Suppressive activity of $CD4^+Foxp3^+$ regulatory T cells in an animal model of spontaneous $CD8^+$ T cell-mediated demyelinating disease

Lee, Crystal

Department of Microbiology and Immunology

McGill University, Montreal

June 2012

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

ABSTRACT

Dr. Fournier's laboratory has generated a mouse strain (L31 mice) that spontaneously develops a $CD8^+$ T cell-mediated demyelinating disease in the central nervous system. In this model of dysregulated costimulation, $CD4^+$ T cells have a regulatory role. A subset of $CD4^+$ regulatory T cells that express the transcription factor Foxp3 have been shown to regulate autoimmune responses. In order to investigate this population's role in disease development, the goal of my M.Sc research project was to functionally characterize the $CD4^+Foxp3^+$ regulatory T cell population in L31 mice.

We found that regulatory T cells from L31 mice were impaired in their ability to suppress the proliferation of effector T cells *in vitro*. In part, this was because B7.2 (CD86) expression impeded regulatory T cell suppressive activity. However, regulatory T cells delayed the onset of neurological symptoms *in vivo*.

Although L31 Treg are not suppressive *in vitro*, our *in vivo* data suggest that they have a regulatory function in L31 disease development. This dichotomy could provide insights into the mechanisms by which these regulatory T cells control disease development in L31 mice.

RÉSUMÉ

Le laboratoire du Dr. Fournier a généré une lignée de souris (les souris L31) qui développe de façon spontanée une maladie du système nerveux central qui conduit à la perte de la gaine de myéline et qui est dépendante de la presence de lymphocytes T. Dans ce modèle les lymphocytes CD8+ sont les cellules effectrices de la maladie tandis que les lymphocytes T CD4+ jouent un rôle régulateur. Il a été démontré qu'une sous-population de lymphocytes T CD4+ qui expriment le facteur de transcription Foxp3 est impliquée dans la regulation des réponses auto-immunes. Afin d'étudier le rôle de cette population dans le développement de la maladie neurologique des souris L31, le but de mon projet de recherche était de caractériser de façon fonctionelle cette sous-population de lymphocytes T CD4+ régulateurs des souris L31.

Nous avons trouvé que les lymphocytes T régulateurs des souris L31 sont altérés dans leur capacité à supprimer la proliferation de cellules T effectrices *in vitro*. Ceci est dû en partie à leur expression elevée de la protein B7.2 (CD86). Cependant, les lymphocytes T régulateurs des souris L31 sont capables de prévenir le développement des symptômes neurologiques *in vivo.*

Donc, bien que les lymphocytes T régulateurs des souris L31 ne sont pas suppresseurs *in vitro,* notre données in vivo suggèrent qu'ils ont une fonction régulatrice dans le développement de la maladie neurologique des souris L31. Cette dichotomie pourrait nous permettre de déterminer les mécanismes utilisés

par ces cellules régulatrices pour contrôler le développement de la maladie neurologique auto-immune dans les souris L31.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Sylvie Fournier for her insight and guidance and for providing me with many opportunities during my masters. Thank you to the members of the Fournier lab Mohamad Habbal, Anthony Rainone, Yunlin Tai and Vlad Dragan for their help, suggestions, support and laughter. Mohamad Habbal assisted with the *in vitro* work done in this thesis and provided valuable insight during the project. Thank you to Sarah Lawrie for sharing her technical expertise. Also, I would like to extend my thanks to the department for their feedback and support. Thank you to the CIHR Neuroinflammation Training Program for their funding and for the chance to be part of an amazing committee. Finally, thank you to my family and friends for their continued support.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

neurological symptoms in L31 mice.

LIST OF ABBREVIATIONS

APC	Antigen presenting cells
BSA	Bovine serum albumin
BTLA	B and T lymphocyte attenuator
CFSE	Carboxyfluorescein succinimidyl ester
CNS	Central nervous system
CSF	Cerebral spinal fluid
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DC	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
FBS	Fetal bovine serum
Foxp3	Forkhead box P3
GVHD	Graft versus host disease
HVEM	Herpes virus entry mediator
IBD	Inflammatory bowel disease
IDO	Indoleamine-2,3-dioxygenase
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
iTreg	Induced regulatory T cells
L31	C57BL/6 B7.2 transgenic line 31
LAG-3	Lymphocyte activation gene-3
mRFP	Monomeric red fluorescent protein

mAb	Monoclonal antibody
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NK	Natural killer cells
NOD	Non-obese diabetic
Nrp-1	Neuropillin
nTreg	Naturally occurring regulatory T cells
P1	Frequency of cells that have proliferated
P2	Frequency of cells that have gone through more than 2 divisions
PBS	Phosphate buffered saline
PLP	Proteolipid protein
RR-MS	Relapsing-remitting multiple sclerosis
SHP-2	Src homology protein 2 domain-containing tyrosine phosphatase 2
TCR	T cell receptor
Tg	Transgenic
Treg	$CD4^{+}Foxp3^{+}$ regulatory T cells
Tresp	Responder T cells

1. INTRODUCTION

1.1 B7/CTLA-4/CD28 pathway

Naïve T cell activation requires two independent signals at the immunological synapse. The first signal involves the interaction between the peptide/MHC complex on antigen presenting cells (APC) with the antigen-specific T cell receptor (TCR) on T cells. However, this signal alone may result in an unresponsive state called clonal anergy (1). A secondary costimulatory signal is required. The most well characterized costimulatory molecule constitutively expressed on T cells is CD28, which interacts with B7 family molecules on APC. Collectively, these two signals result in full T cell activation and are illustrated in Figure 1.

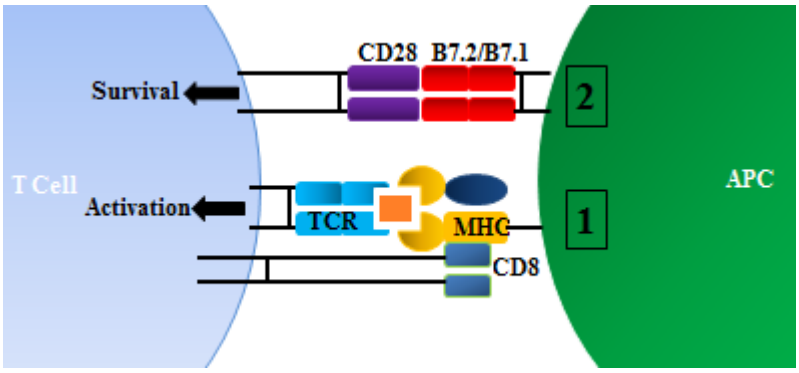

Figure 1: Naïve T cell activation requires two signals. Signal 1 involves the antigen-specific interaction between peptide/MHC complex with the T cell receptor (TCR). Signal 2 includes the interaction between CD28 and B7.1/B7.2.

In order to investigate the role of CD28 in naïve T cell responses, a series of studies explored the proliferative response of T cells from $CD28^{-/-}$ TCR transgenic (Tg) mice (2-4). While $CD28^{+/+}$ TCR Tg controls displayed normal proliferative responses, proliferation of $CD28^{-/-}$ TCR Tg T cells were significantly reduced (5). In addition to failed proliferation, many of the $CD28^{-/-}$ cells had undergone apoptosis. Similarly, normal TCR Tg T cells could not sustain a proliferative response or survive when CD28 interactions were blocked. Therefore, CD28 engagement enhances naïve T cell proliferation and survival. Reports also show that CD28 ligation causes stabilization of IL-2 mRNA, leading to increased IL-2 production (6, 7). IL-2 is an important cytokine needed for cell proliferation and growth. Thus, CD28 ligation may induce proliferation and survival by encouraging the release of cytokines such as IL-2. Consistent with this function, Boise et al. (1995) have shown that CD28 engagement also upregulates anti-apoptotic protein Bcl-xL (8).

While CD28 engagement on T cells is important for delivering signals for T cell survival and proliferation, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is critical for inhibiting T cell responses. This negative regulator is stored intracellularly and is rapidly upregulated on the surface of helper and cytotoxic T cells upon activation (9). $CTLA\text{-}4^{-/-}$ mice develop a fatal lymphoproliferative disorder characterized by infiltration of $CD4^{+}$ T cells into many non-lymphoid tissues, which indicates that CTLA-4 is important in maintaining tolerance to self-antigens (10). CTLA-4 does this through both cell-intrinsic and cell-extrinsic mechanisms. Intrinsically, it has been demonstrated that engagement of CTLA-4

expressed by antigen-specific effector T cells results in decreased IL-2 release, subsequently preventing proliferation and differentiation (11, 12). This may occur through the recruitment of phosphatases such as Src homology protein 2 domain-containing tyrosine phosphatase 2 (SHP-2) to the CTLA-4 cytoplasmic tail, which has been proposed to dephosphorylate CD3 zeta chains and interfere with signals for T cell activation (13, 14). CTLA-4 also competes with CD28 to bind costimulatory ligands at the immunological synapse since CD28 and CTLA-4 bind to the same ligands B7.1 (CD80) and B7.2 (CD86), although CTLA-4 binds with a higher affinity (15). Cell-extrinsic mechanisms are described later with regulatory T cell mechanisms of suppression.

Although engagement of CTLA-4 or CD28 transmits signals to the T cells that express them, B7 molecules have also been shown to transmit signals to APC. For example, B7.2 on B cells can transmit a positive signal that increases IgG1 and IgE production when B7.2 engages CD28 (16, 17). Additionally, engagement of B7.2 on dendritic cells (DC) with CTLA-4 has been shown to induce the release of indoleamine-2,3-dioxygenase (IDO) through an IFNγ-dependent manner (18). IDO is an enzyme that catabolizes tryptophan to by-products that inhibit T cell proliferation. Therefore, bidirectional signalling can occur during B7:CTLA-4/CD28 interactions.

Even though B7 molecules have been established as costimulatory ligands on antigen presenting cells, additional roles for B7.1 and B7.2 expressed on T cells are emerging. In mice, B7.2 is expressed on the resting T cell surface at low levels and is quickly upregulated upon activation, whereas B7.1 is induced after T cells

are activated (19). B7.1 and B7.2 are also found on activated human T cells (20-22). However, upregulation occurs much slower in humans (23). In a mouse model of graft versus host disease (GVHD), T cells with constitutively high expression of B7.2 had a decreased alloresponse (24). In the same study, B7-deficient T cells had an accelerated alloresponse compared to wildtype (WT). This suggested that B7 on T cells can also act as a negative regulator of immune responses.

A key function of host immunity is to differentiate between foreign and self-antigens. Failure to mount an adequate immune response could result in infection, whereas a misguided response against self-antigens leads to autoimmunity. Regulation of T cell responses is critical in mediating this balance. B7/CTLA-4/CD28 molecules play important roles in autoimmunity, which have been demonstrated in various autoimmune murine models. B7/CD28 blockade using CTLA-4Ig, the soluble form of CTLA-4, resulted in decreased disease severity in a murine model of multiple sclerosis called experimental autoimmune encephalomyelitis (25, 26). Similarly, in a spontaneous model of type I diabetes, non-obese diabetic (NOD) mice were resistant to developing disease when treated early with anti-B7.2 mAb (27). CD28-deficient mice were also resistant to disease development in EAE and collagen-induced arthritis (28, 29). Therefore, B7/CD28 interactions are important in the development of autoimmunity.

On the other hand, in vivo blockade of CTLA-4 using anti-CTLA-4 mAb exacerbated EAE (30). NOD mice that express a specific transgenic T cell receptor specific for a pancreatic islet antigen also had worsened diabetes when

CTLA-4 was blocked before the onset of symptoms (31). Thus, in the context of autoimmunity, CTLA-4 is essential to preventing autoreactive immune responses and establishing peripheral tolerance.

Manipulation of the B7/CTLA-4/CD28 pathway clearly has effects in autoimmune models. Regulating the complex interplay between costimulatory molecules and their ligands is therefore an attractive target in the prevention or treatment of autoimmune diseases.

1.2 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). This disease is prominent in developed regions, with the highest prevalence rates in North America, Europe and Australia. It affects women three times more than men and is most commonly diagnosed between 20 and 40 years of age (32). The hallmark characteristics of MS include demyelination and neurodegeneration. Consequently, symptoms involve impaired motor and neurocognitive function. While the disease is not life threatening in the sense that MS patients often have the disease for prolonged periods of time, it has significant debilitating effects on their quality of life.

The most common form of MS is relapsing-remitting MS (RR-MS). About 85-90% of MS patients have this form of the disease which is characterized by episodes of leukocyte infiltration into the CNS, demyelination of white matter and

periods of remission (33). Most of these patients develop secondary progressive MS. In this phase, disease steadily progresses and demyelination in the grey matter is apparent along with axonal degeneration in the white matter (34). The primary progressive form of MS affects approximately 10-15% of patients and involves a steady progression throughout the course of the disease (32). Fulminant MS, also known as Marburg's disease, is an accelerated and rare form of MS which causes severe disability or death in a short time span of weeks to months (35). However, there is some dispute whether Marburg-type MS should be categorized as a separate disease altogether since fulminant patients also develop other conditions not included in the classical MS diagnosis, such as neuromyelitis optica and vascularitis (35).

1.2.1 Pathogenic involvement of T cells in MS

MS is known to be a complex and multi-faceted autoimmune disease with influence from both genetic and environmental factors. The involvement of autoreactive T cells is also implicated in its pathogenesis. Support that $CD4^+$ T cells are primarily involved stems from the abundance of research done using experimental autoimmune encephalomyelitis (EAE), which is the most widely accepted murine model of MS. Susceptible mice immunized with myelin antigen such as myelin oligodendrocyte protein (MOG), myelin basic protein (MBP) or proteolipid protein (PLP) with adjuvant develop Th1/Th17-mediated demyelinating lesions and neurological symptoms. Adoptive transfer of activated $CD4^+$ T cells specific for myelin antigens into a naïve mouse also induces EAE.

