximal lck promoter. The lck promoter was known to be active in the thymocytes, starting at the DN3 stage (92). Essentially all 2D2 TCR-expressing thymocytes in the CD24 transgenic mice expressed high levels of CD24 (**Figure 4**A**)**. Nevertheless, the thymic cellularity (**Figure 4**B) and the

distribution of T cell subsets (**Figure 4**C) were unaffected by CD24 expression in the thymocytes. These results demonstrate that lacking CD24 in the thymocytes is not solely responsible for enhanced clonal deletion.

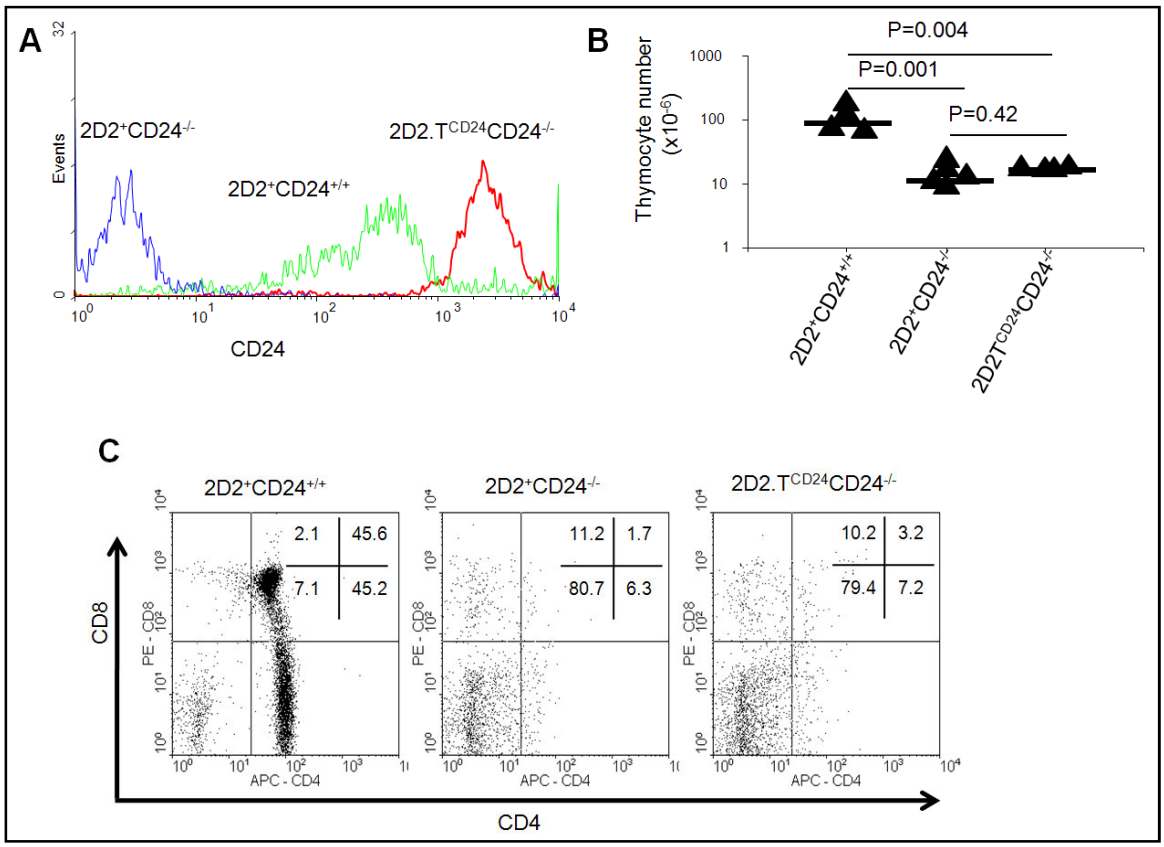

Figure 4: Restoration of CD24 on 2D2 T cells in CD24-deficient mice does not prevent its thymic deletion.

We produced double transgenic mice by breeding $2D2^{+}CD24^{-/-}$ mice with $T^{CD24}CD24^{-/-}$ mice (mice with CD24 expression on T cells only) and produced CD24 deficient mice with CD24 expression on T cells alone ($2D2.T^{CD24}CD24^{-/-}$ mice). A) Phenotypes of three different mice identified by flow cytometry. Single cell suspensions of thymocytes were stained for CD24 and Vα3.2 markers followed by flow cytometry analysis. Data shown were gated on Vα3.2-positive thymocytes. B) Thymocyte numbers in mice with different genotypes. No significant difference was observed between $2D2^{+}CD24^{-/-}$ mice with $2D2.T^{CD24}CD24^{-/-}$ mice. C) Thymocytes from $2D2.T^{CD24}CD24^{-/-}$ mice show a phenotype similar to that seen in $2D2^{+}CD24^{-/-}$ mice.

To determine whether CD24 expressed on bone marrow-derived or non-bone marrow-derived stromal cells is responsible for the enhanced clonal deletion, we established chimeras using bone marrow from $2D2^{+}CD24^{+/+}$ or $2D2^{+}CD24^{-/-}$ mice to reconstitute lethally irradiated WT or CD24-deficient mice. CD24 expression on thymocytes in chimeric mice that received BM from CD24 deficient donors (**Figure 5**A red ($2D2^{+}CD24^{+/+}$ > $CD24^{+/+}$) and blue ($2D2^{+}CD24^{+/+}$ > $CD24^{-/-}$) lines) indicates a nearly complete replacement of the hematopoietic compartment in the chimera mice. In spleens of mice, we detected intermediate to high levels of CD24 expression on $CD11c^{+}$ cells from $2D2^{+}CD24^{+/+}$ > $CD24^{-/-}$ chimeras but not on $CD11c^{+}$ cells from $2D2^{+}CD24^{-/-}$ > $CD24^{+/+}$ chimeras (**Figure 5**B). Thus, the chimeric mice can be used to evaluate the contribution of CD24 in clonal deletion of 2D2 T cells. Mature 2D2 T cells (CD4-single positive) were generated in $2D2^{+}CD24^{+/+}$ > $CD24^{+/+}$ chimeras but not in $2D2^{+}CD24^{-/-}$ > $CD24^{-/-}$ chimeras (**Figure 5**C & D, left and right panels). CD24 expression on the radio-resistant stromal cells (**Figure 5**C & D**,** middle left panel), but not on bone marrow-derived cells (**Figure 5**C & D**,** middle right panel), rescued transgenic $TCR\alpha\beta^{+}$ cells at the $CD4^{+}CD8^{+}$ stage; however, since no mature $CD4^{+}$ 2D2 T cells were generated (**Figure 5**D, middle left), it is likely that CD24 expression on radio-resistant thymic stromal cells alone is not sufficient for rescuing 2D2 T cell deletion. Thus, these bone marrow chimera data suggest that CD24 expression on radio-resistant stromal cells inhibits deletion of transgenic T cells at the $CD4^{+}CD8^{+}$ stage.

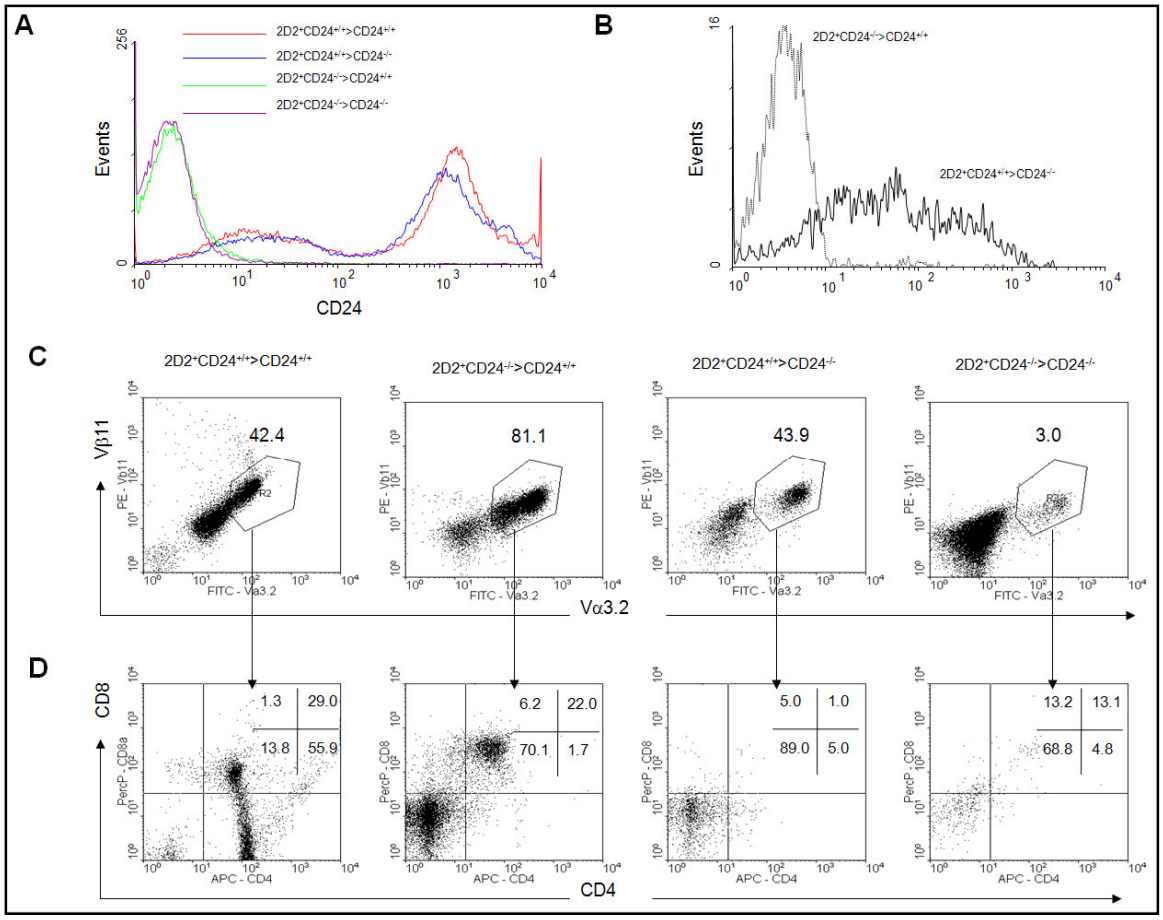

Figure 5: CD24 on radio-resistant thymic stromal cells is necessary but not sufficient for the generation of mature CD4 T cells specific for MOG peptide.

$CD24^{-/-}$ and $CD24^{+/+}$ mice were lethally irradiated (1000-Rad) and reconstituted with $2D2^{+}CD24^{-/-}$ or $2D2^{+}CD24^{+/+}$ donor bone marrow cells. A) CD24 expression on the thymocytes from different bone marrow chimeric mice. B) CD24 expression on splenic $CD11c^{+}$ cells from different bone marrow chimeras. Splenocytes were digested with collagenase IV, and the resulting mononuclear cells were stained for CD11c and CD24 followed by flow cytometry analysis. Data shown were gated on CD11c-positive cells. C) Generation of 2D2 T cells in the thymi of bone marrow chimeras. Thymocytes were stained for Vα3.2, Vβ11, CD4 and CD8 followed by flow cytometry analysis. D) 2D2 T cell subsets in bone marrow chimeras. Data shown were gated on $V\alpha3.2^{+}V\beta11^{+}$ thymocytes. The result shown represents three independent experiments with similar results.

Since thymic epithelial cells (TECs), especially medullary epithelial cells (mTECs), are the major non-hematopoietic cells that are involved in negative selection (91, 93-95), we analyzed expression of CD24 on TECs. More than 50% of CD45-negative thymic stromal cells express CD24 (**Figure 6A**,); almost 100% of medullar epithelial cells ($B7^{+}$) express CD24. These data are consistent with a role of CD24 on radio-resistant thymic epithelial cells. To determine whether CD24-deficiency effects expression of MOG antigen in the thymus, we compared MOG mRNA expression in the thymus and enriched thymic stromal cells. Similar levels of MOG-transcripts were detected in WT and CD24-deficient thymi (**Figure 6B)**, regardless of whether RNA from whole thymus extract or from enriched stromal cells were compared. As such, the function of CD24 is unlikely through regulation for expression of peripheral antigens in the thymus.