There is much less known about the involvement of $CD8^+$ T cells in MS compared to $CD4^+$ T cells. However, appreciation for $CD8^+$ T cells in the pathogenesis of MS is increasing due to several reasons. $CD4^+$ T cells often mediate pathogenic characteristics through the recruitment of other myeloid cells. $CD8^+$ T cells on the other hand, are armed and have the ability to directly engage with cells and inflict damage through cytotoxic mechanisms. Additionally, resident cells of the CNS, except microglia, do not express MHC Class II. Therefore, primary cell types that are susceptible to damage in MS such as oligodendrocytes and neurons can be directly recognized by $CD8^+$ T cells through the expression of MHC Class I. In humans, effector $CD8^+$ T cells have been detected in the cerebral spinal fluid (CSF) of patients with RR-MS (36). Furthermore, oligoclonal expansions of $CD8^+$ memory T cells are more prominent that $CD4^+$ T cells in the cerebral spinal fluid and brain tissue of MS patients (37, 38). In fact, $CD8^+$ T cells predominate over $CD4^+$ T cells in both active and chronic MS lesions (39). Therefore, evidence of $CD8^+$ T cells in human patients provides support that $CD8^+$ T cells may be involved in MS pathology.

Accumulating evidence for the involvement of $CD8^+$ T cells in MS has also been supported by several murine models. $CD8^+$ T cell-mediated EAE has been reported to closely recapitulate MS pathology. MBP-specific $CD8^+$ T cells from mice with EAE conferred neurological symptoms, CNS infiltration and demyelination to naïve recipients (40). Transgenic mouse lines that express OVA on oligodendrocytes present OVA to $CD8^+$ T cells via H2-K MHC Class I (41). These mice spontaneously develop neurological symptoms at 12-19 days of age.

Furthermore, transferring activated OVA-specific $CD8^+$ T cells onto brain slices of mice that express OVA on oligodendrocytes revealed that $CD8^+$ T cells could cause damage to bystander neurons using perforin and granzyme B (42). In another model, transgenic overexpression of PLP in oligodendrocytes results in demyelination, axonal swelling and Wallerian degeneration (43). Crossing the PLP transgenic mice with RAG-1-deficient mice reduced pathology, indicating that symptoms were due to a component of the adaptive immune system. Reconstitution with $CD8^+CD4^-$ bone marrow and not $CD8^-CD4^+$ revived demyelinating symptoms (44). Another model that supports the involvement of $CD8^+$ T cells in demyelination is the L31 model and is described in detail in the following section.

1.3 An animal model of spontaneous $CD8^+$ T cell mediated demyelinating disease: L31 mice

Costimulation can contribute to the development of autoreactive T cells and autoimmunity. It has been suggested that changes in costimulation could play a role in MS (45). This is supported by the L31 transgenic mouse model. L31 mice, which constitutively express high levels of costimulatory ligand B7.2 on their T cells and microglia, spontaneously develop neurological symptoms around 4-5 months of age (46). Neurological symptoms include hind limb splaying, hind limb clasping and poor proprioception when walking on cage bars. These neurological symptoms are associated with demyelination and the infiltration of leukocytes in the spinal cord parenchyma and roots, predominantly in the lumbar region (46).

Although costimulation is a central process that occurs throughout the immune system, histological staining of other organs in preclinical and symptomatic L31 mice, such as the kidney, heart, liver, pancreas, lungs and muscle, showed normal tissue morphology (46). This suggested that the nervous tissue was the primary tissue target affected in L31 mice. Pathology specific to the CNS was partially explained by the dependence of disease on transgene expression in microglia, which are the resident myeloid immune cells of the CNS and have been reported to participate in antigen presentation (47-49). In contrast, L27 mice have transgenic expression of B7.2 on their T cells but not microglia and do not develop neurological symptoms, demonstrating the requirement for B7.2 on microglia for disease development (46).

In this model, disease development is also dependent on the expression of B7.2 on T cells. Transgenic mice deficient in the production of T cells through the deletion of the gene for the β chain of the T cell receptor do not develop neurological symptoms, indicating that the disease is also T-cell dependent (46). Injection of B7.2 transgenic T cells into a $TCR\beta^{-/-}$ L31 recipient, which have transgenic expression of B7.2 on microglia, developed neurological symptoms accompanied with CNS pathology (46). Adoptive transfer of B7.2 transgenic T cells into a wildtype recipient deficient in T cells did not result in disease (46). Collectively, these studies show that neurological symptoms are dependent on transgene expression in both T cells and microglia.

Microglia are activated early in disease development. In L31 mice, microglia characterized by $CD11b^{+}CD45^{int}$ in the CNS showed marked upregulation of

activation markers CD80, CD40, I-A^b and H-$2K^b$ as early as 3 weeks old (50). This microglial activation is prevented in L31 mice that are impaired in IFNγ receptor signalling (50). IFNγ$R^{-/-}$ L31 mice are also resistant to developing neurological symptoms and have a much lower frequency of T cell infiltration in the CNS suggesting that IFNγ receptor signalling is also important in disease development (50).

To further explore the role of T cells in the L31 mouse model, investigation of the two major T cell subsets revealed a cardinal accumulation of highly activated $CD8^+$ T cells in the CNS of symptomatic L31 mice (46). Immunohistochemical analysis showed $CD8^+$ T lymphocytes in close proximity to demyelinated lesions (51). However, it was unknown whether these $CD8^+$ T cells accumulate as they infiltrate into the CNS or if they recognize specific CNS antigen and proliferate within the CNS. To investigate this, L31 mice with a restricted T cell repertoire were generated by crossing L31 mice with an OT-I TCR line, which have T cell receptors specific for ovalbumin. Approximately 95% of the $CD8^+$ T cells in OT-I TCR L31 mice were clonally positive ($V\alpha2^+V\beta5^+$) (50). These mice were resistant to disease development after 12 months of observation, which suggests that a CNS-specific $CD8^+$ T cell response is involved in the accumulation and expansion of $CD8^+$ T cells in the CNS and the development of neurological disease (50).

Given that a restricted T cell repertoire for non-CNS-specific antigen prevented disease development, investigation of CDR3 length distribution of the Vβ chain would reveal a skewed distribution of CDR3 lengths if CNS-specific antigens were involved in $CD8^+$ T cell expansion. While peripheral $CD8^+$ T cells displayed

a Gaussian distribution for all Vβ families similar to controls, $CD8^+$ T cells in the CNS of 7 week old I-$A\beta^{-/-}$ L31 mice had Vβ families which displayed skewed distributions in preclinical L31 mice, suggesting that a CNS-specific antigen response drives $CD8^+$ T cell expansion at an early disease stage (50).

The notion that this disease is dependent on IFNγ receptor signalling and is mediated by activated $CD8^+$ T cells suggested that $CD8^+$ T cells could be involved in MHC-restricted cytotoxic attack as a pathogenic mechanism for demyelination. Upregulation of MHC Class I was evident on oligodendrocytes, which insulate axons by providing myelin sheaths, in the preclinical stage (51). In line with this hypothesis, increased perforin and Fas-L was detected in CNS mononuclear cells and $CD8^+$ T lymphocytes expressed heightened levels of granzyme B and IFNγ (52). $CD8^+$ T cells also expressed CD107a at the cell surface, suggesting degranulation (52). If MHC Class I-restricted cytotoxic attack was a main pathogenic mechanism, it would be expected that L31 mice deficient in either perforin or Fas-L would exhibit less overt disease. Surprisingly, perforin- and Fas-L-deficient L31 mice developed accelerated onset of neurological symptoms accompanied with characteristic predominance of $CD8^+$ lymphocytes in the CNS within 6 weeks of age (52). Therefore, disease pathogenesis does not occur through a perforin- or Fas-dependent manner. Instead, perforin or Fas may be involved in regulatory mechanisms.

In addition to the dramatic accumulation of $CD8^+$ T cells found in the CNS, some $CD4^+$ T cells were also present. In order to study the role of $CD4^+$ T cells in this disease, L31 mice deficient in the gene encoding the β chain of the MHC Class II

molecule I-A were observed for the onset of neurological symptoms. These mice, bred on a C57BL/6 background, are also defective for the MHC Class II molecule I-E. In the absence of MHC Class II molecules, the differentiation of $CD4^+$ T cells in the thymus is impaired and few mature $CD4^+$ T cells are found in peripheral organs. These mice showed accelerated onset of disease and accelerated kinetics compared to L31 mice with functional $CD4^+$ T cells (50). Similarly, L31 mice carrying a deletion of the gene for CD4 also showed similar results (50). These results suggested a pathogenic role for $CD8^+$ T cells and a regulatory role for $CD4^+$ T cells.

1.4 Regulatory T Cells

Several $CD8^+$ and $CD4^+$ regulatory T cell subsets have been described. In mice, a small subset of $CD8^+$ T cells have been shown to restrict autoreactive T cells in autoimmunity though the non-classical MHC Class 1b molecule, Qa-1 (murine counterpart of human HLA-E) (53). Qa-1 deficient mice have exaggerated $CD4^+$ T cell responses and an increased susceptibility to EAE, indicating a role for Qa-1 in immune suppression (54).

$CD8^+CD122^+$ cells have also shown to regulate autoimmunity. Mice deficient in CD122, the β chain of the IL2/IL15 receptor, had an accumulation of activated T cells and abnormally high levels of plasma cells (55). These abnormalities were reversed with the injection of $CD8^+CD122^+$ T cells. Similarly, in an animal model of Graves' disease, systemic administration of anti-CD122 monoclonal antibodies resulted in increased hyperthyroidism (56). Depleting this subset in EAE

increased T cell infiltration and cytokine production in the CNS (57). While the mechanism through which $CD8^+CD122^+$ cells regulate autoimmunity remains to be elucidated, these examples demonstrate a regulatory role for $CD8^+CD122^+$ T cells.

Other $CD8^+$ regulatory T cell subsets have also been described. Human $CD8^+CD28^-$ cells have been shown to suppress alloreactive $CD4^+$ T cell responses in vitro (58, 59). $CD8^+CD28^-Foxp3^+$ T cells have also been shown to secrete suppressor cytokines IL-10 and TGF-β and confer protection in an animal model of myasthenia gravis (60, 61). Regulatory $CD8^+Foxp3^+$ T cells found in both $CD28^+$ and $CD28^-$ populations can be induced by a tolerogenic anti-DNA IgG-derived peptide in an animal model of systemic lupus (62). Another subset of $CD8^+CD11b^+$ T cells has also been shown to regulate a murine model of collagen-induced arthritis (63).

Although CD8 regulatory T cells were the first subset described to induce immune suppression back in the 1970s, research in this area was limited because $CD8^+$ regulatory cells were not as well characterized. In addition to difficulties in characterizing $CD8^+$ regulatory T cells, regulatory cells in the $CD4^+$ T cell compartment were gaining popularity. Since Sakaguchi et al. (1995) characterized a subset of $CD4^+$ regulatory T cells using a component of the IL-2 receptor, CD25, a plethora of research has been done on the $CD4^+CD25^+$ subset (64). However, CD25 also marks activated $CD4^+$ T cells and is therefore not a completely faithful marker of $CD4^+$ regulatory T cells. More recently, discovery of the transcription factor forkhead box P3 (Foxp3) has unlocked a lineage of

$CD4^+$ regulatory T cells, and therefore propelled research in the $CD4^+$ regulatory T cell compartment.

1.4.1 Classification of $CD4^+$ regulatory T cells

A subset of $CD4^+$ T cells known to have a role in regulating immune responses are the $CD4^+Foxp3^+$ regulatory T cells. This population makes up approximately 5-10% of the $CD4^+$ T cells. Natural Treg (nTreg) are committed to their regulatory fate in the thymus and express Foxp3 (65, 66). Adaptive or induced regulatory T cells (iTreg) describe conventional $CD4^+$ T cells that can be induced to express Foxp3 and mediate suppressive effects in the periphery. Other subsets of $CD4^+$ T cells that may have suppressive functions include Tr1 and Th3 T cells. Tr1 cells do not express Foxp3 and secrete suppressive cytokines such as TGF-β (67, 68). Th3 T cells are mostly found in the mucosae and also produce TGF-β, along with IL-4 and IL-10 (67). Classification of $CD4^+$ regulatory T cells is summarized in Figure 2. The focus of this thesis is on $CD4^+Foxp3^+$ regulatory T cells (herein referred to as Treg).

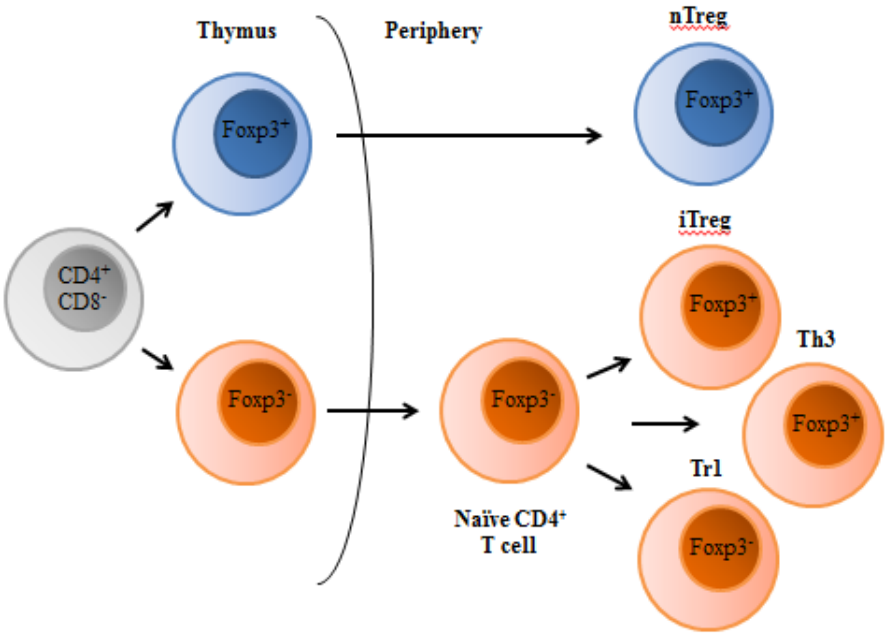

Figure 2: CD4$^+$ regulatory T cell classification. The lineage of regulatory T cells that expresses Foxp3 in the thymus is referred to as natural regulatory T cells (nTreg). Naïve CD4$^+$Foxp3$^-$ T cells can be induced to express Foxp3 (iTreg). Additional CD4$^+$ regulatory T cell subsets include Th3 and Tr1.

1.4.2 Treg characteristics

As their name suggests, Treg are known for their capability to regulate autoreactive T cell responses in a suppressive manner. Characteristics that are unique to this T cell subset describe how they are programmed to carry out their function.

Treg suppressive function is associated with the expression of the transcription factor Foxp3. Mutations in human Foxp3 result in immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (69). A mutation in Foxp3 in mice results in a *scurfy* phenotype, an X-linked mutation that displays multiorgan infiltration of CD4$^+$ T cells and is lethal for hemizygous males (70).

Expression of Foxp3 is important to Treg function since suppressive function is lost in mature Treg when Foxp3 is deficient (71). Furthermore, ectopic expression of Foxp3 on conventional $CD4^+CD25^-$ cells conferred suppressive capabilities and an anergic phenotype (65). Therefore, Foxp3 is a key transcription factor expressed by Treg and is important for suppressive function.

Treg require antigen-specific stimulation for their activation. However, Treg are 10- to 100-fold more sensitive to antigen stimulation than $CD4^+CD25^-$ T cells (72). Treg also differ from conventional $CD4^+$ T cells in their response to stimulation. Although they also require antigen-specific stimulation of the TCR for activation, they are generally anergic *in vitro* and do not proliferate when they are stimulated (73-75). This may be explained by the ability of Foxp3 to bind the transcription factor AP-1, which is involved in regulating T cell proliferation and differentiation (76). Binding of AP-1 hampers its function and therefore may explain why Treg are anergic (76). Furthermore, Foxp3 acts to downregulate cytokine production and Treg are normally restricted in their ability to produce cytokines involved in proliferation such as IL-2, IL-4 and IFNγ (77). However, while Treg are generally considered anergic, addition of exogenous cytokines such as IL-2, IL-4 or IL-15 at high concentrations can induce Treg proliferation (73, 74, 78). Furthermore, the use of TCR transgenic Treg have revealed that $CD4^+CD25^+$ regulatory T cells are capable of proliferation in response to antigens *in vivo* (79, 80). Since Treg do not produce their own IL-2, they rely on exogenous IL-2 and therefore also express high levels of CD25, the IL-2 receptor alpha chain. After Treg are stimulated in an antigen-specific manner, they become

armed to suppress. However, once they are activated they do not require antigen to execute suppression (9, 81).

Treg are key players in regulating autoimmune disease. Several animal models exhibit exacerbated disease when Treg are depleted. Although Treg are key players in regulating autoimmunity, an imbalance of Treg can also influence susceptibility to tumours and pathogens.

1.4.3 Mechanisms of suppression

There are several proposed mechanisms on how Treg can dampen T cell responses. Treg can either act indirectly through targeting APC, or directly on effector T cells.

Since APC function to present antigen to T cells and can provide costimulatory signals for T cell activation, it is not surprising that Treg can target APC in order to regulate immune responses. One such mechanism involves the engagement of CTLA-4 on Treg with B7 molecules on APC and is described in further detail in the next section.

Other indirect mechanisms have also been proposed. Lymphocyte activation gene-3 (LAG-3), a CD4 homologue, expressed on Treg can also interact with MHC Class II on immature dendritic cells (82). This suppresses dendritic cell maturation and therefore their ability to present antigen. Extracellular ATP acts as an indicator of tissue damage and can upregulate costimulatory ligands on DC. Treg can prevent this by catalytically converting ATP to AMP through

sequestering ATP via CD39, which is expressed on all murine Treg and 50% of human Treg (83). Likewise, neuropillin (Nrp-1), a class III semaphorin receptor and coreceptor for vascular endothelial growth factor, is expressed on Treg and acts to prolong interactions between Treg and immature DC (84). When effector T cells are stimulated at low concentrations of antigen, anti-Nrp-1 blocks Treg suppressive activity. It has been suggested that Nrp-1 allows Treg to preferentially interact with DC when antigen is limited and prevents activation of effector T cells.

In addition to acting indirectly on APC, Treg can also directly suppress effector T cells by inducing cell cycle arrest or apoptosis. Some major mechanisms include the secretion of suppressor cytokines, IL-2 inhibition or consumption and cytolysis. Soluble cytokines such as IL-10 and TGF-β are known to be involved in the development of iTreg but have also been shown to mediate cell contact-independent suppression. Both IL-10 and TGF-β are involved in preventing colitis in a model of inflammatory bowel disease and suppressing allergic responses (85-87). IL-35 was more recently discovered and also has potent inhibitory capabilities. It is preferentially expressed by Treg and significantly upregulated during active suppression. In vitro, ectopic expression of IL-35 conferred regulatory activity to naïve T cells and addition of recombinant IL-35 suppressed T cell proliferation (88).

Another contact-independent method of suppression is through regulation of IL-2. Studies have shown that Treg can inhibit IL-2 mRNA production in effector T cells (89, 90). However, another study suggested that Treg can compete for IL-2

through consumption without inhibiting T cell effector production (91). Treg have a high affinity for IL-2 because they express high levels of the IL-2 receptor complex (CD25, CD122 and CD132). Since IL-2 is critical for cell survival and proliferation, depletion of IL-2 from effector T cells impedes their expansion. However, there are some studies in the literature that argue against IL-2 consumption as a major suppression mechanism. For example, Tran et al. (2009) show that adding anti-CD25 antibodies to their system to block IL-2 consumption did not have any effect on suppressive function (92). Even though Treg-mediated suppression through the regulation of IL-2 is under some dispute, it is likely that alterations of IL-2 levels either at the mRNA or protein level is an important mechanism for Treg suppression (93). Particularly, IL-2 consumption has a role in *in vitro* closed systems such as suppression assays (93).

Similar to NK or cytotoxic $CD8^+$ T cells, Treg can induce apoptosis in effector T cells through granzyme-mediated cytolysis. Human Treg can be activated *in vitro* with anti-CD3 and CD46 antibodies to express granzyme A (94). They were able to kill both $CD8^+$ and $CD4^+$ T cells that were activated in a perforin-dependent and Fas-FasL-independent mechanism. Another study showed that activated mouse Treg can upregulate granzyme B and kill through a perforin-independent and granzyme-B-dependent manner (95). Although the mechanisms vary between studies, it appears that Treg have the ability to express cytotoxic molecules under certain conditions.

Galectin-1 may also be involved in Treg-mediated suppression. Blocking galectin-1 in both mouse and human Treg impeded their suppressive capacity

(96). Likewise, galectin-1-deficient mice had reduced Treg activity. This homodimer is part of the β-galactoside binding protein family and binds to glycoproteins such as CD45, CD43 and CD7. Engagement with these glycoproteins induces cell cycle arrest and apoptosis. However, it is not known whether galectin-1 is secreted in close proximity to cells as a soluble cytokine or if it works in a cell-to-cell contact dependent manner.

Various Treg suppressive mechanisms exist and they are not mutually exclusive. There is not a mechanism that is generally considered to be dominant. Rather, depending on the inflammatory context and the experimental model, different mechanisms may prevail over others.

1.4.4 Involvement of costimulatory molecules in Treg mechanisms of suppression

Although there are several proposed mechanisms independent of CD28/B7/CTLA-4, these molecules are also important in Treg function. A key feature of Treg is that they constitutively express CTLA-4. As mentioned, CTLA-4 ligation on antigen-specific effector T cells dampens immune responses through a cell-intrinsic mechanism. It has also been shown that CTLA-4 on Treg attributes cell-extrinsic activity in immune suppression (11). Qureshi et al. (2011) showed that Treg endocytose B7.1 and B7.2 from APC in a CTLA-4-dependent manner (97). As a result, depleting costimulatory ligands on APC restricts engagement of CD28 on effector T cells. Absence of adequate costimulatory signals prevents excessive activation of T cells.

In addition to limiting B7 costimulatory ligands on APC, Treg CTLA-4 engagement with B7 molecules on DC can also induce indoleamine-2,3-dioxygenase release, thereby inhibiting effector T cell proliferation via release of tryptophan by-products (18).

Reports also suggest that engagement of B7 molecules on effector T cells by Treg is involved in downregulating autoreactive responses. Paust et al. (2004) indicated that engagement of B7 molecules on effector T cells by Treg was required for suppression (23). In this study, B7-deficient T cells were resistant to suppression *in vitro*. To test this *in vivo*, multiorgan inflammation was induced by injecting B7-deficient T cells into $Rag2^{-/-}$ BALB/c mice. Despite co-transfer with WT Treg, recipients still developed inflammation, providing consistent results with their *in vitro* data. In a more recent study, May et al. (2007) showed the contrary. *In vitro* suppression assays showed that $B7^{-/-}$ and $B7^{+/+}$ T cells were equally susceptible to Treg-mediated suppression (98). Co-transfer of $B7^{-/-}$ T cells and WT Treg into $Rag1^{-/-}$ C57BL/6J mice suppressed inflammation, showing that B7 on effector T cells and CTLA-4 on Treg interactions were not required for Treg function.

1.4.5 Role of costimulatory molecules in Treg development and homeostasis

CD28 costimulation has a role in Treg thymic development, which is reflected by decreased numbers of $CD4^{+}CD25^{+}$ T cells generated in the thymus in NOD mice that lack CD28 or B7.1/B7.2 and develop accelerated diabetes (81, 99). Tai et al. (2005) reveal two effects of CD28 signalling in Treg thymic development (100). A deficiency in CD28 reduces IL-2 production by $Foxp3^{-}$ cells in the thymus and

periphery, and consequently does not induce upregulation of CD25 and cannot support Treg development (100). While this describes an indirect effect of CD28 on Treg generation, CD28 costimulation is also intrinsically required in the generation of Treg precursors. This was demonstrated when bone-marrow-derived precursors that lack CD28 still do not develop normal levels of Treg even with sufficient IL-2 supply (100).

However, which costimulatory ligand is responsible for this cell-intrinsic requirement for CD28 engagement during thymic development is not clear. NOD mice were more dependent on B7.1 rather than B7.2 during Treg generation (101). Contrastingly, another study on BALB/c mice showed the opposite (102). Furthermore, mice that were heterozygous for B7.1/B7.2 show partial decreases in Treg numbers (99). This suggests that the level of expression of B7 molecules is also important, rather than its absolute presence or absence. While both costimulatory ligands may be important, further studies are needed.

A two-step model has been proposed in attempt to explain Treg development in the thymus that incorporates the requirement for costimulation and IL-2 (103). The first step involves encounter with self-antigen during positive selection in the cortex. Initial models propose that TCR affinity determines cell fate: thymocytes with low-affinity TCR for self-peptide mature into naïve T cells, intermediate-affinity TCR mature into Treg and high affinity TCR are anergic or deleted during negative selection. Secondly, exposure of intermediate-affinity TCR primed cells to IL-2 and B7 ligands from thymic dendritic cells in the medulla is proposed as a second step in determining cell fate. Thymocytes with greater costimulation or IL-

2 affinity may result in switching on Foxp3 expression and regulatory capacity. Absence of IL-2 or costimulatory signals may undergo alternative fates, such as negative selection or maturation as a naïve T cell.

Not only do mice deficient in CD28 have reduced Treg in the thymus, they also have fewer Treg in the periphery (81). As shown through B7.1/B7.2 blockade, continuous expression of CD28 ligands in the periphery is needed for Treg maintenance (99). In humans, CD28 costimulation is essential for Treg expansion and function (104). This leads us to conclude that CD28 is also important for Treg homeostasis in the periphery. However, it is unclear whether CD28 may also regulate Treg population size indirectly through IL-2 production from non-Treg cells, or directly through CD28 engagement on Treg.

1.5 Rationale and goal of M.Sc. project

As discussed previously, our lab has shown that transgenic mice with high expression of B7.2 on their T cells and microglia spontaneously develop neurological symptoms around 4-5 months of age (46). These neurological symptoms were associated with organ-specific demyelination and accumulation of $CD8^+$ T cells in the CNS (46). In this model, both T cells and dysregulated expression of costimulation in the CNS is required for demyelination (46). While the $CD8^+$ T cells are mostly effector memory-like cells, they have an increased ability to cross the blood brain barrier and enter the CNS. Clonal expansion of these $CD8^+$ T cells in the CNS are likely involved as pathogenic mediators of demyelination since disease occurs in the absence of $CD4^+$ T cells (50).

Understanding mechanisms by which pathogenic $CD8^+$ T cells may be regulated in an immune-mediated demyelinating disease could provide valuable insight to both the role of costimulation in immune responses and the regulation of auto-inflammatory responses. Given that L31 mice deficient in the development of $CD4^+$ T cells developed disease with accelerated kinetics (50), we are interested in further investigating a particular subset of $CD4^+$ T cells that may be responsible for this regulatory effect.

While there are many different $CD4^+$ T cells that have regulatory activity, the $CD4^+CD25^+$ regulatory T cell subset is best characterized using established lineage maker, Foxp3. The availability of commercial Foxp3 antibody and the established characterization of this lineage make the $CD4^+CD25^+$ regulatory T cell subset a feasible candidate for investigation. Research has shown that $CD4^+CD25^+$ regulatory T cells are involved in mediating peripheral tolerance to autoreactive T cells as depletion of Treg exacerbates autoimmunity and transfer of Treg can reduce autoimmune disease (72). Furthermore, $CD4^+CD25^+$ regulatory T cells have been shown to be able to regulate $CD8^+$ T cell responses *in vitro* and *in vivo* (90, 105-107).