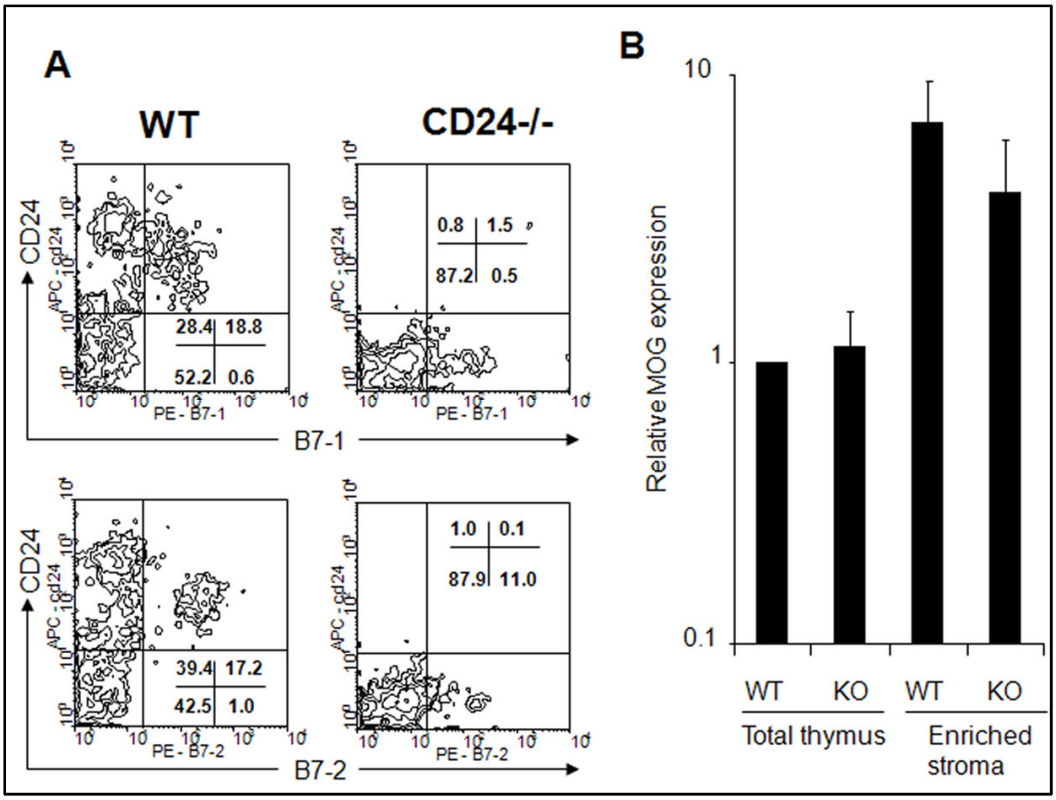

Figure 6: Expression of CD24 and MOG in thymic stromal cells.
A)Thymi from $CD24^{-/-}$ and $CD24^{+/+}$ C57BL6 mice were digested with collagenase, cell suspensions were enriched for stromal cells and were then stained for CD45, CD24 and B7-1 or B7-2 markers. Data shown were gated on $CD45^{-}$ cells. B) Real time PCR was used to examine MOG gene expression in total thymocytes and enriched thymic stromal cells. Three experiments were performed with similar results.

4.3 CD24-deficiency does not alter development of T cells specific for foreign antigen

As a comparison to the development of autoreactive transgenic T cells, we also studied development of transgenic T cells specific for ovalbumin (OTII – Vα2 Vβ5) in WT and CD24-deficient host. The TCR distribution (**Figure 7**A), thymic cellularity (**Figure 7**B) and subset distribution (**Figure 7**C) are similar in $OTII^{+}CD24^{+/+}$ and $OTII^{+}CD24^{-/-}$ transgenic mice. Moreover, the OTII T cells in the periphery are fully functional (**Figure 7**D). These results demonstrate that CD24 does not have a general effect on the development of all transgenic T cells. Moreover, since the development of the OTII T cells requires positive selection (96), our data emphasize that the CD24 gene does not regulate positive selection.

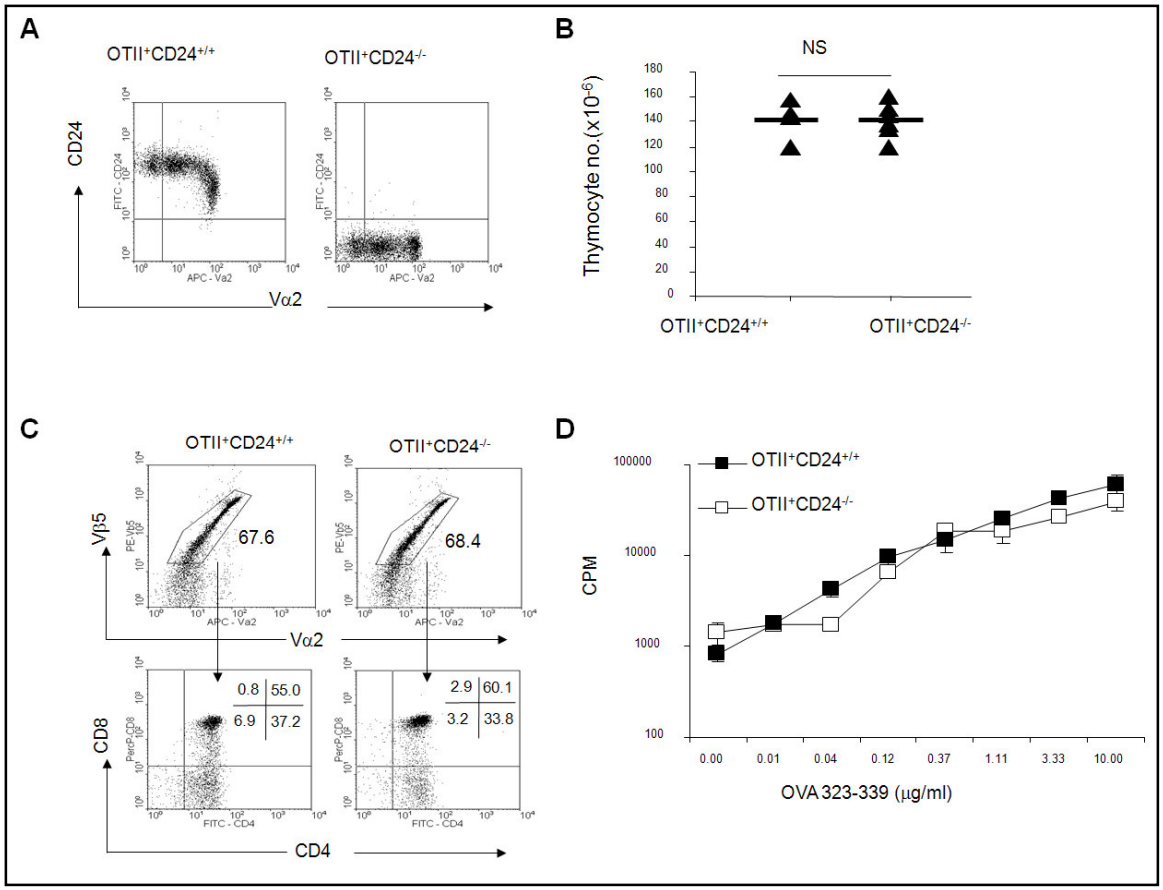

Figure 7: CD24 expression is not required for the thymic generation of CD4 T cells specific for ovalbumin (OVA).

OTII TCR transgenic mice were bred with $CD24^{-/-}$ mice for two generations. The resulting $OTII^{+}CD24^{-/-}$ mice were compared with wild type mice for thymocyte development. A) CD24 expression in the thymocytes of OTII TCR transgenic mice with different genotypes. B) Summary of thymocyte numbers in mice of different genotypes. C) Flow cytometry analysis of the thymi of OTII TCR transgenic mice with or without CD24. $OTII^{+}CD24^{+/+}$ and $OTII^{+}CD24^{-/-}$ mice revealed similar generation of OTII T cells. D) Splenocytes from $OTII^{+}CD24^{+/+}$ and $OTII^{+}CD24^{-/-}$ mice show similar proliferation in response to OVA peptide. Data presented in **A**, **C and D** represent five independent experiments with similar results.

4.4 CD24-deficiency increases the efficiency of clonal deletion of viral super antigen (VSA)-reactive T cells in the thymus

BALB/c mice have integrated mouse mammary tumor provirus types 6, 8, and 9 into their genome and are subject to negative selection. As a result, the majority of T cells expressing Vβ3, Vβ5, Vβ11, and Vβ12 have been deleted (97, 98). The VSAs react with the variable regions of the TCR β chain (Vβ). Since T cells expressing any given Vβ can be monitored by flow cytometry, quantitation of the frequencies of these T cells is a useful assay available to evaluate clonal deletion in mice without a transgenic TCR. We therefore generated $CD24^{-/-}$BALB/c mice by five generations of marker-assisted backcross to achieve nearly 100% of the BALB/c genome. The $CD24^{+/-}$BALB/c were intercrossed, and the $CD24^{+/+}$ and $CD24^{-/-}$ littermates were compared for the Vβ3, 5, 8, 11 and 12 in order to determine whether CD24-deficiency increased the efficacy of clonal deletion. VSA-reactive T cells among CD4 or CD8 single positive thymocytes from $CD24^{+/+}$ or $CD24^{-/-}$ BALB/c mice were quantitated (**Figure 8**A). We observed significantly reduced frequencies of Vβ3, 5, 11 and 12 positive CD4 single positive thymocytes (**Figure 8**B); Vβ3 and 12 positive CD8 single positive thymocytes were also significantly reduced (**Figure 8**B). These data demonstrate that CD24-deficiency increased the efficiency of clonal deletion of VSA-reactive T cells. Therefore, the function of CD24 is not limited to MOG-specific autoreactive T cells. Furthermore, it is physiologically relevant as they are not an artifact of transgenic αβ expression.

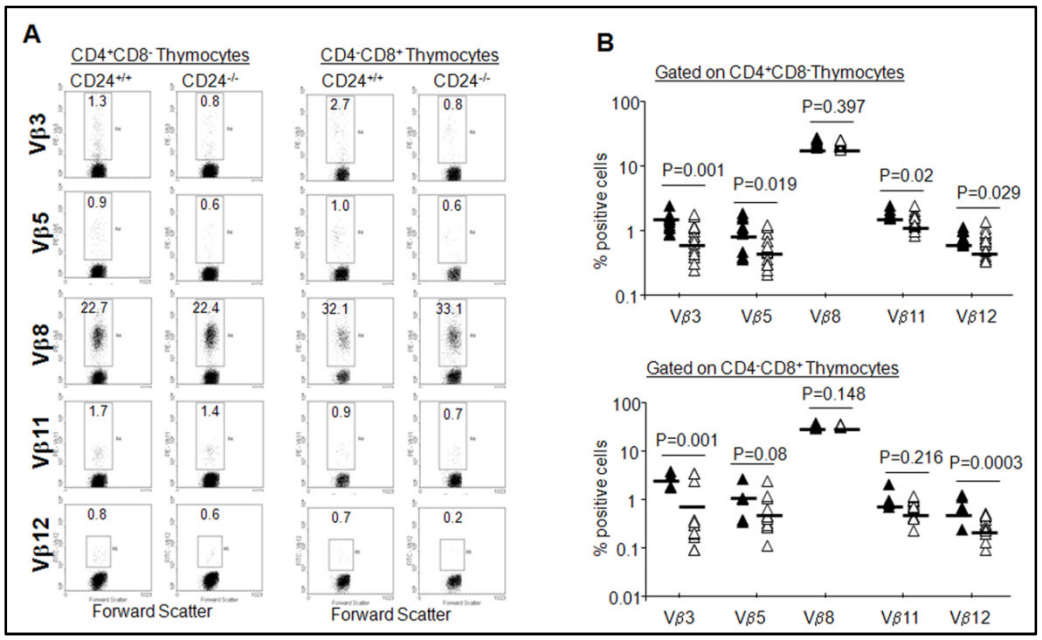

Figure 8: Frequencies of viral super antigen reactive T cells are reduced in CD24$^{-/-}$BALB/c mice.

CD24$^{+/-}$BALB/c mice were bred with CD24$^{+/-}$BALB/c mice and CD24$^{+/+}$BALB/c and CD24$^{-/-}$BALB/c mice were generated. Thymocytes from mice (4-5 weeks old) were stained for CD4, CD8 and a single Vβ. Frequencies of each type of Vβ^{+} cells (Vβ3, 5, 8, 11, 12) were quantitated. A total of 200,000 cells were harvested for each sample. Students' t test was used for statistical analysis. A) representative flow cytometry profile of CD4^{+}CD8^{-} and CD4^{-}CD8^{+} thymocytes from one pair of mice is shown. B) Summary of frequencies of VSAg-reactive CD4^{+}CD8^{-} and CD4^{-}CD8^{+} thymocytes. Each triangle represents a number from a single mouse. A closed triangle (▲) represents a number from CD24$^{+/+}$BALB/c mouse; an open triangle (△) represents a number from CD24$^{-/-}$BALB/c mouse. Student's t test was used for the comparison.