Initial characterization of Treg in L31 mice has shown that $CD4^+Foxp3^+$ regulatory T cells are increased in frequency and absolute number in the periphery (51). They also harbour a highly activated phenotype characterized by several activation markers ($CD44^{hi}$, $CD69^{hi}$, $CD103^{hi}$ $CD25^{hi}$, CTLA-4^{hi}, $CD62L^{low}$) (51). Consistent with a more activated phenotype, L31 Treg are also more potent producers of IL-10 (51). Since activated Treg are known to be potent suppressors,

the goal of my M.Sc. project was to functionally characterize L31 Treg by evaluating their suppressive activity both *in vitro* and *in vivo*.

2. MATERIALS AND METHODS

2.1 Animal subjects

L31 mice with the B7.2 transgene expressed under the control of the MHC class I promoter and Igμ enhancer were backcrossed on a C57BL/6 background and maintained (108). Genotyping was done by collecting blood from the tail vein into Alsever's solution (114mM dextrose, 27mM $Na_3C_6H_5O_7 \cdot H_2O$, 71mM NaCl). Red blood cells were lysed by treatment with ACK cell lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2-EDTA) and washed with 1X phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4) containing 1% fetal bovine serum (FBS) (GIBCO) and 0.1% NaN_3. Samples were blocked with 2.4G2 and stained with anti-mouse B7.2 (eBioscience). High expression of B7.2 was determined using flow cytometry. C57BL/6 mice without the transgene were used as wildtype (WT) controls. All animal procedures were in accordance with guidelines of the Canadian Council on Animal Care, as approved by the animal care committee of McGill University.

2.2 Preparation of cell suspension

To isolate lymph node cells, mice were anaesthetized with isofluorane (Baxter) and euthanized using cervical dislocation. Inguinal, axillary and mesenteric lymph nodes were extracted and pooled into complete RMPI 1640 medium (GIBCO) supplemented with 10% FBS, 50μM B-mercaptoethanol (Bioshop), 100U/ml

penicillin (Sigma) and 100µg/ml streptomycin (GibcoBRL). Cells were mechanically dissociated through a 70µm cell strainer (BD Falcon). Spleens were extracted the same way but were also treated with ACK cell lysis buffer. Samples were counted using a Bright-Line hemacytometer (Sigma), with exclusion of dead cells using 0.4% trypan blue staining (Sigma).

2.3 Primary T cell isolation by magnetic cell separation

$CD8^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were isolated using magnetic beads (Miltenyi Biotech). Lymph node cell suspensions were labelled with anti-CD8a antibody conjugated to magnetic beads in MACS buffer (1x PBS with 0.5% bovine serum albumin (BSA) and 2mM EDTA, pH 7.3) and incubated for 15 minutes at 4°C on a metal rack. Cells were washed with MACS buffer and centrifuged at 1200 rpm for 5 minutes and passed through a magnetic column (LS, Miltenyi Biotech). $CD8^-$ cells were then incubated with a biotinylated antibody cocktail including anti-CD8, anti-CD11b, anti-CD45R, anti-CD49b and anti-Ter-119. Cells were subsequently incubated with anti-biotin microbeads and PE-conjugated anti-CD25 antibody and then passed through an LD magnetic column (Miltenyi Biotech). The column was discarded and the cells in the flow-through were incubated with anti-PE microbeads. These cells were passed through an MS column in order to separate $CD4^+CD25^+$ and $CD4^+CD25^-$ cells (Miltenyi Biotech).

To increase the purity of the $CD4^+$ populations, $CD4^+CD25^+$ cells were passed through an additional MS column. $CD4^+CD25^-$ cells were subject to an additional

positive selection by incubation with anti-CD4 microbeads and isolated using an LS column. Purity aliquots of cell samples were taken before and after isolation and read by flow cytometry. $CD4^+CD25^+$ cells were consistently ≥ 75% pure for Foxp3. $CD4^+CD25^-$ and $CD8^+$ Tresp consistently had purities of ≥ 90%.

2.4 Primary T cell isolation by flow cytometry cell sorting

Mice on a C57BL/6 background that expressed monomeric red fluorescent protein (mRFP) in cells that express Foxp3 (Jackson Laboratories) were crossed with L31 mice. Cells from these mice were prepared as mentioned above and enriched for $CD4^+$ T cells using magnetic beads (Miltenyi Biotech). $mRFP^+$ cells were isolated using flow cytometry cell sorting on a BD FACSAria.

2.5 Preparation of irradiated splenocytes

Spleen cell suspensions from C57BL/6 mice were irradiated with 3000rad using the GammaCell 1000 (Atomic Energy of Canada Limited, Radiochemical Company). These cells were used as a source of antigen presenting cells in the suppression assay.

2.6 Responder T cell labelling with CFSE

Cells were washed with PBS supplemented with 5% fetal bovine serum (FBS) and resuspended at a concentration of 7 million cells per ml of PBS with 5% FBS. For 1ml of volume, cells were labelled with 0.5µl of 0.5mM CFSE (Invitrogen)

and constantly shaken for 3 minutes. Labelling was quenched by adding equal volume of FBS and washed once with PBS 5% FBS. Samples were subsequently washed twice with complete RPMI medium at 1200 rpm for 5 minutes at 21°C. All labeling was done in the dark and samples were covered with foil.

2.7 Suppression assay

Isolated T cell populations were diluted to desired concentrations in complete RPMI 1640 medium and plated into round-bottom tissue culture-treated 96-well plates (Becton Dickinson) at $[1 \times 10^5 \text{ cells/ml}]_f$. Treg and Tresp were plated at a 1:1 ratio. Irradiated splenocytes were added at $[4 \times 10^5 \text{ cells/ml}]_f$ to all conditions. Anti-CD3 mAb $[0.5\mu g/ml]_f$ (BioLegend) was added to stimulate cells. Samples were incubated at 37°C at 5% CO_2 for 72 hours.

2.8 Addition of blocking antibodies

L31 Treg were treated with either anti-B7.2 antibody or ratIgG for 30 minutes at 4°C at a concentration of 100µg/ml for $4x10^5$ Treg. Excess antibody was then washed out with PBS, and cells were resuspended in complete RPMI 1640 medium to desired concentration and plated in suppression assay as described above.

For the addition of anti-B7.2 antibody directly to the suppression assay for use of a positive control, antibody was added to stimulated culture at $[50\mu g/ml]_f$.

To confirm the suppressive effects of blocking B7.2 on L31 Treg were due to blocking B7.2 on the Treg, and not B7.2 blocking antibody having a direct effect on other cell types in culture, the same quantity of B7.2 mAb used to pre-treat L31 Treg (5µg) was added directly to stimulated culture for a final concentration of [1.25µg/ml]. This "theoretical maximum" was determined by calculating how much anti-B7.2 mAb would be in cell culture if 100% bound to L31 Treg during pre-treatment.

2.9 Flow cytometry staining

Suppression assay samples were collected into FACS tubes, washed with PBS and labelled with LIVE/DEAD amine-reactive violet viability marker according to the manufacturer's protocol (Invitrogen). Cells were then blocked with 2.4G2 and flow cytometry staining was then done for cell surface markers (anti-mouse CD4 or CD8).

Purity samples of one million cell aliquots were placed into FACS tubes (BD Falcon) and blocked with 2.4G2 for 30 minutes at 4°C. Cells were then labeled with primary antibodies specific for cell surface molecules (anti-mouse CD4, CD25, CD8, CD44 and/or CD62L). Intracellular staining for Foxp3 was done using the Foxp3 staining kit (eBioscience). Following permeabilization and fixation, samples were blocked with 2.4G2 and stained with anti-mouse FoxP3 (BioLegend).

Samples were acquired on the BD Fortessa (BD Biosciences) with FACSDiva software. Data was analyzed using FlowJo 7.6.5.

2.10 Adoptive transfer

Treg were isolated using magnetic beads as described above and injected intravenously through the tail vein into age and sex matched $CD4^{-/-}$ L31 recipients that were 6-8 weeks old. Recipients and non-injected controls were monitored weekly for the onset of neurological symptoms on a binary scale. Mice were considered symptomatic if they displayed hind limb clasping, splaying and poor proprioception when walking on cage bars.

2.11 Statistical analysis

Percent suppression was calculated using the formula:

$$\text{Percent suppression} = 1 - \left(\frac{\%\ \text{proliferation of stimulated Tresp in the presence of Treg}}{(\%\ \text{proliferation of stimulated Tresp} - \%\ \text{proliferation of unstimulated Tresp})}\right) \times 100$$

Data are expressed as mean ± standard deviation, which were calculated using GraphPad Prism5. Statistical difference between groups was calculated using student's T test with values of $p \leq 0.05$ considered as statistically significant.

3. RESULTS

3.1 In vitro suppressive activity of L31 Treg on WT Tresp

In order to functionally characterize the suppressive activity of L31 Treg *in vitro*, we used a standard suppression assay as described in section 2.7. In brief, responder T cells (Tresp) were stimulated with an anti-CD3 mAb to cross-link the T cell receptor, and in the presence of irradiated APC. The proliferation was tracked by labelling Tresp with carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye that is incorporated into the cytoplasm by linking amino groups to form stable covalent bonds (109). Florescence from labelled cells is detected by flow cytometry. When labelled cells divide, the concentration of CFSE is diluted in the daughter cells and the frequency of cells that enter cell cycle can be tracked. Furthermore, the number of cell divisions can also be determined. To assess the suppressive capacity of Treg, the proliferation of Tresp was compared in the presence and absence of Treg. The cell culture conditions are summarized in Table 1.

Table 1: Suppression assay: cell culture conditions

	Cell culture components				
Condition	Tresp	APC	Anti-CD3	WT Treg	L31 Treg
Unstimulated	+	+	-	-	-
Stimulated	+	+	+	-	-
Stimulated + WT* Treg	+	+	+	+	-
Stimulated + L31° Treg	+	+	+	-	+

*WT=C57BL/6
°L31=C57BL/6 B7.2 transgenic line 31

3.1.1 Suppressive activity of L31 Treg on WT $CD4^+CD25^-$ Tresp

It is now clearly established that WT Treg are potent suppressors of WT Tresp *in vitro* (73, 74, 78, 110, 111). To validate our culture conditions, we monitored the effects of WT Treg on WT $CD4^+CD25^-$ Tresp (herein referred to as $CD4^+$ Tresp). In the absence of anti-CD3 mAb, Tresp cells did not proliferate, as expected (Figure 3A). Addition of anti-CD3 mAb allowed robust proliferation of Tresp (Figure 3B), as indicated by 93.5 ± 3.4% of cells that proliferated (P1) and 70.4 ± 7.7% of cells that went through more than 2 divisions (P2) (n=3). As expected, WT Treg effectively suppressed the proliferation of WT Tresp. Indeed, a marked decrease in the frequency of cells that proliferated and went through more than 2 cell divisions was observed (Figure 3C). Only 29.9 ± 7.1% of the Tresp proliferated and only 8.4 ± 3.0% of the cells went through more than 2 divisions ($p \leq 0.001$, n=3).

Compared to WT Treg, L31 Treg were clearly not as effective at suppressing Tresp proliferation (Figure 3D). In the presence of L31 Treg, 71.3 ± 13.4% of cells proliferated and 28.4 ± 10.1% divided further, which is significantly higher than in the presence of WT Treg ($p \leq 0.01$, n=3). Although L31 Treg exhibited some suppressive capacity, with a percent suppression of Tresp of 22.6 ± 11.6%, it was markedly lower than WT Treg, which suppressed 67.4 ± 7.3% of Tresp (Figure 3E) ($p \leq 0.01$, n=3). The proliferation frequencies of cells that entered cycle and cells that went through more than 2 cell divisions are summarized in Figure 3F. These data indicate that L31 Treg are impaired in their ability to suppress WT $CD4^+$ Tresp.

3.1.2 Suppressive activity of L31 Treg on $CD8^+$ WT Tresp

Since $CD8^+$ T cells are the main pathogenic T cell subset in the L31 model, we investigated the effect of L31 Treg on WT $CD8^+$ Tresp as this would reflect the ability of L31 Treg to regulate a $CD8^+$ T cell-mediated immune response. In the absence of regulatory T cells, 76.9 ± 4.6% of the stimulated WT $CD8^+$ T cell responders proliferated with 51.6 ± 11.3% of cells going through more than 2 divisions (Figure 4B, 4F). Addition of WT Treg resulted in a decreased proliferation of Tresp with only 59.6 ± 4.5% of cells entering cycle ($p \leq 0.001$, n=4) and 25.5 ± 8.6% of cells that divided further than 2 divisions ($p \leq 0.01$, n=4) (Figure 4C, 4F). Of note, WT Treg did not suppress $CD8^+$ WT Tresp as effectively as $CD4^+$ WT Tresp (compare Figures 3C and 4C).

Surprisingly, in comparison with WT Treg, L31 Treg showed an opposite effect on WT $CD8^+$ Tresp. Instead of having a suppressive effect, L31 Treg promoted proliferation of WT $CD8^+$ Tresp with P1 and P2 values of 93.8 ± 2.5% and 74.6 ± 2.8%, respectively (Figure 4D, 4F) ($p \leq 0.05$, n=4). Percent suppression of Tresp with WT Treg was 19.1 ± 4.3% compared to -27.8 ± 9.2% with L31 Treg (Figure 4E) ($p \leq 0.01$, n=4). These data indicate that WT and L31 Treg behave differently on WT $CD8^+$ T cell responders. Furthermore, not only are L31 Treg unable to suppress, they promote proliferation of these Tresp.