4.5 CD24-deficiency nullifies pertussis toxin-induced experimental autoimmune encephalomyelitis

In order to understand the immunological consequence of CD24-mediated clonal deletion for autoimmune diseases, we compared the function and pathogenicity of 2D2 T cells developed in the presence or absence of CD24 gene. In spleens of $2D2^{+}CD24^{+/+}$ mice, the $V\alpha3.2^{+}V\beta11^{+}$ population of cells are mainly $CD4^{+}$, while the $V\alpha3.2^{+}V\beta11^{+}$ cells from $2D2^{+}CD24^{-/-}$ mice do not express CD4 (**Figure 9**A), thus, no mature 2D2 T cells ($CD4^{+}V\alpha3.2^{+}V\beta11^{+}$) are present in the spleens of $2D2^{+}CD24^{-/-}$ mice (**Figure 9**B). Immunostaining of the $V\alpha3.2^{+}V\beta11^{+}$ T cells from $2D2^{+}CD24^{-/-}$ mice suggests that the majority of the transgenic T cells are of a naïve phenotype, as is reflected by high expression of CD62L and low or no expression of CD25 and CD69 (**Figure 9**C). TCR expression levels on 2D2 T cells from $2D2^{+}CD24^{-/-}$ mice were down-regulated compared to 2D2 T cells from $2D2^{+}CD24^{+/+}$ mice (**Figure 9**C). Thus it is likely that those TCR-positive T cells in the peripheral lymphoid organs of $2D2^{+}CD24^{-/-}$ mice were anergic.

Functional analysis suggests that splenocytes from $2D2^{+}CD24^{-/-}$ mice failed to respond to MOG peptide stimulation whereas splenocytes from $2D2^{+}CD24^{+/+}$ mice had a vigorous proliferative response to MOG peptide even at (1.0 – 10 μg) (**Figure 10**A). This difference is not attributable to co-stimulatory activity of CD24 on APC as purified T cells from WT and $CD24^{-/-}$ 2D2 transgenic mice exhibit the same difference when WT APC were used (**Figure 10**B).

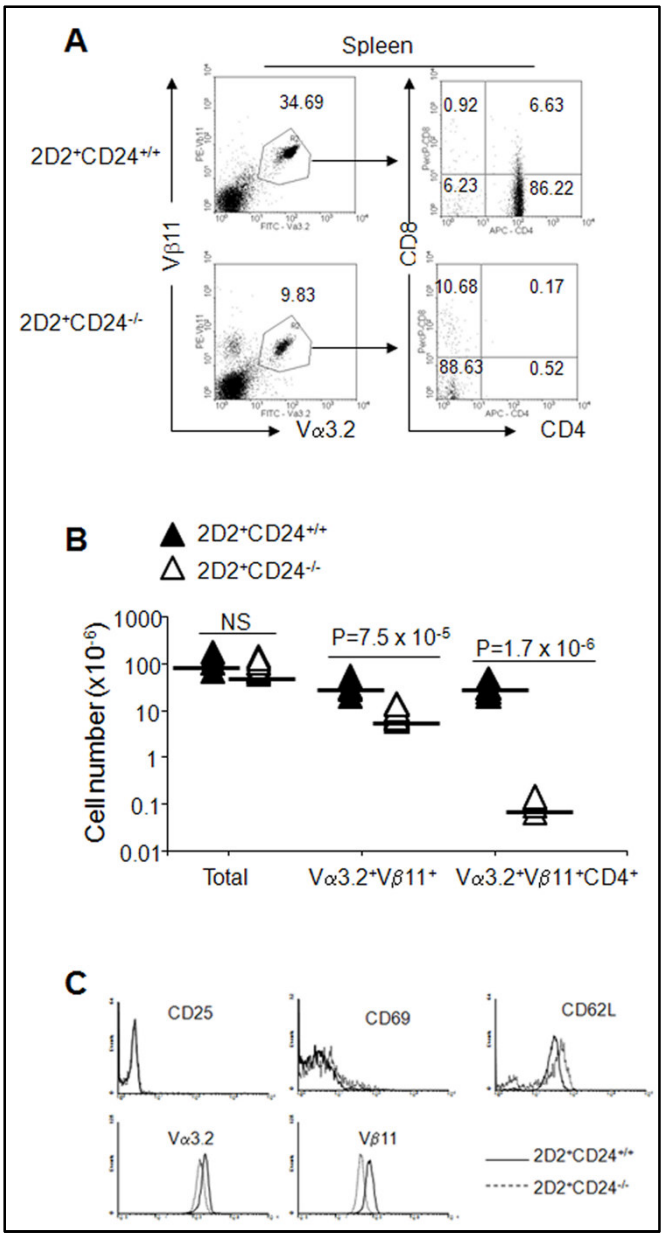

Figure 9: Phenotype analysis of 2D2 T cells in the peripheral lymphoid organs of mice with or without CD24.

A) Phenotypes of 2D2 T cells in the peripheral lymphoid organs. Splenocytes were stained for different cell surface markers and flow cytometry was used to analyze the stained splenocytes. Data represent at least five experiments with similar results. The $V\alpha3.2^{+}V\beta11^{+}$ cells in the peripheral lymphoid organs of $2D2{+}CD24^{-/-}$ mice are mainly $CD4^{-}CD8^{-}$. B) The peripheral lymphoid organs (spleen) of $2D2^{+}CD24^{-/-}$ mice contain no mature 2D2 ($CD4^{+}V\alpha3.2^{+}V\beta11^{+}$) T cells. Significant, but reduced numbers of $V\alpha3.2^{+}V\beta11^{+}$ cells were detected in $2D2^{+}CD24^{-/-}$ mice. Splenocytes were stained for different cell surface markers and numbers of each subset of cells were calculated. Each triangle denotes a number from one single mouse. Thick lines represent median numbers. Student's t test was used for statistical analysis. C) $V\alpha3.2^{+}V\beta11^{+}$ cells in the peripheral lymphoid organs of $2D2^{+}CD24^{-/-}$ mice are of a naïve phenotype with down-regulated TCR expression. Cell suspensions of spleen were stained for Vα3.2, Vβ11 and one of the other cell surface markers. Data shown were gated on $V\alpha3.2^{+}V\beta11^{+}$ cells. Three independent experiments were done with similar results.

2D2 T cells are encephalitogenic, as the injection of pertussis toxin induces EAE in 2D2 TCR transgenic mice (83). We therefore used this model to determine the immunological consequence of CD24-mediated escape of autoreactive T cells. Two doses of pertussis toxin at day 0 and day 2 induced severe EAE symptoms (**Figure 11**A) in 7 out of 10 $2D2^{+}CD24^{+/+}$ mice, with disease onset at about day 10, peaked at about day 18 and persisted over the observation period.

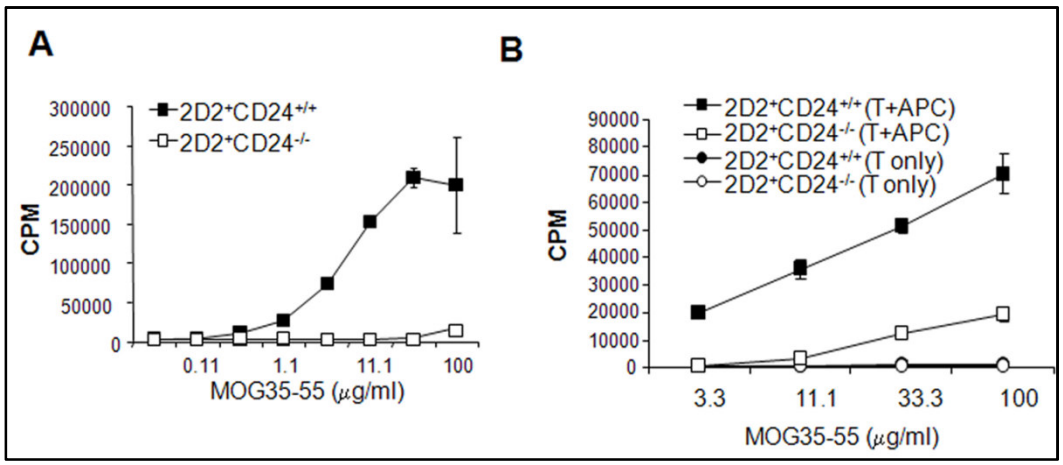

Figure 10: $V\alpha3.2^{+}V\beta11^{+}$ cells in the peripheral lymphoid organs of $2D2^{+}CD24^{-/-}$ mice failed to proliferate in response to their cognate peptide antigen

Splenocytes from mice with different genotypes were cultured in the presence of titrated MOG peptide. ^{3}H-Tridium assay was used to quantify DNA synthesis in response to MOG peptide stimulation. A) Splenocytes from $2D2^{+}CD24^{+/+}$ mice proliferated vigorously to MOG peptide stimulation, while splenocytes from $2D2^{+}CD24^{-/-}$ mice failed to respond to MOG peptide. B) Purified 2D2 T cells from $2D2^{+}CD24^{-/-}$ mice failed to respond to MOG antigen. 2D2 T cells from $2D2^{+}CD24^{-/-}$ and $2D2^{+}CD24^{+/+}$ mice were purified from spleens by negative selection using Dynabeads. Equal numbers of purified 2D2 T cells were then used as responders, irradiated splenocytes were used as antigen presenting cells (APC). Since the two lines representing purified T cells only are overlapping, only three lines can be seen in this figure. Data presented in **A** and **B** represents three independent experiments with similar results.

In contrast, only 1 out of 10 $2D2^{+}CD24^{-/-}$ mice that received the same dose of pertussis toxin developed EAE. Histology analysis further revealed that inflammatory cells infiltrated the cerebellum and spinal cords of $2D2^{+}CD24^{+/+}$ mice and caused demyelination, while the cerebellum and spinal cords of $2D2^{+}CD24^{-/-}$ mice were largely devoid of inflammation and demyelination (**Figure 11**B, C). We also detected severe inflammation in the optic nerves of $2D2^{+}CD24^{+/+}$ mice but not in optic nerves of $2D2^{+}CD24^{-/-}$ mice (**Figure 11**D). Therefore, CD24-mediated escape of clonal deletion preserved autopathogenic T cells that can potentially cause autoimmune diseases under the condition of inflammatory insults.

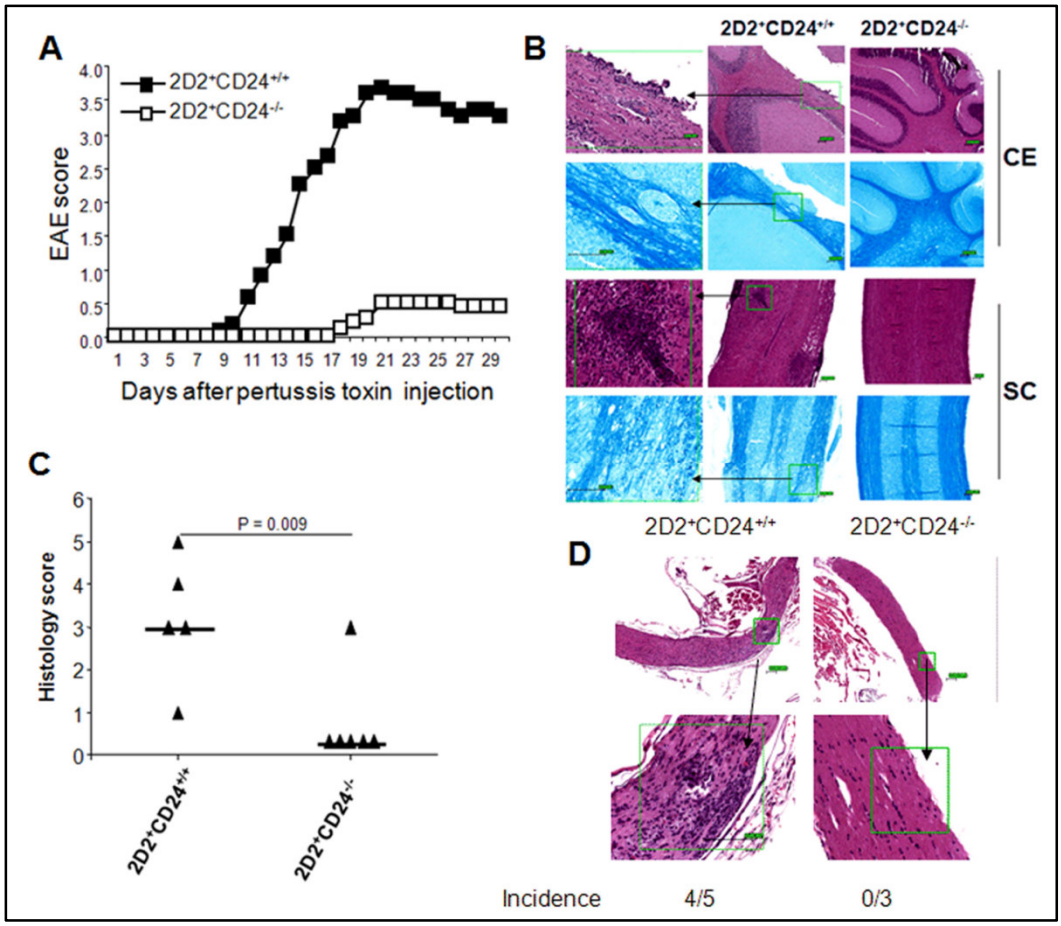

Figure 11: CD24-deficiency prevents EAE in 2D2 TCR transgenic mice.

We induced EAE in mice with different genotypes by pertussis toxin injection (200 ng i.v. on day 0 and 2). A) $2D2^{+}CD24^{+/+}$ mice developed severe EAE while $2D2^{+}CD24^{-/-}$ mice show no signs of EAE, with the sole exception of one $2D2^{+}CD24^{-/-}$ mouse which became severely disabled later. B) H&E and fast blue stainings of cerebellum and spinal cords revealed severe inflammation and demyelination in $2D2^{+}CD24^{+/+}$ but not in $2D2^{+}CD24^{-/-}$ mice. Blown up sections are shown to the left. C) Summary of histology scores in each group. The median score for $2D2^{+}CD24^{+/+}$ mice was 3.0 and only one $2D2^{+}CD24^{-/-}$ mouse reached a score of 3.0. D) H&E staining of the optical nerves revealed $2D2^{+}CD24^{+/+}$ but not $2D2^{+}CD24^{-/-}$ mice have inflammation in response to pertussis toxin (bottom row shows the blow up images).

CHAPTER 5:

CD24 ON THE CENTRAL NERVOUS SYSTEM ANTIGEN PRESENTING CELLS ENHANCES EFFECTOR FUNCTION OF MYELIN ANTIGEN SPECIFIC T CELLS.

5.1 Over-expression of CD24 in the CNS enhances EAE progression

Studies from our lab have shown that $CD24^{-/-}$ mice are resistant to T cell adoptive transfer-induced EAE (15). Bone marrow chimeric mice in which CD24 deficient recipients received WT donor cells (WT > $CD24^{-/-}$) become sensitive to the induction of adoptive transfer EAE, although the disease severity is significantly reduced compared to WT > WT chimeras. Both WT > $CD24^{-/-}$ and WT > WT chimeras developed EAE around day 10 after T cell transfer. The lower severity in WT > $CD24^{-/-}$ mice suggests that CD24 on the CNS parenchymal cells contributes to EAE development.

To understand how CD24 on the CNS cells contributes to EAE development, we have produced CD24-transgenic mice in which CD24 transgene expression is under the control of the GFAP promoter ($Astro^{CD24}$). See Materials and Methods chapter for transgene construct information. We obtained a strain of mice with abundant expression of CD24 in the CNS (**Figure 12**), in contrast to the CNS of non-transgenic mice. CD24 expression colocalized with astrocytes ($GFAP^{+}$), but

not with other CNS cells such as neurons (NeuN^{+}), microglia (IB4^{+}), nor oligodendrocytes (CNPase^{+}) (data not shown).

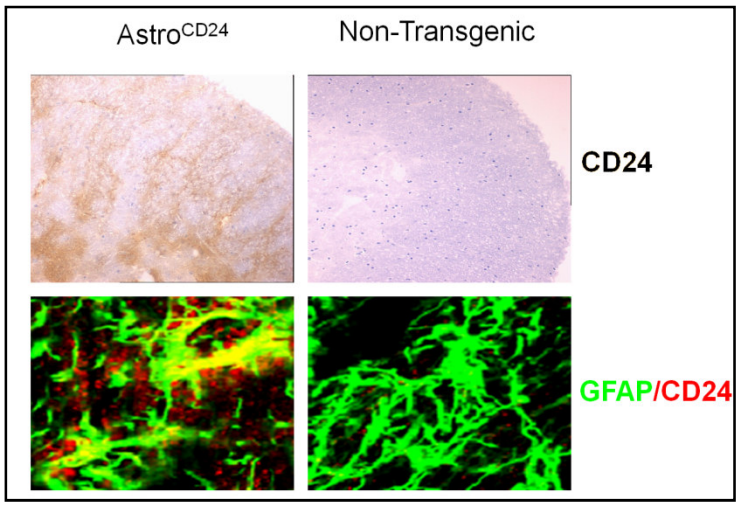

Figure 12: Production of Astro^{CD24} mice
The transgenic vector contained a 2.2-kb human GFAP promotor, 1.8-kb cDNA, for mouse CD24 and a fragment of the mouse protamine-1 gene that supplies an intron to stabilize 3'-UTR and a polyadenylation signal. The DNA used for microinjection of fertilized eggs from C57BL/6 mice is a 4.6-kb fragment. Confocal microscopy verified expression of CD24 in the spinal cords of Astro^{CD24} mice but not in non-transgenic WT mice. The Abs used for CD24 immunohistochemistry were biotinylated CD24 (M1/69; BD Pharmingen), followed by HRP-streptavidin (BD Pharmingen), and color was developed by using 3, 3'-diaminobenzidine as substrate. For confocal analysis, biotinylated CD24 and Alexa-633-streptavidin (Molecular Probes) were used to label CD24, and Alexa-488-GFAP (Molecular Probes) were used to label astrocytes. Spinal cord tissue sections (10 µm) were analyzed.

We induced EAE in Astro^{CD24} and non-transgenic littermates with MOG_{35-55} peptide and PTx. Disease symptoms were evident in both groups of mice within 2 weeks post immunization with MOG peptide and pertussis toxin peaking between days 17 and 25. Recover from paralysis after day 20 occurred in both groups of mice, however Astro^{CD24} mice did not recover as well, as paralysis in

Astro^{CD24} mice persisted throughout the observation period (**Figure 13**A). The mean accumulating scores and mean maximal scores were significantly different between these two groups ($p < 0.05$), while other parameters such as mean onset days and incidences were not (data not shown). Thus, Astro^{CD24} mice had enhanced EAE progression. Inflammatory lesions could be detected in all spinal cord histological sections in both groups of mice on day 50 (**Figure 13**B). Inflammatory infiltration crossing the gray matter of the spinal cord was detected in Astro^{CD24} mice (**Figure 13**B – Bottom Left), but in non-transgenic mice, infiltrates were located in proximity to menenges (**Figure 13**B – Top Left) and sometimes non-detectable in spinal cords. Large areas of demyelination could be detected in the spinal cords of Astro^{CD24} mice (**Figure 13**B – Bottom Middle), whereas only little or focal demyelination was detected in the spinal cords of non-transgenic mice (**Figure 13**B – Top Middle). Axonal staining of the spinal cord sections suggested that broad areas of axonal damage were present in Astro^{CD24} mice (**Figure 13**B – Bottom Right), while non-transgenic mice usually show axonal damage limited to the submeninges area(**Figure 13**B – Top Right). Quantitation of inflammation, demyelination, and axonal degeneration areas revealed significant differences between AstroCD24 and non-transgenic (**Figure 13**C). Thus, Astro^{CD24} mice suffered enhanced EAE progression with broad demyelination and axonal damage.

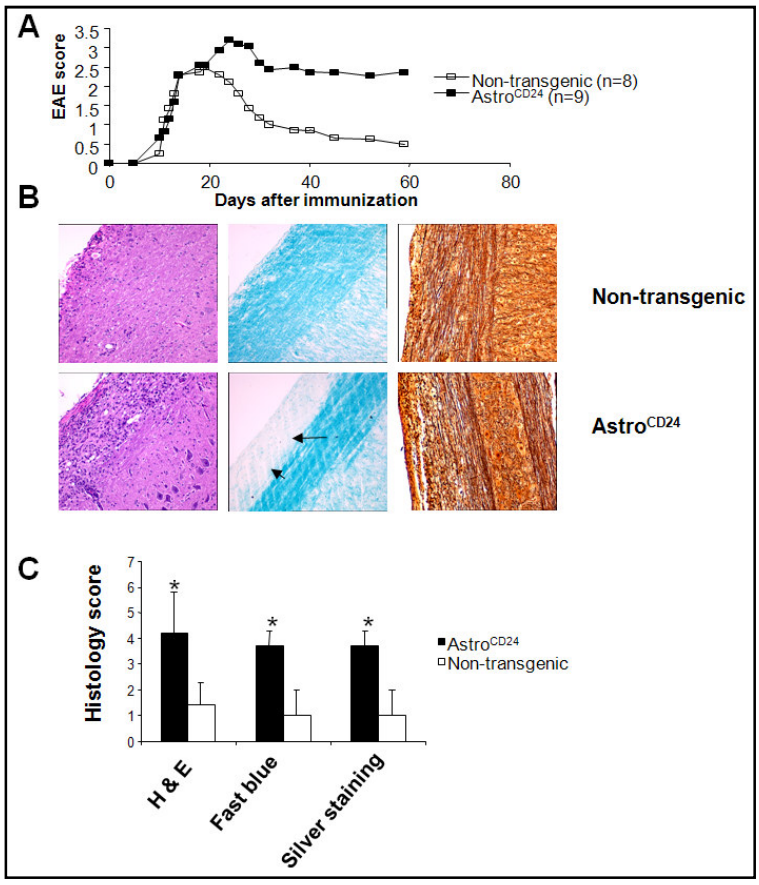

Figure 13: Astro^{CD24} mice had enhanced EAE progression
A) Astro^{CD24} mice and their nontransgenic littermates were immunized with MOG peptide and pertussis toxin. EAE signs were scored every 2–3 days. B) Histology of spinal cords from Astro^{CD24} mice and controls. At day 50, after EAE induction, mice were sacrificed, spinal cords were fixed in formalin, and tissues were processed for H&E (*left panel*), Luxol fast blue (*middle panel*), and Biechowsky silver stainings (*right panel*). Original photographs were taken at x200. C) Seminal quantitative summary of inflammation, demyelination, and axonal damage. To calculate the extent of spinal cord pathology in mice, an interactive digital analysis system and camera lucida attached to a photomicroscope (Zeiss) were used. The percentages of spinal cord inflammation, demyelination, and axonal damage per mouse were calculated by first determining the total white matter area for all spinal cord sections and by manually tracing the regions. Next, the areas of spinal cord inflammation, demyelination, and axonal damage were determined by manually tracing each of the sections. Pathological changes of each spinal cord were scored as follows: 0, no changes; 1, focal area involvement; 2, <5% of total myelin area involvement; 3, 5–10% of total myelin area involvement; 4, 10–20% of total myelin area involvement; and 5, >20% of total myelin area involvement. Inflammation was evaluated on H&E slides, TG = 5, NTG = 6; demyelination was evaluated on myelin-stained slides, n = 3 for both groups; axonal damage was evaluated on silver-stained slides, n = 3 for both groups. *, $p < 0.05$. Mann-Whitney U test was used for the comparison.

5.2 Enhanced EAE progression in Astro^{CD24} mice is associated with increased expression of pro-inflammatory cytokines in the CNS.

To evaluate if CD24 over-expression by astrocytes results in a T cell population more optimally primed for damaging affects, we measured cytokine gene expressions at day 20 (peak of EAE), and day 40 (EAE recovery) by using real-time PCR (RT-PCR). On day 20, the spinal cords of Astro^{CD24} mice had increases in the expression of IL17 ($p<0.01$) and decrease in the expression of IFNγ genes ($p < 0.01$) (**Figure 14)**. On day 40, the spinal cords of Astro^{CD24} mice IL17 and IFNγ continued to be elevated ($p<0.01$), while IL12, and TNFα also become elevated compared to WT ($p<0.05$).

Enhanced EAE signs and myelin/axonal damage in Astro^{CD24} mice may be a result of a more aggressive T cell population during EAE development. We compared expression of cytokine genes in the spinal cords of Astro^{CD24} mice with non-transgenic mice at day 20 (peak of EAE) and day 40 (EAE recovery) by using realtime PCR (RT-PCR). On day 20 (**Figure 14** Top Row), dramatic up-regulation of cytokine genes, including IL-10, IL-12, IL-17, IFN-γ, and TNF-α, but not IL-4, were detected in both groups of mice (**Figure 14**). IL-17 was significantly increased in the spinal cords of Astro^{CD24} mice ($p < 0.01$) compared to non-transgenic mice. Additionally IFN-γ significantly reduced in Astro^{CD24} mice ($p < 0.01$) compared to non-transgenic mice. No significant differences were detected among other cytokine genes between Astro^{CD24} and non-transgenic mice. On day 40 (**Figure 14** Bottom Row) after EAE induction, the majority of

cytokine genes measured was dramatically lower compared with day 20 in both groups of mice; however, the expression of IL-12, IL-17, IFN-γ, and TNF-α was still significantly higher in the spinal cords of Astro^{CD24} mice in comparison with non-transgenic mice. Up-regulation of IL-10 and IL-4 genes was also observed in both groups of mice on day 40, but no significant differences were observed between Astro^{CD24} and non-transgenic mice.