3.1.3 Suppressive activity of electronic cell sorted L31 Treg on WT $CD8^+$ Tresp

Our method of cell isolation with magnetic beads selects cells based on the expression of cell surface markers CD4 and CD25 (Section 2.3). The main marker for Treg is Foxp3, which is intracellular. Both WT and L31 Treg isolated by magnetic beads using the CD4 and CD25 markers were ≥ 75% positive for Foxp3. We therefore used an alternate method to isolate L31 Treg to address whether the enhanced proliferation of WT $CD8^+$ Tresp was due to the ~25% cell contaminants. To this end, mice expressing the fluorescent protein mRFP in cells which express Foxp3 were crossed with L31 mice to generate $mRPF^+Foxp3^+$L31 mice. Cells were isolated from these mice and sorted by electronic cell sorting as described in section 2.4 to yield a higher purity of Treg specifically selected using the intracellular marker Foxp3.

In the suppression assay, about 97.2% of stimulated WT $CD8^+$ Tresp cells proliferated in the presence of electronic cell sorted Treg (Figure 5C), which was a slightly greater frequency compared to the stimulated condition with 93.4% of cells that proliferated (Figure 5B). However, the effect is more drastic when the frequency of cells that went through more than 2 divisions is considered. These data show that L31 Treg isolated using electronic cell sorting yielded similar results to L31 Treg isolated with magnetic columns.

3.1.4 Effect of B7.2 transgene expression on L31 Treg suppressive activity

In order to assess the contribution of the expression of the B7.2 transgene to the impaired suppressive ability of L31 Treg, B7.2 on L31 Treg was blocked using anti-B7.2 mAb to mask its interactions with the its receptors. L31 Treg were pre-treated with ratIgG or anti-B7.2 mAb and then used in the suppression assay. Only 15.9 ± 3.3% of WT $CD4^+$ Tresp proliferated in the presence of anti-B7.2-treated L31 Treg, which was remarkably less compared to untreated L31 Treg (Figure 6). Similarly, suppressive activity of L31 Treg on $CD8^+$ WT Tresp was restored when B7.2 on L31 Treg was blocked as only 42 ± 5.5% of WT $CD8^+$ Tresp proliferated compared to 93.1 ± 0.8% of Tresp that proliferate in the presence of untreated L31 Treg (Figure 7) ($p \leq 0.01$, n=3). Notably, more suppression was seen with treated L31 Treg than WT Treg on the same $CD8^+$ WT Tresp (compare Figures 7C and 4C) suggesting that L31 Treg have the potential to be more potent suppressors than their wildtype counterparts. Thus, masking B7.2 interactions on L31 Treg restores their suppressive activity on WT $CD4^+$ and $CD8^+$ Tresp and therefore indicates that high B7.2 expression on L31 Treg interferes with Treg suppression.

It is possible that blocking B7.2-mediated costimulation would prevent Tresp proliferation. We therefore wanted to assess whether the restored suppressive activity of anti-B7.2 treated L31 Treg was due to blocking B7.2 expressed by L31 Treg, rather than blocking B7.2-mediated costimulation of Tresp. To address this, anti-B7.2 mAb was added directly to stimulated cell culture conditions at 50µg/ml to demonstrate that blocking B7.2-mediated costimulation will prevent Tresp

proliferation. Indeed, adding B7.2 at 50µg/ml directly to Tresp prevented their proliferation (data not shown). We also added directly to the culture of Tresp the "theoretical" maximum amount of mAb that binds the L31 Treg as described in section 2.8. Figure 8B shows that when this amount of mAb was added directly to the cell culture, only a slight inhibition of the proliferation was observed. In contrast, when the same amount of mAb was used to pre-treat L31 Treg, much more inhibition of proliferation was observed (Figure 8C). These data are consistent with the interpretation that the suppression observed with pre-treated L31 Treg results from the blocking of B7.2 expressed on L31 Treg, and not from the mAb directly blocking B7.2-mediated costimulation of Tresp.

3.2 In vitro suppressive activity of L31 Treg on L31 Tresp

3.2.1 L31 $CD4^+CD25^-$ T cell responders are refractory to Treg-mediated suppression

To get a better understanding of the key players interacting in the L31 model, we evaluated the effect of L31 Treg on L31 Tresp, which also express the B7.2 transgene. Stimulated L31 $CD4^+$ Tresp showed 91.4 ± 3.1% of cells entered cycle and 66.8 ± 14.8% went through more than 2 divisions (Figure 9B, 9F). In the presence of WT Treg in cell culture, 74.4 ± 6.4% of Tresp proliferated, whereas 35.4 ± 11.4% of cells went through more than 2 divisions. Thus, WT Treg had minimal suppressive effects on L31 $CD4^+$ Tresp (Figure 9) ($p \leq 0.05$, n=3). Of note, the same WT Treg were potent suppressors of WT Tresp (Figure 3C). L31

Treg were slightly more suppressive than WT Treg with average P1 and P2 values at 65.4 ± 14.7% and 29.1 ± 12.8% respectively. However, differences in the percent suppression between WT and L31 Treg did not reach statistical significance (Figure 9E, 9F) ($p > 0.05$, n=3). As it was observed that WT Treg could not suppress L31 $CD4^+$ Tresp proliferation as effectively as WT Tresp, and that suppressive capacity between both WT and L31 Treg were not significantly different we conclude that L31 $CD4^+$ Tresp are resistant to Treg-mediated suppression in these conditions.

3.2.2 L31 $CD8^+$ T cell responders are refractory to Treg-mediated suppression

Stimulated L31 $CD8^+$ Tresp exhibited low CFSE expression as shown by a CFSE peak shifted to the left, indicating that all cells had gone through robust proliferation (Figure 10B). WT Treg, which were shown to suppress WT $CD8^+$ Tresp (Figure 4C), were unable to suppress L31 $CD8^+$ Tresp. Close to 100% of cells proliferated in all stimulated conditions, regardless of co-culture with regulatory T cells (Figure 10F). Similarly, L31 Tresp showed an almost identical proliferation profile in the presence of L31 Treg (Figure 10C, 10D). Since there were no significant differences between the suppressive capacity of WT and L31 Treg, these results indicate that $CD8^+$ L31 Tresp were also refractory to Treg-mediated suppression.

3.3 In vivo suppressive activity of L31 Treg

Although L31 Treg were not suppressive *in vitro*, we wanted to assess their role *in vivo* before eliminating them as prospective regulators of L31 disease. Either WT or L31 Treg were purified using magnetic columns and injected intravenously through the tail vein into $CD4^{-/-}$ L31 mice. Recipients were monitored weekly for the onset of neurological symptoms as described in section 2.10 and compared to non-injected controls. Mice that received WT Treg displayed a delayed onset of disease development compared to controls (Figure 11). Strikingly, recipients with L31 Treg did not develop neurological symptoms after 28 weeks of observation. These data also show that L31 Treg are able to regulate disease development and delay the onset of neurological symptoms *in vivo*. In addition, these data further support the notion that L31 Treg may have the capacity to be more potent suppressors than WT Treg.

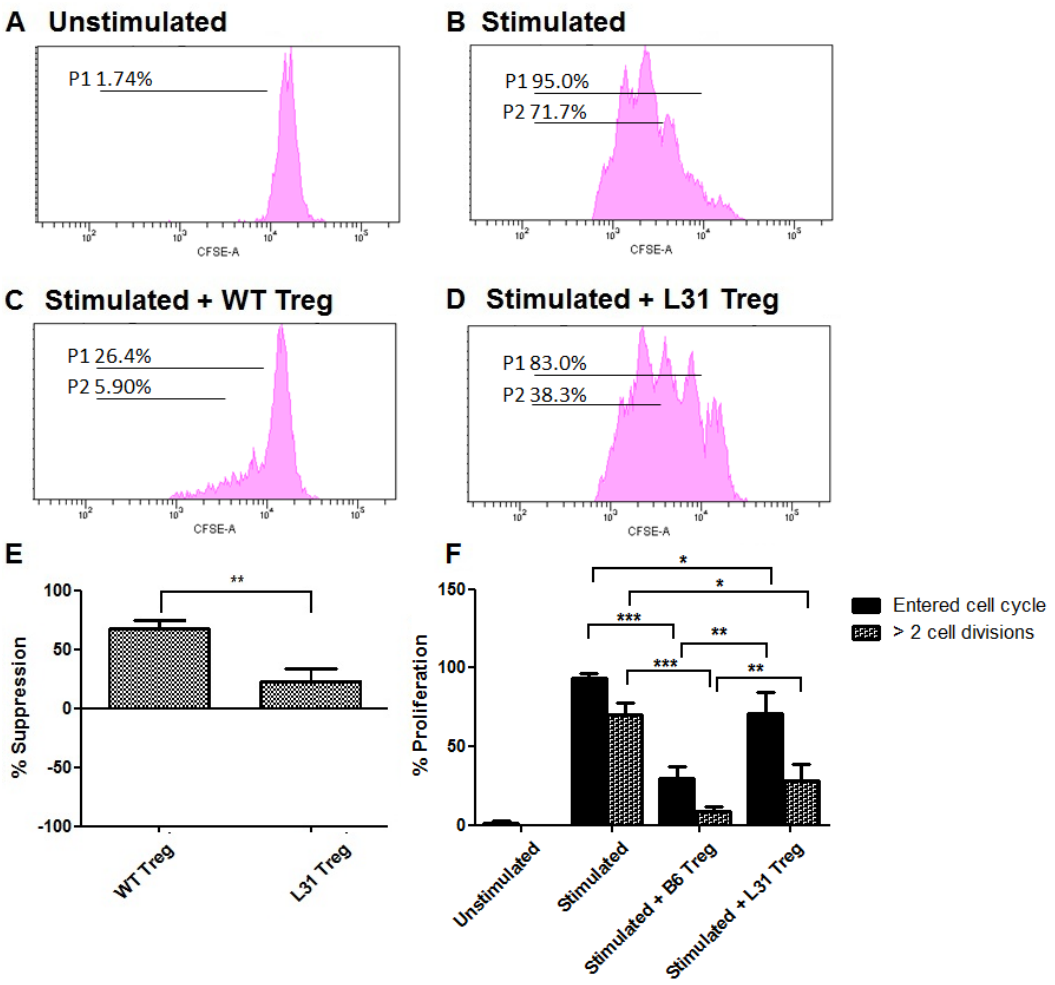

Figure 3: L31 Treg are impaired in their ability to suppress WT CD4$^+$CD25$^-$ T cell responders. (A-D) Representative flow cytometry histograms of WT CD4$^+$CD25$^-$ T cell proliferation. Cells are gated on CD4$^+$CFSE$^+$ with the exclusion of dead cells. P1 shows the frequency of cells that entered cell cycle and P2 indicates the frequency of cells that had undergone more than 2 cell divisions. Data shown are representative of three independent experiments. (E) Percent suppression of WT CD4$^+$CD25$^-$ Tresp (F) Percent proliferation of Tresp that entered cell cycle or went through more than 2 cell divisions in various conditions. Data shown are the mean of three independent experiments, error bars indicate standard deviation; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

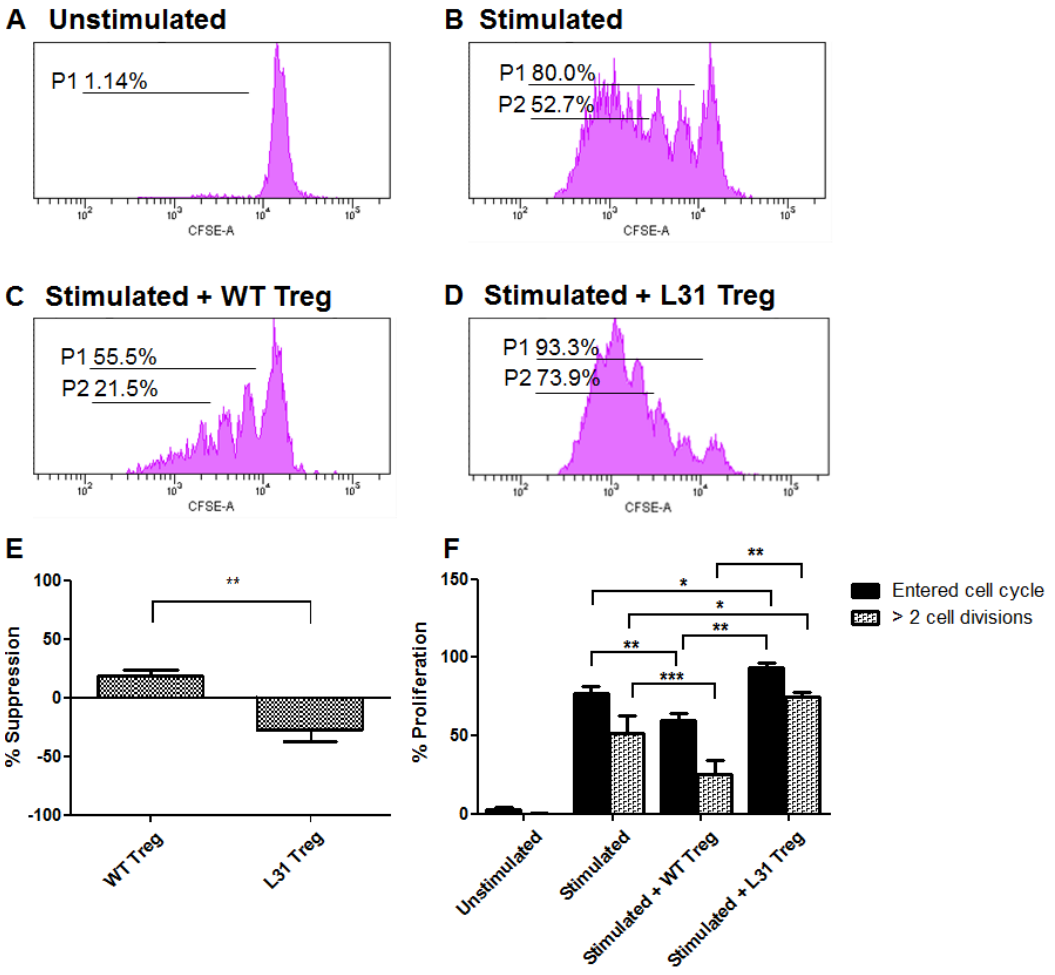

Figure 4: L31 Treg promote proliferation of WT CD8$^+$ T cell responders. (A-D) Representative flow cytometry histograms of WT CD8$^+$ T cell proliferation. Cells are gated on CD8$^+$ with the exclusion of dead cells. P1 shows the frequency of cells that entered cell cycle and P2 indicates the frequency of cells that had undergone more than 2 cell divisions. Data shown are representative of four independent experiments. (E) Percent suppression of WT CD8$^+$ Tresp (F) Percent proliferation of Tresp that entered cell cycle or went through more than 2 cell divisions in various conditions. Data shown are the mean of four independent experiments, error bars indicate standard deviation; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