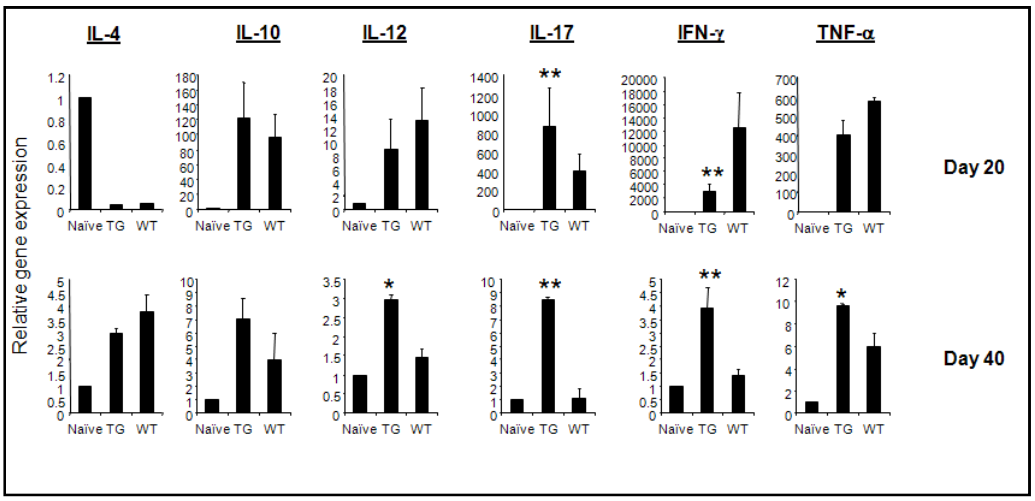

Figure 14: Assessment of cytokine gene expression in the spinal cords of Astro^{CD24} mice and controls by real-time PCR

We first induced EAE in Astro^{CD24} (TG) mice and their WT littermates by active immunization. On days 20 (peak of EAE) and 40 (recovery of EAE), we sacrificed mice, and total RNA was prepared from diseased mice and naive C57BL/6 mice. Real-time PCR was used to quantify cytokine gene expression. The HPRT gene was amplified and served as endogenous control. Each sample was assayed in triplicate, and experiments were repeated twice. The relative amounts of mRNA were calculated by plotting the Ct (cycle number), the average relative expression was determined by the comparative method ($2^{-\Delta\Delta Ct}$), and cytokine gene expression in naive spinal cord was set as 1. Three mice per group were used and data were expressed as mean ± SD. (** $P < 0.01$, * $P < 0.05$)

The most recent research in EAE pathogenesis has revealed that IL-17-producing T cells, but not IFN-γ producing T cells, are linked with pathogenicity in the CNS (32, 99). Moreover, the development of Th17 cells is negatively regulated by Th1 cells (99, 100). Therefore, we directly quantitated Th1 vs Th17 cells in the CNS of Astro^{CD24} mice and non-transgenic mice induced with EAE. On day 20, the CNS of Astro^{CD24} mice had more Th17 cells than the CNS of non-transgenic mice. However, there was no statistical difference in Th1 cell numbers between the two groups (**Figure 15**). Thus, the intracellular cytokine-staining data correlated with real-time PCR data and suggested that over-expression of CD24 in the CNS increased Th17 but not Th1 responses. We also detected similar numbers of IFN-g/IL17 double-positive cells in the spinal cords of both groups of mice. IFN-γ/IL17 double-positive cells have been detected by other groups in the spinal cords of EAE mice (101, 102); the significance of these cells remains unclear.

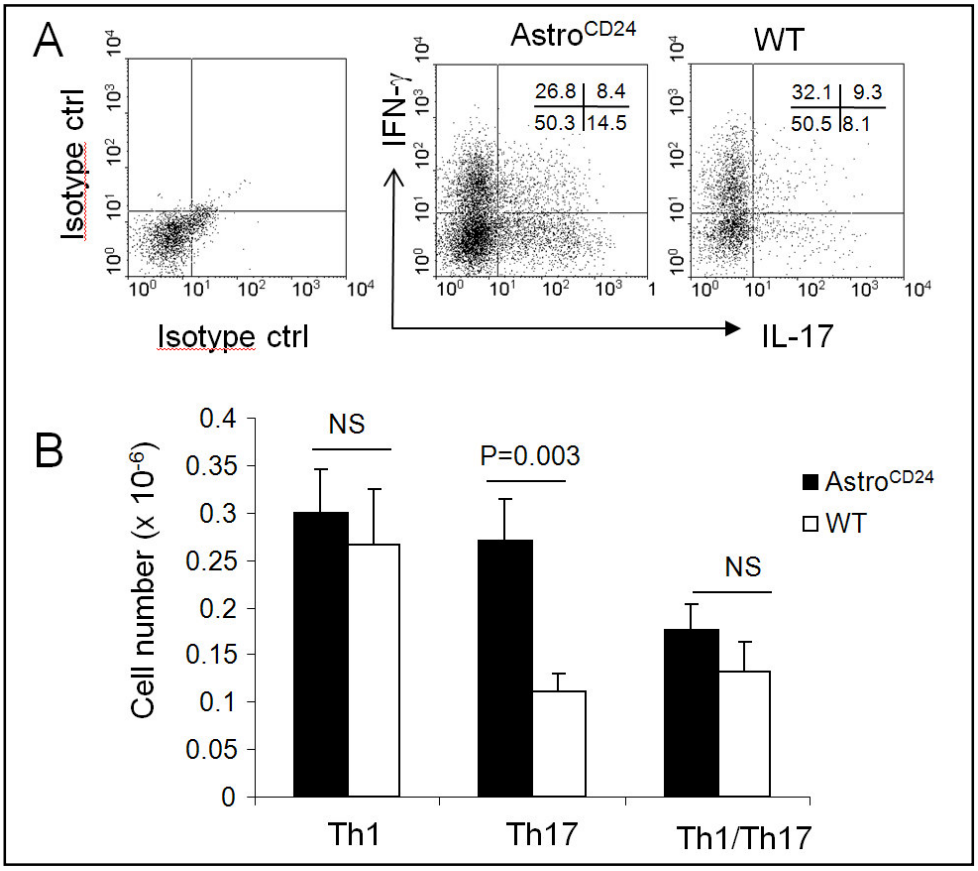

Figure 15: Th1 and Th17 cells in the spinal cords of Astro^{CD24} mice
EAE was induced in Astro^{CD24} and control mice by active immunization. On day 20 after immunization, we sacrificed mice and perfused mice with PBS (pH 7.4), and we then isolated the CNS-infiltrating cells from the brain and spinal cord of each mouse. Cells were incubated with PMA and ionomycin for 5 h. During the last 2 h of culture, Golgi^{Stop} (BD Pharmingen) was added into the cell culture. Cells were first stained for CD4 followed by intracellular cytokine staining. We performed intracellular staining for cytokines by using a kit from BD Pharmingen. The following Abs were used for the staining: anti-CD4 FITC (GK1.4; BD Pharmingen), anti-IFN-γ allophycocyanin (XMG1.2; BD Pharmingen), anti-IL-17 PE (TC11-18H10; BD Pharmingen), and rat IgG1 isotype control PE or allophycocyanin (BD Pharmingen). Cells were analyzed on a FACSCalibur flow cytometer. A) Example of intracellular cytokine staining. B) Quantitation of Th1, Th17, and Th1/Th17 cells among total CD4 cells. Student's *t* test was used for the statistical analysis. Four mice per group were quantitated. Data are representative of at least three experiments with similar results.

5.3 Over-expression of CD24 enhances local T cell proliferation in the CNS

To determine if CD24 over-expression in the CNS enhances T cell proliferation, thereby increasing EAE severity, we quantitated total $CD4^+$ T cell numbers in the CNS of $Astro^{CD24}$ mice vs. non-transgenic mice at day 20 after of EAE induction. We injected 1 mg of BrdU i.p. into each mouse and twelve hours later evaluated $CD4^+$ T cells in the CNS of $Astro^{CD24}$ and WT mice. We detected significantly increased numbers of $CD4^+$ T cells in the CNS of $Astro^{CD24}$ mice compared to non-transgenic mice (**Figure 16**A). The $CD4^+$ T cells in the CNS of $Astro^{CD24}$ mice were proliferating more vigorously (19.7% $BrdU^+$) than in non-transgenic mice (6.4% BrdU) (**Figure 16**B&C).

To evaluate the hypothesis that CD24 expression on astrocytes is sufficient to support proliferation of T cells, we bred $Astro^{CD24}$ mice with CD24-deficient mice to produce $Astro^{CD24}CD24^{-/-}$ mice in which CD24 is over expressed on astrocytes, but absent on all other cells. We cultured astrocytes from $Astro^{CD24}CD24^{-/-}$ and $CD24^{-/-}$ newborn (day 2) mice, and stimulated them with IFNγ (100 U/ml) for 72 hours. We then performed a proliferation assay with MOG peptide and purified 2D2 TCR transgenic T cells to evaluate the proliferation potential of CD24 on astrocytes. After 5 days, $Astro^{CD24}CD24^{-/-}$ astrocytes were observed to support proliferation of 2D2 T cells (**Figure 17**A), while $CD24^{-/-}$ astrocytes did not support 2D2 T cell proliferation. Furthermore the proliferation of 2D2 T cells by

Astro^{CD24}/CD24$^{-/-}$ astrocytes can be blocked by a CD24 specific antibody (20C9) (15) (**Figure 17**B).

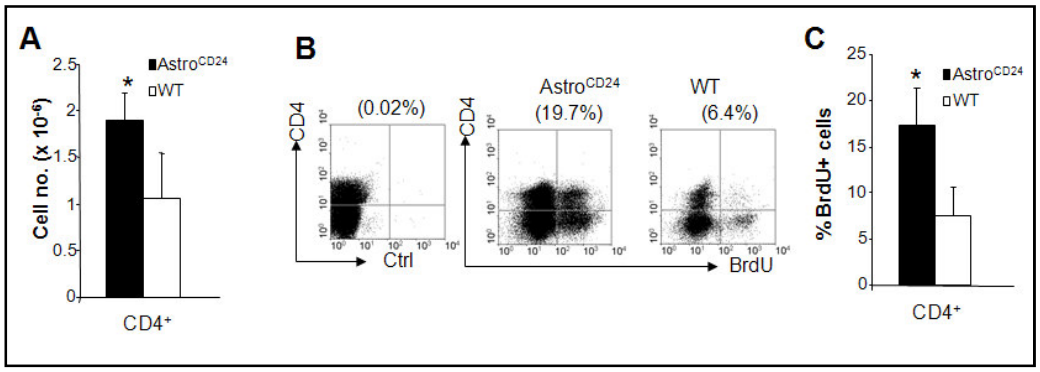

Figure 16: Over-expression of CD24 in the CNS enhances CD4 T cell proliferation in the CNS

A) Quantization of total CD4$^+$ T cells in the CNS of mice with EAE. EAE was induced in Astro^{CD24} and control mice by active immunization. On day 20 after immunization, we sacrificed mice and perfused mice with PBS (pH 7.4). We then isolated the CNS-infiltrating cells from the brain and spinal cord of each mouse. Cells were stained for CD4 marker, and total CD4$^+$ T cells were quantitated by flow cytometry. Data were expressed as mean ± SD, with five mice per group being used. *, $p < 0.05$, Student's *t* test was used. B) Analysis of BrdU incorporation in the CNS infiltrating cells. On day 19, we injected 1 mg of BrdU (BD Pharmingen) i.p. into each mouse. Twelve hours later, we sacrificed mice and isolated CNS-infiltrating cells from each mouse. We performed BrdU staining by using a kit from BD Pharmingen. C) Quantitation of BrdU incorporation in CD4_T cells. Data were expressed as mean ± SD, $n = 5$/group. *, $p < 0.05$, Student's *t* test was used.

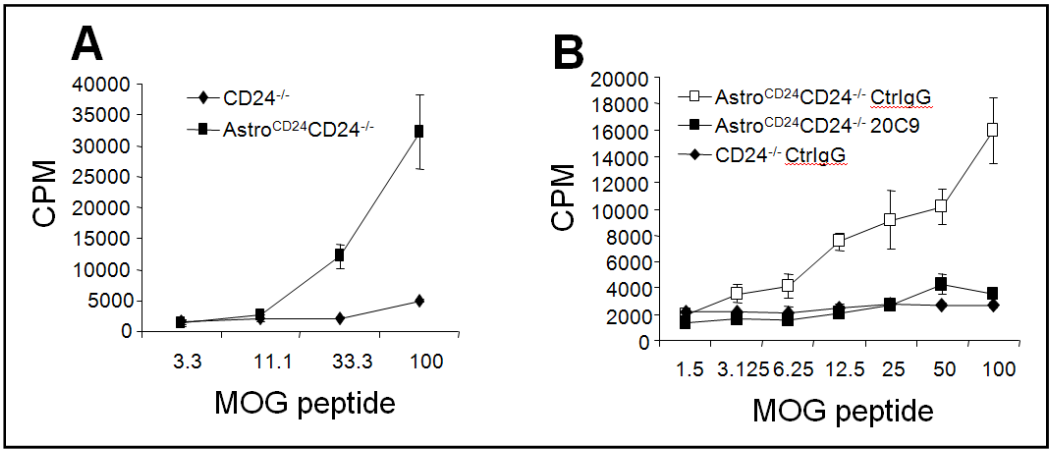

Figure 17: Transgenic expression of CD24 in CD24-deficient astrocytes promotes their co-stimulatory activity to MOG-specific T cells

We have bred Astro^{CD24} mice with CD24$^{-/-}$ mice and generated mice with CD24 expression only on astrocytes (Astro^{CD24}CD24$^{-/-}$). Astrocytes were generated from newborn (day 2) mice with CD24$^{-/-}$ or Astro^{CD24}CD24$^{-/-}$ genotypes. A) Astrocytes from CD24$^{-/-}$ and Astro^{CD24}CD24$^{-/-}$ mice were tested for their ability to stimulate MOG specific 2D2 T cells to proliferate in the presence of MOG peptide ([^{3}H] thymidine incorporation assay). Data shown were after 5 days co-culture and represents four experiments with similar results. B) Anti-CD24 Ab 20C9 blocked the stimulatory effects of Astro^{CD24}CD24$^{-/-}$ astrocytes. 20C9 Ab or control IgG (10 µg/ml) was used in the culture.