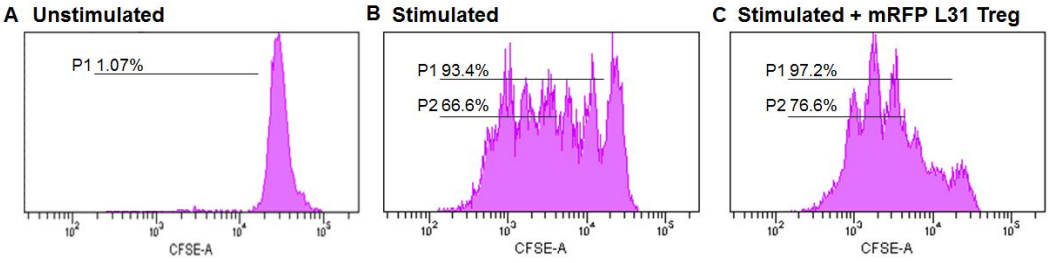

Figure 5: L31 Treg isolated via electronic cell sorting also promote proliferation of WT CD8$^+$ T cell responders. (A-C) Flow cytometry histograms of WT CD8$^+$ T cell proliferation. Cells are gated on CD8$^+$CFSE$^+$ with the exclusion of dead cells. P1 shows the frequency of cells that entered cell cycle and P2 indicates the frequency of cells that had undergone more than 2 cell divisions. Data shown is representative of one experiment.

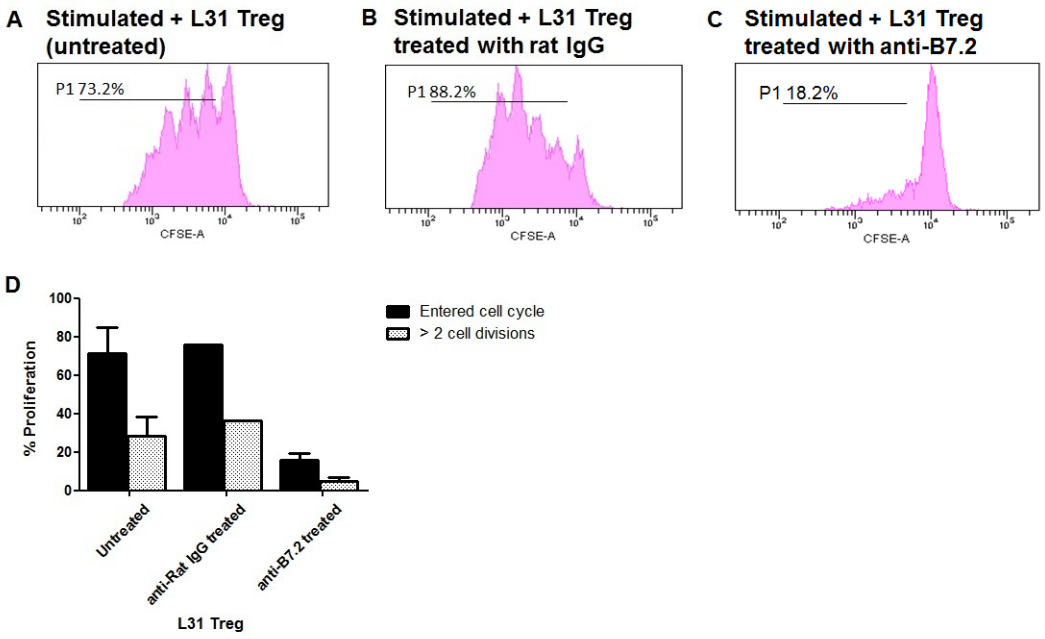

Figure 6: B7.2 impedes L31 Treg ability to suppress WT $CD4^+CD25^-$ T cell responders. (A-C) Flow cytometry histograms of WT $CD4^+CD25^-$ T cell proliferation. Cells are gated on $CD4^+CFSE^+$ with the exclusion of dead cells. P1 shows the frequency of cells that entered cell cycle. Data shown are representative of two independent experiments. (D) Percent proliferation of Tresp that entered cell cycle or went through more than 2 cell divisions in the presence of L31 Treg. Data shown are the mean of two independent experiments, error bars indicate standard deviation.

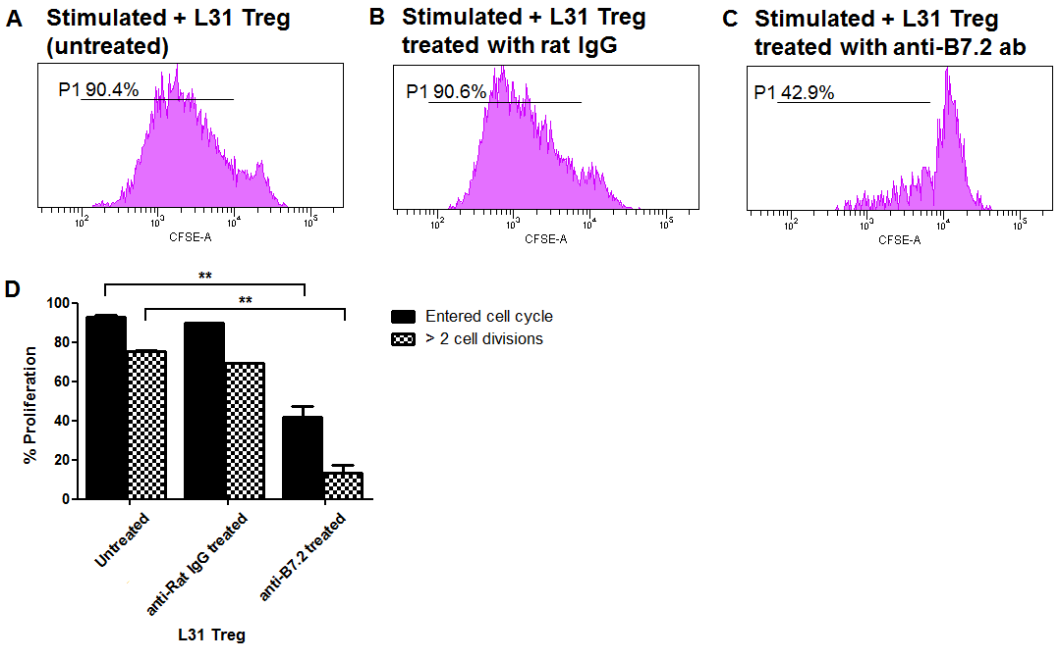

Figure 7: B7.2 impedes L31 Treg ability to suppress WT CD8$^+$ T cell responders. (A-C) Flow cytometry histograms of WT CD8$^+$ T cell proliferation. Cells are gated on CD8$^+$CFSE$^+$ with the exclusion of dead cells. P1 shows the frequency of cells that entered cell cycle. Data shown are representative of three independent experiments. (D) Percent proliferation of Tresp that entered cell cycle or went through more than 2 cell divisions in the presence of L31 Treg. Data shown are the mean of three independent experiments, error bars indicate standard deviation; ** $p \leq 0.01$.

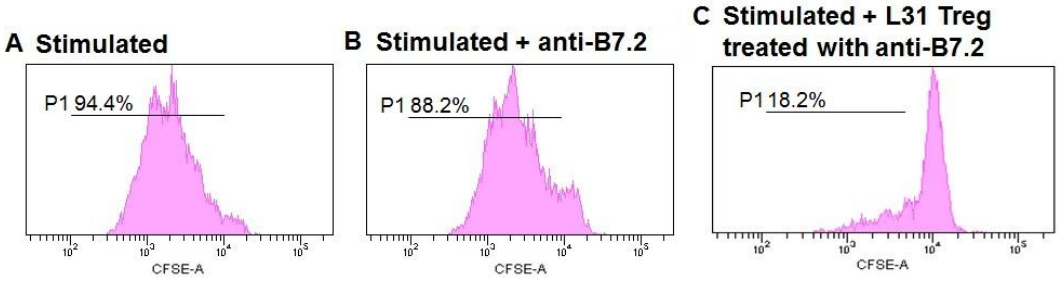

Figure 8: Suppression observed from L31 Treg treated with anti-B7.2 mAb is not due to B7.2-mediated costimulation. (A-C) Flow cytometry histograms of WT $CD4^{+}CD25^{-}$ T cell proliferation. Cells are gated on $CD4^{+}CFSE^{+}$ with the exclusion of dead cells where P1 shows the frequency of cells that entered cell cycle. (B) The same amount of anti-B7.2 antibody used to pre-treat L31 Treg was added directly to stimulated culture in the absence of L31 Treg.

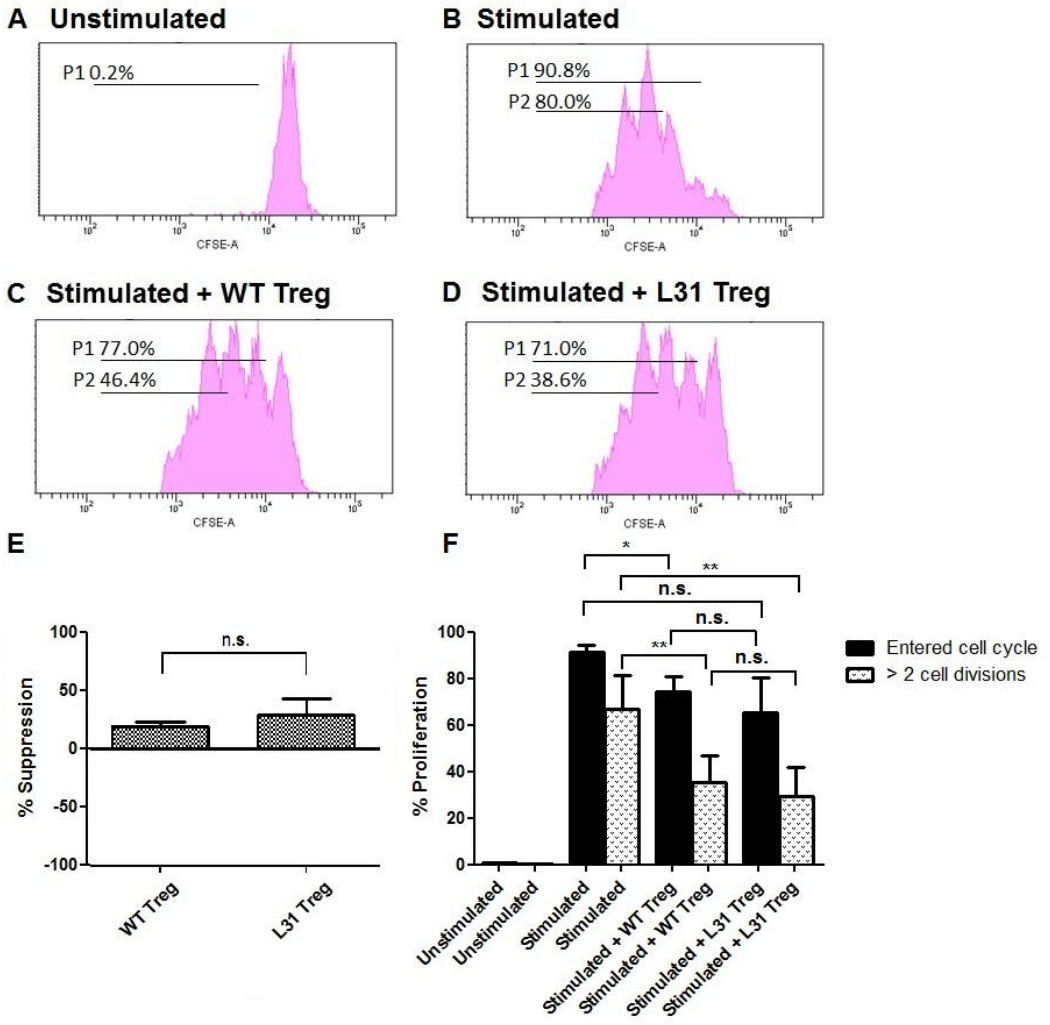

Figure 9: L31 CD4$^+$CD25$^-$ T cell responders are refractory to Treg-mediated suppression. (A-D) Representative flow cytometry histograms of L31 CD4$^+$CD25$^-$ T cell proliferation. Cells are gated on CD4$^+$CFSE$^+$ with the exclusion of dead cells. P1 shows the frequency of cells that entered cell cycle and P2 indicates the frequency of cells that had undergone more than 2 cell divisions. Data shown is representative of three independent experiments. (E) Percent suppression of L31 CD4$^+$CD25$^-$ Tresp (F) Percent proliferation summary of Tresp that entered cell cycle or went through more than 2 cell divisions in various conditions. Data shown are the mean of three independent experiments, error bars indicate standard deviation; * $p \leq 0.05$; ** $p \leq 0.01$; n.s. indicates that there were no significant differences between groups.