CHAPTER 6:

CONCLUSIONS AND DISCUSSION

6.1 CD24 controls thymic generation of self reactive T cells

We have demonstrated that CD24 actively inhibits clonal deletion of autoreactive T cells, as CD24 deficient mice exhibit enhanced clonal deletion. The phenomenon of enhanced deletion is observed in transgenic T cells specific for MOG as well as in polyclonal T cells specific for VSAs. These data establish that CD24 mediated active mechanisms exist to enable the selection of autoreactive T cells, and rescuing them from negative selection. The biological benefits of CD24 mediated inhibition of thymic negative selection are unclear. It is conceivable, that CD24-mediated protection of self-reactive T cells may be required for the immune system to preserve anti-tumor immunity, since the majority of tumor antigens are self antigens.

6.2 The cellular basis of CD24 inhibition of negative selection

Recent studies demonstrate that both DP and SP thymocytes are targets of negative selection (93, 94, 103, 104). mTECs can synthesize peripheral antigens and present them on MHC class II molecules thereby playing a central role in negative selection. In addition to mTECs, dendritic cells can import peripheral antigens to the thymus and present the self antigens to the immature thymocytes (105-108). Our results demonstrate the importance of TECs in

rescuing DP thymocytes, however CD4 SP thymocytes are not rescued in bone marrow chimeric mice with CD24 expression only TECs (**Figure 5**). So CD24 on dendritic cells may also provide a signal inhibiting clonal deletion, but further experiments are required to support this hypothesis.

6.3 Signal pathway of CD24 action in the thymus

The molecule mechanisms of how CD24 inhibits negative selection remains to be determined. Increased understanding of the signaling pathways that regulate negative selection has recently been achieved (109). c-Jun NH2-terminal kinase (JNK) is activated in DP thymocytes *in vivo* in response to signals that initiate negative selection (110). Studies have revealed that the JNK pathway is required for the deletion of DP thymocytes by apoptosis in response to TCR-derived signals (110-112). JNK is a signaling molecule downstream of kinase MINK and upstream of pro-apoptotic molecule Bim (109, 113). All three molecules have been shown to be critically involved in promoting negative selection (110, 114, 115), thus, it is interesting to determine if CD24 directly affects activation/induction of JNK/Bim signaling pathways during negative selection.

6.4 A hypothetical model of CD24 action in the thymic generation of autoreactive T cells

We suggest a model (**Figure 18**) that CD24 provides a survival signal blocking apoptosis and allowing immature thymocytes differentiation and progression into mature SP T cells. A default pathway of apoptosis exists for T cells that engage their TCR with a self peptide. CD24 on APCs will interact with CD24

ligand on T cells providing an inhibitory signal to the apoptotic pathways. In the absence of CD24 signaling, DP T cells that interact with self antigen will be eliminated leaving only immature T cells to populate the periphery.

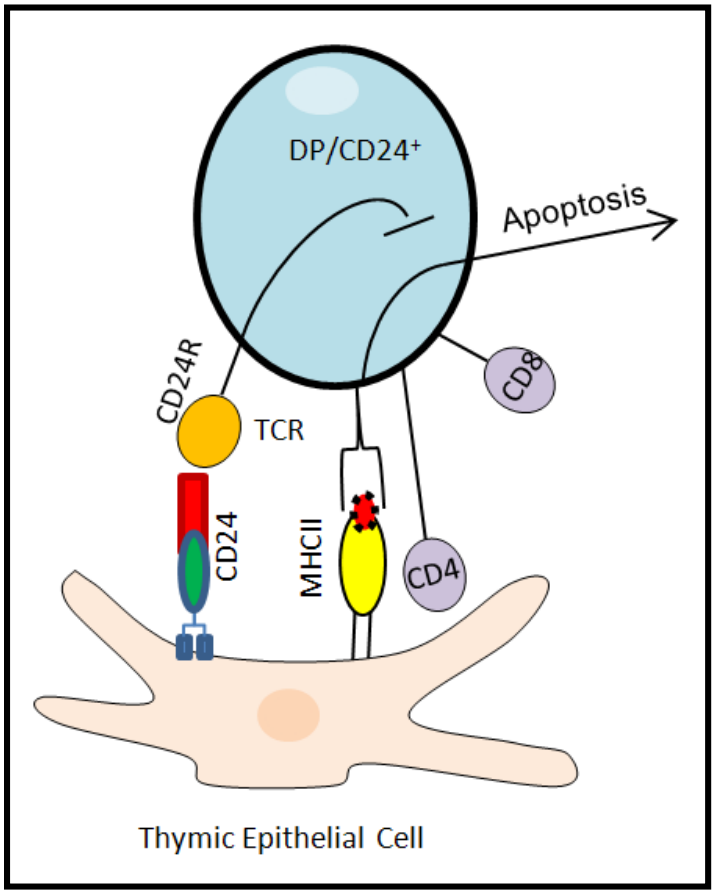

Figure 18: CD24 expression on Thymic Epithelial Cells Provides a Signal to Developing T Cells Inhibiting Apoptosis.
MHC II presentation of self autoantigens by TECs engages TCRs on naïve Thymocytes inducing a default pathway of apoptosis. Co-stimulation by CD24 on TECs with CD24R (unknown ligand) on naïve thymocytes provides an inhibitory signal blocking apoptosis.

6.5 The role of CD24 in the CNS in EAE development

We have tested the hypothesis that abundant CD24 in the CNS co-stimulates myelin antigen specific T cells and promotes their effector function. Several lines of evidence support this hypothesis. Over-expression of CD24 on astrocytes

enhanced the progression of EAE (**Figure 13**). The Enhanced EAE progression was associated with higher expression of cytokine genes such as IL-17 in the spinal cords of Astro^{CD24} mice compared with nontransgenic mice. The CNS of Astro^{CD24} mice accumulated higher numbers of total CD4^{+} T cells and Th17 cells compared with non transgenic mice. Moreover, BrdU incorporation assay revealed that CD4^{+} T cells from the CNS of Astro^{CD24} mice underwent more proliferation compared with that of non transgenic mice. We also showed that transgenic expression of CD24 in CD24- deficient astrocytes significantly enhanced their co-stimulatory activity to MOG-specific, TCR-transgenic 2D2 T cells *in vitro*. CD24 is not required for the differentiation of Th17 in peripheral lymphoid organs (unpublished data), however it is not clear if CD24 co-stimulation directly causes CD4^{+} T cells to differentiate into Th17 cells in the CNS. Further studies are required to clarify this issue.

6.6 A hypothetical model of CD24 action in the CNS during EAE development

We propose a model (**Figure 19**) that CNS APCs provide a necessary CD24 signal to pathogenic T cells, which allows optimal proliferation and persistence of T cells in the CNS. Over-expression of CD24 on APCs provides a stronger co-stimulatory signal enhancing the proliferation and survival of pathogenic autoreacitve T cells in the CNS.

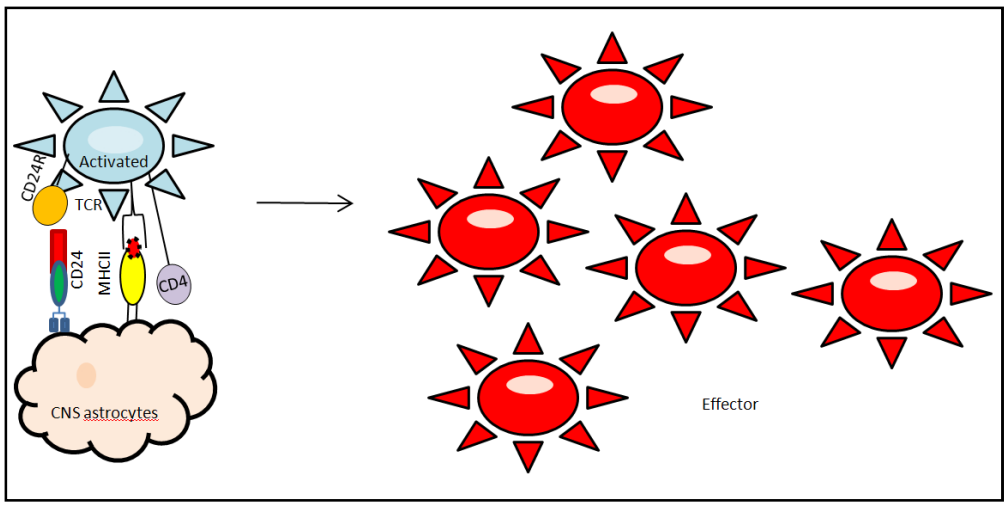

Figure 19: CD24 Expression on Central Nervous Systems Antigen Presenting Cells Provides a Co-stimulatory Signal to T cells Enabling the Proliferation and Survival of Effectors Cells.
Co-stimulation by CD24 on astrocytes with CD24R (unknown ligand) on activated myelin specific T cells can optimally activate CNS antigen specific T cells.

6.7 Significance

CD24 is a critical checkpoint for the pathogenesis of autoimmune diseases (116, 117). Mice with targeted mutation of CD24 are resistant to EAE (14, 118, 119). Moreover, CD24 polymorphism affects risk of both organ-specific and systemic autoimmune disease in human (79, 82). Our previous studies have demonstrated several mechanisms by which CD24 facilitates autoimmune diseases, including expansion of autoreactive T cells in the target organ (15, 120) and regulation of homeostatic proliferation (121, 122). Our study presented here suggests a novel mechanism by which CD24 mediates pathogenesis of autoimmune diseases, namely the escape of autoreactive T cells from clonal deletion.

6.8 Future Prospective

Based on this study, a therapeutic that could block the signaling associated with CD24 would be beneficial to the amelioration of EAE and presumable MS in humans. CD24's ligand is currently unknown, so a soluble CD24 protein was developed, and has been shown to drastically reduce EAE symptoms when administered 8-10 days after the induction of EAE(14). The soluble CD24 protein is comprised of the mature murine CD24 protein and the signal protein fused to a human IgG1 Fc fragment denoted HSAIg. Secreted as a homodimer, HSAIg presumably will bind with the ligand of CD24 (CD24L) thus blocking CD24 associated co-stimulatory signaling. The HSAIg method of action hypothetically should mimic a CD24 deficient mouse.

During the course of MS, activation of myelin antigen specific T cells should exhaust the naïve population of potentially autoreactive T cells. The generation of a new pool of naïve autoreactive T cells would be affected by the presence of the soluble HSAIg. Based on the data presented in this study, autoreactive T cells should be eliminated and the periphery should become depleted of autoreactive T cells specific for myelin antigen. Assuming the peripheral pool of naïve autoreactive T cells is depleted, the soluble HSAIg should provide a long term protection from future relapses.

During an inflammatory process, the BBB should allow access of the soluble HSAIg into the CNS thus blocking the action of CD24 on thymocytes. CD24 deficient mice are highly resistant to the induction of EAE(14), furthermore

adoptive transfer studies indicated that activated T Cells expressing CD24 will traffic to the CNS but not become re-activated (15) and subsequently undergoing apoptosis. As shown in this study, over-expression of CD24 on astrocytes resulted in a more severe course of EAE. Taken together, one could predict that HSAIg would have a similar method of action as the CD24 deficient mice and block the activation of myelin specific T cells in the CNS. Once the short lived activated T cells are exhausted, no newly activated T cells should be generated and the inflammation response should subside. Based on the data found in this study, the HSAIg looks to be a promising therapeutic for the treatment of MS.

LIST OF REFERENCES

1. Noseworthy JH, Gold R, Hartung HP. 1999. Treatment of multiple sclerosis: recent trials and future perspectives. *Curr Opin Neurol* 12:279-293.

2. Hohlfeld R, Wekerle H. 2004. Autoimmune concepts of multiple sclerosis as a basis for selective immunotherapy: from pipe dreams to (therapeutic) pipelines. *Proc Natl Acad Sci U S A.* 2004:14599-14606.

3. McFarland HF, Martin R. 2007. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* 8:913-919.

4. Hafler, DA. 2004. Multiple sclerosis. *J Clin Invest* 113:788-794.

5. Zamvil SS, Steinman L. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu Rev Immunol* 8:579-621.

6. Kuchroo VK, Anderson AC, Waldner H, Munder M, Bettelli E, Nicholson LB. 2002. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu Rev Immunol* 20:101-123.

7. Thomas M. Rivers, M.D., D.H. Sprunt, M.D.,A~D G. P. Berry, M.D. 1933. Observations on Attempts to Produce Acute Disseminated Encephalomyelitis in Monkeys. *The Journal of Experimental Medicine,* 58:39-53.

8. Lassmann, H. 2007. Experimental models of multiple sclerosis. *Rev Neurol (Paris).* 163:651-655.

9. Brabb T, Goldrath AW, von Dassow P, Paez A, Liggitt HD, Goverman J. 1997. Triggers of autoimmune disease in a murine TCR-transgenic model for multiple sclerosis. *J Immunol* 159:497-507.

10. Racke MK, Hu W, Lovett-Racke AE. 2005. PTX cruiser: driving autoimmunity via TLR4. *Trends Immunol* 26:289-291.

11. El Behi M, Dubucquoi S, Lefranc D, Zéphir H, De Seze J, Vermersch P, Prin L. 2005. New insights into cell responses involved in experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol Lett.* 96:11-26.

12. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. 2000. Multiple sclerosis. *N Engl J Med.* 343:938-952.

13. Liu JQ, Carl JW Jr, Joshi PS, RayChaudhury A, Pu XA, Shi FD, Bai XF. 2007. CD24 on the resident cells of the central nervous system enhances experimental autoimmune encephalomyelitis. *J Immunol* 178:6227-6235.

14. Bai XF, Liu JQ, Liu X, Guo Y, Cox K, Wen J, Zheng P, Liu Y. 2000. The heat-stable antigen determines pathogenicity of self-reactive T cells in experimental autoimmune encephalomyelitis. *J Clin Invest* 105:1227-1232.

15. Bai XF, Li O, Zhou Q, Zhang H, Joshi PS, Zheng X, Liu Y, Wang Y, Zheng P, Liu Y. 2004. CD24 controls expansion and persistence of autoreactive T cells in the central nervous system during experimental autoimmune encephalomyelitis. *J Exp Med* 200:447-458.

16. Boehm T, Bleul CC. 2006. Thymus-homing precursors and the thymic microenvironment. *Trends Immunol* 27:477-484.

17. PJ., Haley. 2003. Species differences in the structure and function of the immune system. *Toxicology* 188:49-71.

18. Kato, S. 1997. Thymic microvascular system. *Microsc Res Tech.* 38:287-299.

19. Pearse, G. 2006. Normal structure, function and histology of the thymus. *Toxicol Pathol.* 34:504-514.

20. G., Pearse. 2006. Normal structure, function and histology of the thymus. *Toxicol Pathol.* 34:504-514.

21. Jolicoeur C, Hanahan D, Smith KM. 1994. T-cell tolerance toward a transgenic beta-cell antigen and transcription of endogenous pancreatic genes in thymus. *Proc Natl Acad Sci U S A.* 91:6707-6711.

22. Anderson AC, Nicholson LB, Legge KL, Turchin V, Zaghouani H, Kuchroo VK. 2000. High frequency of autoreactive myelin proteolipid protein-specific T cells in the periphery of naive mice: mechanisms of selection of the self-reactive repertoire. *J Exp Med* 191:761-770.

23. Klein L, Klugmann M, Nave KA, Tuohy VK, Kyewski B. 2000. Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. *Nat Med* 6:56-61.

24. Fowlkes BJ, Schweighoffer E. 1995. Positive selection of T cells. *Curr Opin Immunol.* 7:188-195.

25. Capone M, Hockett RD Jr, Zlotnik A. 1998. Kinetics of T cell receptor beta, gamma, and delta rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44(+)CD25(+) Pro-T thymocytes. *Proc Natl Acad Sci U S A.* 95:12522-12527.

26. Lind EF, Prockop SE, Porritt HE, Petrie HT. 2001. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J Exp Med* 194:127-134.

27. Lohr J, Knoechel B, Nagabhushanam V, Abbas AK. 2005. T-cell tolerance and autoimmunity to systemic and tissue-restricted self-antigens. *Immunol Rev.* 204:116-127.

28. Wilczynski JR, Radwan M, Kalinka J. 2008. The characterization and role of regulatory T cells in immune reactions. *Front Biosci* 1:2266-2274.

29. Girvin AM, Dal Canto MC, Rhee L, Salomon B, Sharpe A, Bluestone JA, Miller SD. 2000. A critical role for B7/CD28 costimulation in experimental autoimmune encephalomyelitis: a comparative study using costimulatory molecule-deficient mice and monoclonal antibody blockade. *J Immunol* 164:136-143.

30. Chang TT, Jabs C, Sobel RA, Kuchroo VK, Sharpe AH. 1999. Studies in B7-deficient mice reveal a critical role for B7 costimulation in both induction and effector phases of experimental autoimmune encephalomyelitis. *J Exp Med* 190:377-340.

31. Constant SL, Bottomly K. 1997. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol* 15:297-322.

32. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233-240.

33. McKenzie BS, Kastelein RA, Cua DJ. 2006. Understanding the IL-23-IL-17 immune pathway. *Trends Immunol* 27:17-23.

34. Lafaille JJ, Keere FV, Hsu AL, Baron JL, Haas W, Raine CS, Tonegawa S. 1997. Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J Exp Med* 186:307-312.

35. Aharoni R, Teitelbaum D, Leitner O, Meshorer A, Sela M, Arnon R. 2000. Specific Th2 cells accumulate in the central nervous system of mice protected against experimental autoimmune encephalomyelitis by copolymer 1. *Proc Natl Acad Sci U S A.* 97:11472-11477.

36. Kniesel U, Wolburg H. 2000. Tight junctions of the blood-brain barrier. *Cell Mol Neurobiol* 20:57-76.

37. Broadwell RD, Baker BJ, Ebert PS, Hickey WF. 1994. Allografts of CNS tissue possess a blood-brain barrier: III. Neuropathological, methodological, and immunological considerations. *Microsc Res Tech.* 27:471-494.

38. Akiyama H, Kondoh T, Kokunai T, Nagashima T, Saito N, Tamaki N. 2000. Blood-brain barrier formation of grafted human umbilical vein endothelial cells in athymic mouse brain. *Brain Res* 858:172-176.

39. Kuchler-Bopp S, Delaunoy JP, Artault JC, Zaepfel M, Dietrich JB. 1999. Astrocytes induce several blood-brain barrier properties in non-neural endothelial cells. *Neuroreport* 10:1347-1353.

40. Allt G, Lawrenson JG. 1997. Is the pial microvessel a good model for blood-brain barrier studies? *Brain Res Brain Res Rev* 24:67-76.

41. Kerfoot SM, Kubes P. 2002. Overlapping roles of P-selectin and alpha 4 integrin to recruit leukocytes to the central nervous system in experimental autoimmune encephalomyelitis. *J Immunol* 169:1000-1006.

42. Battistini L, Piccio L, Rossi B, Bach S, Galgani S, Gasperini C, Ottoboni L, Ciabini D, Caramia MD, Bernardi G, Laudanna C, Scarpini E, McEver RP, Butcher EC, Borsellino G, Constantin G. 2003. CD8+ T cells from patients with acute multiple sclerosis display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1. *blood* 101:4775-4782.

43. Xu H, Manivannan A, Jiang HR, Liversidge J, Sharp PF, Forrester JV, Crane IJ. 2004. Recruitment of IFN-gamma-producing (Th1-like) cells into the inflamed retina in vivo is preferentially regulated by P-selectin glycoprotein ligand 1:P/E-selectin interactions. *J Immunol* 172:3215-3224.

44. Cross AH, Cannella B, Brosnan CF, Raine CS. 1990. Homing to central nervous system vasculature by antigen-specific lymphocytes. I. Localization of 14C-labeled cells during acute, chronic, and relapsing experimental allergic encephalomyelitis. *Lab Invest* 63:162-170.

45. WF., Hickey. 1991. Migration of hematogenous cells through the blood-brain barrier and the initiation of CNS inflammation. *Brain Pathol* 1:97-105.

46. Yeager MP, DeLeo JA, Hoopes PJ, Hartov A, Hildebrandt L, Hickey WF. 2000. Trauma and inflammation modulate lymphocyte localization in vivo: quantitation of tissue entry and retention using indium-111-labeled lymphocytes. *Crit Care Med* 28:1477-1482.

47. Slavin AJ, Soos JM, Stuve O, Patarroyo JC, Weiner HL, Fontana A, Bikoff EK, Zamvil SS. 2001. Requirement for endocytic antigen processing and influence of invariant chain and H-2M deficiencies in CNS autoimmunity. *J Clin Invest* 108:1133-1139.

48. Kuchroo VK, Anderson AC, Waldner H, Munder M, Bettelli E, Nicholson LB. 2002. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu Rev Immunol* 20:101-123.

49. Flügel A, Berkowicz T, Ritter T, Labeur M, Jenne DE, Li Z, Ellwart JW, Willem M, Lassmann H, Wekerle H. 2001. Migratory activity and functional changes of

green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. *Immunity* 14:547-560.

50. Targoni OS, Baus J, Hofstetter HH, Hesse MD, Karulin AY, Boehm BO, Forsthuber TG, Lehmann PV. 2001. Frequencies of neuroantigen-specific T cells in the central nervous system versus the immune periphery during the course of experimental allergic encephalomyelitis. *J Immunol* 166:4757-4764.

51. Kawakami N, Lassmann S, Li Z, Odoardi F, Ritter T, Ziemssen T, Klinkert WE, Ellwart JW, Bradl M, Krivacic K, Lassmann H, Ransohoff RM, Volk HD, Wekerle H, Linington C, Flügel A. 2004. The activation status of neuroantigen-specific T cells in the target organ determines the clinical outcome of autoimmune encephalomyelitis. *J Exp Med* 199:185-197.

52. Kornek B, Storch MK, Weissert R, Wallstroem E, Stefferl A, Olsson T, Linington C, Schmidbauer M, Lassmann H. 2000. Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. *Am J Pathol* 157:267-276.

53. Mancardi G, Hart B, Roccatagliata L, Brok H, Giunti D, Bontrop R, Massacesi L, Capello E, Uccelli A. 2001. Demyelination and axonal damage in a non-human primate model of multiple sclerosis. *J Neurol Sci* 184:41-49.

54. Bauer J, Bradl M, Hickley WF, Forss-Petter S, Breitschopf H, Linington C, Wekerle H, Lassmann H. 1998. T-cell apoptosis in inflammatory brain lesions: destruction of T cells does not depend on antigen recognition. *Am J Pathol* 153:715-724.

55. Schmied M, Breitschopf H, Gold R, Zischler H, Rothe G, Wekerle H, Lassmann H. 1993. Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis. Evidence for programmed cell death as a mechanism to control inflammation in the brain. *Am J Pathol* 143:446-452.

56. Nimmerjahn A, Kirchhoff F, Helmchen F. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308:1314-1318.

57. Linthicum DS, Munoz JJ, Blaskett A. 1982. Acute experimental autoimmune encephalomyelitis in mice. I. Adjuvant action of Bordetella pertussis is due to vasoactive amine sensitization and increased vascular permeability of the central nervous system. *Cell Immunol* 73:299-310.

58. Sedgwick JD, Ford AL, Foulcher E, Airriess R. 1998. Central nervous system microglial cell activation and proliferation follows direct interaction with tissue-infiltrating T cell blasts. *J Immunol* 160:5320-5330.

59. Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, Svendsen CN, Mucke L, Johnson MH, Sofroniew MV. 1999. Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron* 23:297-308.

60. Nikcevich KM, Gordon KB, Tan L, Hurst SD, Kroepfl JF, Gardinier M, Barrett TA, Miller SD. 1997. IFN-gamma-activated primary murine astrocytes express B7 costimulatory molecules and prime naive antigen-specific T cells. *J Immunol* 158:614-621.

61. Matsumoto Y, Ohmori K, Fujiwara M. 1992. Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under in vivo-mimicking conditions. *Immunology* 76:209-216.

62. Aloisi F, Ria F, Columba-Cabezas S, Hess H, Penna G, Adorini L. 1999. Relative efficiency of microglia, astrocytes, dendritic cells and B cells in naive CD4+ T cell priming and Th1/Th2 cell restimulation. *Eur J Immunol.* 29:2705-2714.

63. Wong GH, Bartlett PF, Clark-Lewis I, Battye F, Schrader JW. 1984. Inducible expression of H-2 and Ia antigens on brain cells. *Nature* 310:688-691.

64. Satoh J, Lee YB, Kim SU. 1995. T-cell costimulatory molecules B7-1 (CD80) and B7-2 (CD86) are expressed in human microglia but not in astrocytes in culture. *Brain Res* 704:92-96.

65. Springer T, Galfrè G, Secher DS, Milstein C. 1978. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *European Journal of Immunology* 8:539-551.