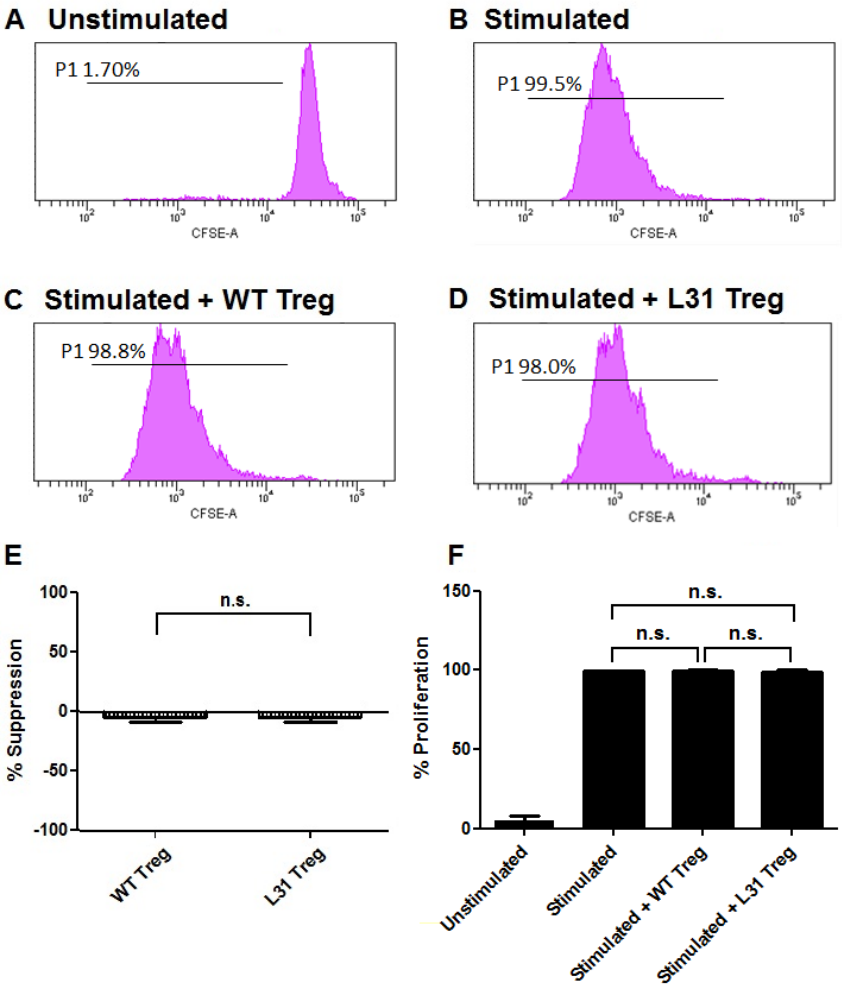

Figure 10: L31 CD8$^+$ responder T cells are refractory to Treg-mediated suppression. (A-D) Representative flow cytometry histograms of L31 CD8$^+$ T cell proliferation. Cells are gated on CD8$^+$CFSE$^+$ with the exclusion of dead cells. P1 shows the frequency of cells that entered cell cycle. Data shown is representative of three independent experiments. (E) Percent suppression of L31 CD8$^+$ Tresp (F) Percent proliferation of Tresp that entered cell cycle in various conditions. Data shown are the mean of three independent experiments, error bars indicate standard deviation; n.s. indicates that there were no significant differences between groups.

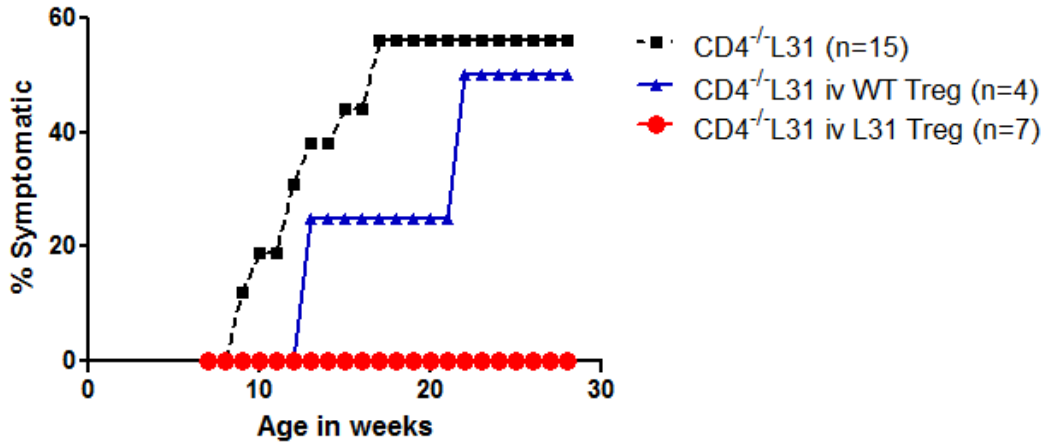

Figure 11: L31 Treg are suppressive *in vivo* and delay the onset of neurological symptoms in L31 mice. Cells were extracted from WT or L31 mice and Treg were isolated using magnetic beads. Cells were warmed at 37°C for 30 minutes and injected intravenously into 6-8 week old $CD4^{-/-}$ L31 recipients. Mice were observed weekly for hind limb clasping. $CD4^{-/-}$ L31, non-injected controls (n=15); $CD4^{-/-}$ L31 iv WT Treg, recipients that received WT Treg intravenously (n=4); $CD4^{-/-}$ L31 iv L31 Treg, recipients that received L31 Treg intravenously (n=7). All mice were monitored during the same time period for a total of 28 weeks.

4. DISCUSSION/CONCLUSION

In this thesis, we functionally characterized the Treg population in L31 mice, a model of spontaneous $CD8^+$ T cell-mediated demyelinating disease, in order to assess L31 Treg suppressive capacity and role in disease development. The approaches we used involved standard *in vitro* suppression assays and adoptive transfer experiments. Our results revealed that L31 Treg were not suppressive compared to WT *in vitro*. Blocking B7.2 on L31 Treg restored its ability to suppress, thereby indicating that B7.2 plays a role in hindering Treg suppressive activity. *In vivo,* L31 Treg delayed the onset of neurological symptoms in this autoimmune mouse model.

Data showed that B7.2 on L31 Treg impeded suppressive activity. To explain this, B7.2 on Treg is impairing suppressive mechanisms or is providing signals that result in T cell responder proliferation. The known receptors of B7.2, CD28 and CTLA-4, are expressed by both Treg and T cell responders in our system. These *in vitro* results may be explained by understanding the interactions of these molecules. We propose two hypotheses that take these interactions into consideration: 1) High expression of B7.2 on L31 Treg interferes with CTLA-4 suppressive function and 2) L31 Treg act as a source of costimulatory ligands for CD28 on Tresp.

Several mechanisms of suppression that involve the engagement of CTLA-4 have been proposed. Treg can indirectly suppress Tresp proliferation by sequestering

costimulatory ligands on APC via ligation of CTLA-4 (97). Engagement of Treg CTLA-4 with its ligand prevents APC from providing costimulatory signals to responder T cells, which consequently results in decreased proliferation of Tresp (97). Bidirectional signalling may also occur since CTLA-4 engagement with B7.2 on DC has been reported to cause the release of IDO and inhibit T cell proliferation (18). Another possibility is that CTLA-4 could be suppressing via interaction with B7 molecules directly on Tresp. This mechanism has not been well established, as research shows conflicting results between Paust et al. (2004) which claims B7 molecules on Tresp are required for Treg-mediated suppression and May et al. (2007) which argue that B7-deficient Tresp are just as susceptible to suppression as B7-sufficient Tresp (23, 98). There are several possibilities by which Treg can suppress through CTLA-4.

High expression of B7.2 on L31 Treg may be interfering with CTLA-4 on Treg and hindering its ability to carry out suppressive function. Diverting this interaction by treatment with anti-B7.2 mAb thereby allows CTLA-4 to interact with its ligands on other cells, such as APC or Tresp. This hypothesis could be tested by blocking CTLA-4 expression on L31 Treg. Treating L31 Treg with anti-CTLA-4 mAb prior to the suppression assay or treating them with siRNA would block CTLA-4-mediated suppression and indicate if L31 Treg are suppressing in a CTLA-4-dependent manner *in vitro*.

The idea that B7.2 expression on Treg can interfere with CTLA-4 function may have physiological relevance such that B7.2 could act as a competitive inhibitor of CTLA-4 binding in *trans*. This may occur in *cis,* in which cell surface molecules

on the same cell surface interact, and could add another layer of complexity to the CTLA-4/B7/CD28 system. In L31 Treg, regulating CTLA-4 would alter Treg ability to suppress in a CTLA-dependent manner. Cell surface molecule interactions in *cis* have been reported in NK cell receptors that are specific for MHC-I (112). More recently, tumour necrosis factor family receptor herpes virus entry mediator (HVEM) on T cells has been shown to engage ligand B and T lymphocyte attenuator (BTLA) in both *cis* and *trans* (113). In this study, along with other examples, it was proposed that *cis* binding acts as a competitive inhibitor for *trans* interactions (114). This hypothesis would also be consistent with the more activated phenotype of L31 Tresp, which also constitutively express B7.2. If B7.2 was interfering with CTLA-4 down regulating signals during T cell activation, activation signals could ensue and therefore Tresp would also have a lower activation threshold in the absence of dampening signals.

In the second hypothesis, the expression of B7.2 on L31 Treg could be providing an additional source of costimulatory ligand and interact with CD28 on Tresp. This would result in an increased proliferation of responder T cells. Blocking B7.2 interactions on L31 Treg could prevent this interaction. Therefore, when B7.2 is blocked there would be less proliferation of Tresp in the absence of additional costimulatory signals. Addition of irradiated L31 effector T cells to stimulated Tresp and measuring Tresp proliferation would inform us if T cells high in B7.2 expression could act as a source of costimulatory ligands for labelled T cell responders.

Our data also show that Treg more efficiently suppress $CD4^+$ Tresp than $CD8^+$ Tresp. This has been suggested by other groups, such as May et al. (2007) who induced a wasting disease by injecting T cells into $Rag2^{-/-}$ mice (98). When they co-transferred T cells with WT Treg, disease symptoms were prevented. Difference in the $CD4^+$ T cell proliferation with and without Treg was much greater than that of $CD8^+$ T cells. Therefore, the group suggested that Treg are more efficient suppressors of $CD4^+$ than $CD8^+$ T cells.

Although total cell numbers in cultures were not determined, there were some noticeable observational differences between cell culture conditions. In general, unstimulated cell cultures had relatively few viable cells compared to cells in stimulated culture conditions. This was expected as activation with CD28 costimulation has been shown to enhance the survival of T cells (115). L31 $CD8^+$ Tresp were generally much more proliferative and had greater total cell numbers than corresponding WT $CD8^+$ Tresp cultures. This was reflected in the percentage of cells that entered cell cycle. Stimulated L31 $CD8^+$ Tresp had proliferated several times as indicated by a CFSE peaks shifted to the left (Figure 10B). Also, culture conditions that contained L31 Treg commonly had greater total cell numbers than conditions that contained WT Treg. This was consistent with the observation that L31 Treg promote proliferation of Tresp and is apparent in L31 $CD8^+$ Tresp with L31 Treg (compare Figures 4C and 4D).

L31 Tresp were refractory to Treg-mediated suppression in the same experimental conditions as WT Tresp. An explanation for these observations could be the difference in the activation state of WT and L31 Tresp. Baecher-Allan et al.

(2001) stimulated $CD4^+CD25^-$ cells using different strengths of activation (111). Co-cultured with regulatory $CD4^+CD25^+$ cells, the group showed more proliferation of $CD4^+CD25^-$ Tresp in conditions that were more strongly activated, thereby demonstrating that strongly activated Tresp are less responsive to suppression (111). When we examined the activation profile of $CD8^+$ Tresp, indeed we found that L31 $CD8^+$ Tresp had more activated $CD62L^{low}CD44^{hi}$ cells compared to WT (data not shown). These effector memory cells could have a lower threshold for activation than naïve T cells. Therefore, if the pool of cells isolated for L31 Tresp contained more activated cells with a lower threshold for stimulation, submitting these cells to the same activation conditions as WT Tresp could result in a much more robust proliferation profile and thus a refractory response to Treg-mediated suppression. To further investigate the proliferation profiles of these populations, responder T cells could be electronically sorted using markers for CD62L and CD44 and submitted to the suppression assay. Differences in the proliferation profile could then be compared between activated $CD62L^{low}CD44^{hi}$ and naïve $CD62L^{hi}CD44^{low}$ T cell responders.

Treg also require antigenic stimulation in order to mediate suppression. Baecher-Allan et al. (2001) also characterized Treg suppressive capacity when Treg were activated with varying strengths (111). Quite different from that of Tresp, Treg were the most suppressive when activated with a low activation signal (111). Strong activating signals rendered Treg less suppressive. These results are consistent with Treg function in down regulation of immune responses to weak activation signals to prevent unwanted response to autologous antigens. In the

context of an infection where strong activation signals may be present, Treg activated with strong stimuli are less suppressive and therefore allow Tresp proliferation and activation to fight infection. While L31 Treg also have a more activated phenotype (51), perhaps they also have a lower threshold for activation compared to WT. Therefore, in the presence of a relatively strong activation signal, L31 Treg are less suppressive. In our suppression assay, the concentration of anti-CD3 used to stimulate cells was based on the relative proliferation/suppression of WT responder T cells. With insight to the strength of activation signals and thresholds of activation, it would be interesting to decrease the concentration of anti-CD3 used in the suppression assay, or to use a weaker method of stimulation for L31 Tresp and L31 Treg.