66. Hubbe M, Altevogt P. 1994. Heat-stable antigen/CD24 on mouse T lymphocytes: evidence for a costimulatory function. *Eur J Immunol.* 24:731-737.

67. Zhou Q, Wu Y, Nielsen PJ, Liu Y. 1997. Homotypic interaction of the heat-stable antigen is not responsible for its co-stimulatory activity for T cell clonal expansion. *Eur J Immunol.* 27:2524-2528.

68. Dalley JA, Bulleid NJ. 2003. How does the translocon differentiate between hydrophobic sequences that form part of either a GPI (glycosylphosphatidylinositol)-anchor signal or a stop transfer sequence? *Biochem Soc Trans.* 31:1257-1259.

69. Liu Y, Jones B, Aruffo A, Sullivan KM, Linsley PS, Janeway CA Jr. 1992. Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. *J Exp Med* 175:437-445.

70. De Bruijn ML, Peterson PA, Jackson MR. 1996. Induction of heat-stable antigen expression by phagocytosis is involved in in vitro activation of unprimed CTL by macrophages. *J Immunol* 156:2686-2692.

71. Hernández-Campo PM, Almeida J, Matarraz S, de Santiago M, Sánchez ML, Orfao A. 2007. Quantitative analysis of the expression of glycosylphosphatidylinositol-anchored proteins during the maturation of different hematopoietic cell compartments of normal bone marrow. *Cytometry B Clin Cytom.* 72:34-42.

72. Shirasawa T, Akashi T, Sakamoto K, Takahashi H, Maruyama N, Hirokawa K. 1993. Gene expression of CD24 core peptide molecule in developing brain and developing non-neural tissues. *Dev Dyn.* 198:1-13.

73. Poncet C, Frances V, Gristina R, Scheiner C, Pellissier JF, Figarella-Branger D. 1996. CD24, a glycosylphosphatidylinositol-anchored molecules is transiently expressed during the development of human central nervous system and is a marker of human neural cell lineage tumors. *Acta Neuropathol.* 91:400-408.

74. Magnaldo T, Barrandon Y. 1996. CD24 (heat stable antigen, nectadrin), a novel keratinocyte differentiation marker, is preferentially expressed in areas of the hair follicle containing the colony-forming cells. *J Cell Sci* 109:3035-3045.

75. Maric D, Kaiserlian D, Croitoru K. 1996. Intestinal epithelial cell line induction of T cell differentiation from bone marrow precursors. *Cell Immunol* 172:172-179.

76. Larkin M, Knapp W, Stoll MS, Mehmet H, Feizi T. 1991. Monoclonal antibodies VIB-E3, IB5 and HB9 to the leucocyte/epithelial antigen CD24 resemble BA-1 in recognizing sialic acid-dependent epitope(s). Evidence that VIB-E3 recognizes NeuAc alpha 2-6GalNAc and NeuAc alpha 2-6Gal sequences. *Clin Exp Immunol* 85:536-541.

77. Mehmet H, Larkin M, Tang PW, Lebien TW, Feizi T. 1990. Monoclonal antibody BA-1 to the human B lymphocyte marker CD24 recognizes a sialic acid (N-acetylneuraminic acid) dependent epitope in multi-valent display on peptide. *Clinical and Experimental Immunology* 81:489-495.

78. Weber E, Lehmann HP, Beck-Sickinger AG, Wawrzynczak EJ, Waibel R, Folkers G, Stahel RA. 1993. Antibodies to the protein core of the small cell lung cancer workshop antigen cluster-w4 and to the leucocyte workshop antigen CD24 recognize the same short protein sequence leucine-alanine-proline. *Clin Exp Immunol.* 93:279-285.

79. Wang L, Lin S, Rammohan KW, Liu Z, Liu JQ, Liu RH, Guinther N, Lima J, Zhou Q, Wang T, Zheng X, Birmingham DJ, Rovin BH, Hebert LA, Wu Y, Lynn DJ, Cooke G, Yu CY, Zheng P, Liu Y. 2007. A dinucleotide deletion in CD24 confers protection against autoimmune diseases. *PLoS Genet.* 6:508-517.

80. Dalley JA, Bulleid NJ. 2003. The endoplasmic reticulum (ER) translocon can differentiate between hydrophobic sequences allowing signals for glycosylphosphatidylinositol anchor addition to be fully translocated into the ER lumen. *J Biol Chem.* 278:51749-51757.

81. Hough MR, Rosten PM, Sexton TL, Kay R, Humphries RK. 1994. Mapping of CD24 and homologous sequences to multiple chromosomal loci. *Genomics* 22:154-161.

82. Zhou Q, Rammohan K, Lin S, Robinson N, Li O, Liu X, Bai XF, Yin L, Scarberry B, Du P, You M, Guan K, Zheng P, Liu Y. 2003. CD24 is a genetic modifier for risk and progression of multiple sclerosis. *Proc Natl Acad Sci U S A.* 100:15041-15046.

83. Bettelli E, Pagany M, Weiner HL, Linington C, Sobel RA, Kuchroo VK. 2003. Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med* 197:1073-1081.

84. Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A. 1994. GFAP promoter directs astrocyte-specific expression in transgenic mice. *J Neurosci* 14:1030-1037.

85. Besnard F, Brenner M, Nakatani Y, Chao R, Purohit HJ, Freese E. 1991. Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein. *J Biol Chem.* 266:18877-18883.

86. Masood K, Besnard F, Su Y, Brenner M. 1993. Analysis of a segment of the human glial fibrillary acidic protein gene that directs astrocyte-specific transcription. *J Neurochem* 61:160-166.

87. Fierz W, Endler B, Reske K, Wekerle H, Fontana A. 1985. Astrocytes as antigen-presenting cells. I. Induction of Ia antigen expression on astrocytes by T cells via immune interferon and its effect on antigen presentation. *J Immunol* 134:3785-3793.

88. Giulian D, Baker TJ. 1986. Characterization of ameboid microglia isolated from developing mammalian brain. *J Neurosci* 6:2163-2178.

89. Carl JW Jr, Liu JQ, Joshi PS, Yin L, Zhen X, Whitacre CC, Liu Y, Bai XF. Autoreactive T Cells Escape Clonal Deletion in the Thymus by a CD24-Dependent Pathway. *J Immunol*.

90. Delarasse C, Daubas P, Mars LT, Vizler C, Litzenburger T, Iglesias A, Bauer J, Della Gaspera B, Schubart A, Decker L, Dimitri D, Roussel G, Dierich A, Amor S, Dautigny A, Liblau R, Pham-Dinh D. 2003. Myelin/oligodendrocyte glycoprotein-deficient (MOG-deficient) mice reveal lack of immune tolerance to MOG in wild-type mice. *J Clin Invest* 112:544-554.

91. Derbinski J, Schulte A, Kyewski B, Klein L. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol* 2:1032-1039.

92. Shimizu C, Kawamoto H, Yamashita M, Kimura M, Kondou E, Kaneko Y, Okada S, Tokuhisa T, Yokoyama M, Taniguchi M, Katsura Y, Nakayama T. 2001. Progression of T cell lineage restriction in the earliest subpopulation of murine adult thymus visualized by the expression of lck proximal promoter activity. *Int Immunol* 13:105-117.

93. Sprent J, Kishimoto H. 2002. The thymus and negative selection. *Immunol Rev.* 185:126-135.

94. von Boehmer H, Kisielow P. 2006. Negative selection of the T-cell repertoire: where and when does it occur? *Immunol Rev.* 209:284-289.

95. Hanahan, D. 1998. Peripheral-antigen-expressing cells in thymic medulla: factors in self-tolerance and autoimmunity. *Curr Opin Immunol.* 10:656-662.

96. Kawahata K, Misaki Y, Yamauchi M, Tsunekawa S, Setoguchi K, Miyazaki J, Yamamoto K. 2002. Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. *J Immunol* 168:4399-4405.

97. Abe R, Foo-Phillips M, Hodes RJ. 1991. Genetic analysis of the Mls system. Formal Mls typing of the commonly used inbred strains. *Immunogenetics* 33:62-73.

98. Pullen AM, Wade T, Marrack P, Kappler JW. 1990. Identification of the region of T cell receptor beta chain that interacts with the self-superantigen MIs-1a. *Cell* 61:1365-1374.

99. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.

100. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005:11.

101. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121-1133.

102. Bailey SL, Schreiner B, McMahon EJ, Miller SD. 2007. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4+ T(H)-17 cells in relapsing EAE. *Nat Immunol* 8:172-180.

103. Sprent, J., and H. Kishimoto. 2002. The thymus and negative selection. *Immunol Rev* 185:126-135.

104. von Boehmer, H., and P. Kisielow. 2006. Negative selection of the T-cell repertoire: where and when does it occur? *Immunol Rev* 209:284-289.

105. Pugliese A, Brown D, Garza D, Murchison D, Zeller M, Redondo MJ, Diez J, Eisenbarth GS, Patel DD, Ricordi C. 2001. Self-antigen-presenting cells expressing diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs. *J Clin Invest* 107:555-564.

106. Throsby M, Homo-Delarche F, Chevenne D, Goya R, Dardenne M, Pleau JM. 1998. Pancreatic hormone expression in the murine thymus: localization in dendritic cells and macrophages. *Endocrinolgy.* 139:2399-2406.

107. Throsby, M., F. Homo-Delarche, D. Chevenne, R. Goya, M. Dardenne, and J. M. Pleau. 1998. Pancreatic hormone expression in the murine thymus: localization in dendritic cells and macrophages. *Endocrinology* 139:2399-2406.

108. Pugliese, A., D. Brown, D. Garza, D. Murchison, M. Zeller, M. J. Redondo, J. Diez, G. S. Eisenbarth, D. D. Patel, and C. Ricordi. 2001. Self-antigen-presenting cells expressing diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs. *J Clin Invest* 107:555-564.

109. Sohn SJ, Thompson J, Winoto A. 2007. Apoptosis during negative selection of autoreactive thymocytes. *Curr Opin Immunol.* 19:510-515.

110. Rincón M, Whitmarsh A, Yang DD, Weiss L, Dérijard B, Jayaraj P, Davis RJ, Flavell RA. 1998. The JNK pathway regulates the In vivo deletion of immature CD4(+)CD8(+) thymocytes. *J Exp Med* 188:1817-1830.

111. Sabapathy K, Kallunki T, David JP, Graef I, Karin M, Wagner EF. 2001. c-Jun NH2-terminal kinase (JNK)1 and JNK2 have similar and stage-dependent roles in regulating T cell apoptosis and proliferation. *J Exp Med* 193:317-328.

112. Behrens A, Sabapathy K, Graef I, Cleary M, Crabtree GR, Wagner EF. 2001. Jun N-terminal kinase 2 modulates thymocyte apoptosis and T cell activation through c-Jun and nuclear factor of activated T cell (NF-AT). *Proc Natl Acad Sci U S A.* 98:1769-1774.

113. Palmer, E. 2005. Signaling negative selection: is MINK the missing link? *Nat Immunol* 6:9-10.

114. McCarty N, Paust S, Ikizawa K, Dan I, Li X, Cantor H. 2005. Signaling by the kinase MINK is essential in the negative selection of autoreactive thymocytes. *Nat Immunol* 6:65-72.

115. Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM, Strasser A. 2002. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* 415:922-926.

116. Liu Y, Zheng P. 2007. CD24: a genetic checkpoint in T cell homeostasis and autoimmune diseases. *Trends Immunol* 28:315-320.

117. Liu, Y., and P. Zheng. 2007. CD24: a genetic checkpoint in T cell homeostasis and autoimmune diseases. *Trends Immunol* 28:315-320.

118. Bai, X. F., J. Q. Liu, X. Liu, Y. Guo, K. Cox, J. Wen, P. Zheng, and Y. Liu. 2000. The heat-stable antigen determines pathogenicity of self-reactive T cells in experimental autoimmune encephalomyelitis. *J Clin Invest* 105:1227-1232.

119. Liu, J. Q., J. W. Carl, Jr., P. S. Joshi, A. Raychaudhury, X. A. Pu, F. D. Shi, and X. F. Bai. 2007. CD24 on the Resident Cells of the Central Nervous System Enhances Experimental Autoimmune Encephalomyelitis. *J Immunol* 178:6227-6235.

120. Bai, X. F., O. Li, Q. Zhou, H. Zhang, P. S. Joshi, X. Zheng, Y. Liu, Y. Wang, and P. Zheng. 2004. CD24 controls expansion and persistence of autoreactive T cells in the central nervous system during experimental autoimmune encephalomyelitis. *J Exp Med* 200:447-458.

121. Li O, Chang X, Zhang H, Kocak E, Ding C, Zheng P, Liu Y. 2006. Massive and destructive T cell response to homeostatic cue in CD24-deficient lymphopenic hosts. *J Exp Med* 203:1713-1720.

122. Li O, Zheng P, Liu Y. 2004. CD24 expression on T cells is required for optimal T cell proliferation in lymphopenic host. *J Exp Med* 200:1083-1089.