Based on their activated phenotype we hypothesized that L31 Treg would have the potential to be more potent suppressors than WT Treg. L31 Treg treated with anti-B7.2 mAb suppressed WT $CD8^+$ Tresp proliferation to a greater extent than WT Treg (Figures 4C and 7C). Moreover, disease onset was delayed for a longer time period in recipients that received L31 Treg than mice injected with WT Treg (Figure 11). While these examples support the notion that L31 Treg have the capacity to be effective suppressors, additional experiments are required. For example, since WT Treg also express B7.2 (19), the proliferation of Tresp in the presence of WT Treg treated with anti-B7.2 mAb would need to be assessed. In vitro suppression assays would also need to be conducted with various ratios of Tresp:Treg in order to compare the relative suppressive activity.

While L31 Treg were not suppressive *in vitro*, our *in vivo* data suggests otherwise. Adoptive transfer of Treg into $CD4^{-/-}$ L31 recipients resulted in a delayed onset of neurological symptoms. Several studies have indicated that Treg suppress through a contact-dependent manner *in vitro*. This has been demonstrated by the abrogation of suppression with the insertion of a semipermeable transwell membrane between activated Tresp and Treg (73, 89, 110). This dichotomy between *in vitro* and *in vivo* data may suggest that Treg-mediated suppression occurs through a cell contact-independent manner in the L31 model, such as through the secretion of suppressive cytokines. This is supported by previous data in which a greater frequency of L31 Treg produce IL-10 (51). Investigation of disease development *in vivo* by generating L31 mice deficient in IL-10 production would provide insight to the role of regulatory cytokines in L31 mice. Furthermore, injecting IL-10-deficient L31 Treg into $CD4^{-/-}$ L31 mice and monitoring their disease development could also provide insight to contact-independent mechanisms of suppression.

$CD4^{-/-}$ L31 recipients that received an intravenous injection of L31 Treg did not develop neurological symptoms after 28 weeks of observation. However, L31 mice that have Treg spontaneously develop neurological symptoms. This difference could be explained by the injection of Treg into lymphopenic mice results in Treg proliferation (116). A greater overall ratio of Treg to pathogenic cell types compared to L31 mice could therefore prevent the onset of disease symptoms. Another possibility is that other $CD4^{+}$ T cell subsets may have a pathogenic role on disease development. Injection of purified Treg into CD4-

deficient L31 mice could therefore amplify regulatory effects on disease development in the absence of other pathogenic subsets.

Data *in vitro* have revealed that L31 Tresp were refractory to Treg-mediated suppression. However, since *in vivo* data show that Treg are suppressive, it is unknown what cell type is the target of Treg-mediated suppression *in vivo*. While $CD8^+$ T cells are the main pathogenic T cell subset in the model, either $CD8^+$ T cells are being targeted through a cell-contact independent mechanism or another cell type may be targeted. Treg have been reported to suppress proliferation of autologous T cells by targeting APC. One of the primary cell types involved in antigen presentation and costimulation in the L31 model are the CNS immune resident microglia. While they are also required for disease development (46), microglia are a likely source of antigen presentation and costimulation for $CD8^+$ T cells. In a model of Theiler's murine encephalomyelitis virus-induced demyelinating disease, which is a murine demyelination model which exhibits a form of chronic-progressive MS, activated microglia were reported to express effector molecules such as IL-1, IL-6, TNF-α and iNOS (47). This study suggested that microglia were competent cells capable of antigen presentation and effector demyelination. This example supports the possibility that microglia may be a target for Treg-mediated suppression, as microglia may be involved in activation and expansion of pathogenic $CD8^+$ T cells or directly involved in providing effector molecules for demyelination. To test the possibility that Treg could target microglia in the L31 mouse model, suppression assays using isolated

microglia as APC could be performed followed by measurement of responder cell proliferation and supernatant cytokine levels.

Overall, L31 Treg are not suppressive *in vitro* but *in vivo* data shows that L31 Treg have a regulatory role in the L31 mouse model. This dichotomy suggests that Treg have a role in regulating disease development in the L31 model, however, the mechanism by which L31 Treg mediate their suppressive effects remains to be elucidated. Further *in vivo* studies would confirm L31 Treg suppressive capabilities, such as monitoring disease development in L31 mice deficient in Foxp3. Another *in vivo* study involves the use of an inflammatory bowel disease (IBD) model in which co-transfer of IBD-inducing effector T cells with WT Treg into RAG-deficient animals prevents the onset of colitis.

In summary, the functional behaviour of Treg that have constitutive expression of B7.2 was investigated. *In vitro* findings provide insight to the potential role of B7.2 and its effects on Treg activation and suppressive ability through interactions with CTLA-4/CD28. Furthermore, *in vivo* data suggests that Treg are involved in disease regulation in an animal model of spontaneous $CD8^+$ T cell-mediated demyelinating disease. Taken together, these data explore new roles for B7.2 on T cells and the role of Treg in the context of autoimmunity.

5. REFERENCES

1. Schwartz RH. 1990. A cell culture model for T lymphocyte clonal anergy. *Science* 248: 1349-56
2. Murphy KM, Weaver CT, Elish M, Allen PM, Loh DY. 1989. Peripheral tolerance to allogeneic class II histocompatibility antigens expressed in transgenic mice: evidence against a clonal-deletion mechanism. *Proc Natl Acad Sci U S A* 86: 10034-8
3. Sha WC, Nelson CA, Newberry RD, Kranz DM, Russell JH, Loh DY. 1988. Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. *Nature* 335: 271-4
4. Dent AL, Matis LA, Hooshmand F, Widacki SM, Bluestone JA, Hedrick SM. 1990. Self-reactive gamma delta T cells are eliminated in the thymus. *Nature* 343: 714-9
5. Sperling AI, Bluestone JA. 1996. The complexities of T-cell co-stimulation: CD28 and beyond. *Immunol Rev* 153: 155-82
6. Fraser JD, Irving BA, Crabtree GR, Weiss A. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* 251: 313-6
7. Jenkins MK, Taylor PS, Norton SD, Urdahl KB. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J Immunol* 147: 2461-6
8. Boise LH, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T, Thompson CB. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* 3: 87-98
9. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775-87
10. Friedline RH, Brown DS, Nguyen H, Kornfeld H, Lee J, Zhang Y, Appleby M, Der SD, Kang J, Chambers CA. 2009. CD4+ regulatory T cells require CTLA-4 for the maintenance of systemic tolerance. *J Exp Med* 206: 421-34
11. Ise W, Kohyama M, Nutsch KM, Lee HM, Suri A, Unanue ER, Murphy TL, Murphy KM. 2010. CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms. *Nat Immunol* 11: 129-35
12. Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. 1994. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* 1: 793-801
13. Marengere LE, Waterhouse P, Duncan GS, Mittrucker HW, Feng GS, Mak TW. 1996. Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. *Science* 272: 1170-3
14. Lee KM, Chuang E, Griffin M, Khattri R, Hong DK, Zhang W, Straus D, Samelson LE, Thompson CB, Bluestone JA. 1998. Molecular basis of T cell inactivation by CTLA-4. *Science* 282: 2263-6

15. Collins AV, Brodie DW, Gilbert RJ, Iaboni A, Manso-Sancho R, Walse B, Stuart DI, van der Merwe PA, Davis SJ. 2002. The interaction properties of costimulatory molecules revisited. *Immunity* 17: 201-10
16. Podojil JR, Kin NW, Sanders VM. 2004. CD86 and beta2-adrenergic receptor signaling pathways, respectively, increase Oct-2 and OCA-B Expression and binding to the 3'-IgH enhancer in B cells. *J Biol Chem* 279: 23394-404
17. Podojil JR, Sanders VM. 2003. Selective regulation of mature IgG1 transcription by CD86 and beta 2-adrenergic receptor stimulation. *J Immunol* 170: 5143-51
18. Grohmann U, Orabona C, Fallarino F, Vacca C, Calcinaro F, Falorni A, Candeloro P, Belladonna ML, Bianchi R, Fioretti MC, Puccetti P. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat Immunol* 3: 1097-101
19. Greenwald RJ, Freeman GJ, Sharpe AH. 2005. The B7 family revisited. *Annu Rev Immunol* 23: 515-48
20. Azuma M, Yssel H, Phillips JH, Spits H, Lanier LL. 1993. Functional expression of B7/BB1 on activated T lymphocytes. *J Exp Med* 177: 845-50
21. Jeannin P, Herbault N, Delneste Y, Magistrelli G, Lecoanet-Henchoz S, Caron G, Aubry JP, Bonnefoy JY. 1999. Human effector memory T cells express CD86: a functional role in naive T cell priming. *J Immunol* 162: 2044-8
22. Sansom DM, Hall ND. 1993. B7/BB1, the ligand for CD28, is expressed on repeatedly activated human T cells in vitro. *Eur J Immunol* 23: 295-8
23. Paust S, Lu L, McCarty N, Cantor H. 2004. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci U S A* 101: 10398-403
24. Taylor PA, Lees CJ, Fournier S, Allison JP, Sharpe AH, Blazar BR. 2004. B7 expression on T cells down-regulates immune responses through CTLA-4 ligation via T-T interactions [corrections]. *J Immunol* 172: 34-9
25. Oliveira-dos-Santos AJ, Ho A, Tada Y, Lafaille JJ, Tonegawa S, Mak TW, Penninger JM. 1999. CD28 costimulation is crucial for the development of spontaneous autoimmune encephalomyelitis. *J Immunol* 162: 4490-5
26. Cross AH, Girard TJ, Giacoletto KS, Evans RJ, Keeling RM, Lin RF, Trotter JL, Karr RW. 1995. Long-term inhibition of murine experimental autoimmune encephalomyelitis using CTLA-4-Fc supports a key role for CD28 costimulation. *J Clin Invest* 95: 2783-9
27. Lenschow DJ, Ho SC, Sattar H, Rhee L, Gray G, Nabavi N, Herold KC, Bluestone JA. 1995. Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. *J Exp Med* 181: 1145-55
28. 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: 733-40

29. Tada Y, Nagasawa K, Ho A, Morito F, Ushiyama O, Suzuki N, Ohta H, Mak TW. 1999. CD28-deficient mice are highly resistant to collagen-induced arthritis. *J Immunol* 162: 203-8
30. Karandikar NJ, Vanderlugt CL, Walunas TL, Miller SD, Bluestone JA. 1996. CTLA-4: a negative regulator of autoimmune disease. *J Exp Med* 184: 783-8
31. Luhder F, Hoglund P, Allison JP, Benoist C, Mathis D. 1998. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates the unfolding of autoimmune diabetes. *J Exp Med* 187: 427-32
32. Sospedra M, Martin R. 2005. Immunology of multiple sclerosis. *Annu Rev Immunol* 23: 683-747
33. Witte ME, Geurts JJ, de Vries HE, van der Valk P, van Horssen J. 2010. Mitochondrial dysfunction: a potential link between neuroinflammation and neurodegeneration? *Mitochondrion* 10: 411-8
34. Hafler DA. 2004. Multiple sclerosis. *J Clin Invest* 113: 788-94
35. Elenein RG, Sharer LR, Cook SD, Pachner AR, Michaels J, Hillen ME. 2011. A second case of Marburg's variant of multiple sclerosis with vasculitis and extensive demyelination. *Mult Scler* 17: 1531-8
36. Jilek S, Schluep M, Rossetti AO, Guignard L, Le Goff G, Pantaleo G, Du Pasquier RA. 2007. CSF enrichment of highly differentiated CD8+ T cells in early multiple sclerosis. *Clin Immunol* 123: 105-13
37. Monteiro J, Hingorani R, Peroglizzi R, Apatoff B, Gregersen PK. 1996. Oligoclonality of CD8+ T cells in multiple sclerosis. *Autoimmunity* 23: 127-38
38. Jacobsen M, Cepok S, Quak E, Happel M, Gaber R, Ziegler A, Schock S, Oertel WH, Sommer N, Hemmer B. 2002. Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients. *Brain* 125: 538-50
39. Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, Friese M, Schroder R, Deckert M, Schmidt S, Ravid R, Rajewsky K. 2000. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med* 192: 393-404
40. Huseby ES, Liggitt D, Brabb T, Schnabel B, Ohlen C, Goverman J. 2001. A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. *J Exp Med* 194: 669-76
41. Na SY, Cao Y, Toben C, Nitschke L, Stadelmann C, Gold R, Schimpl A, Hunig T. 2008. Naive CD8 T-cells initiate spontaneous autoimmunity to a sequestered model antigen of the central nervous system. *Brain* 131: 2353-65
42. Gobel K, Melzer N, Herrmann AM, Schuhmann MK, Bittner S, Ip CW, Hunig T, Meuth SG, Wiendl H. 2010. Collateral neuronal apoptosis in CNS gray matter during an oligodendrocyte-directed CD8(+) T cell attack. *Glia* 58: 469-80
43. Ip CW, Kroner A, Bendszus M, Leder C, Kobsar I, Fischer S, Wiendl H, Nave KA, Martini R. 2006. Immune cells contribute to myelin

degeneration and axonopathic changes in mice overexpressing proteolipid protein in oligodendrocytes. *J Neurosci* 26: 8206-16
44. Leder C, Schwab N, Ip CW, Kroner A, Nave KA, Dornmair K, Martini R, Wiendl H. 2007. Clonal expansions of pathogenic CD8+ effector cells in the CNS of myelin mutant mice. *Mol Cell Neurosci* 36: 416-24
45. Chitnis T, Khoury SJ. 2003. Role of costimulatory pathways in the pathogenesis of multiple sclerosis and experimental autoimmune encephalomyelitis. *J Allergy Clin Immunol* 112: 837-49; quiz 50
46. Zehntner SP, Brisebois M, Tran E, Owens T, Fournier S. 2003. Constitutive expression of a costimulatory ligand on antigen-presenting cells in the nervous system drives demyelinating disease. *FASEB J* 17: 1910-2
47. Mack CL, Vanderlugt-Castaneda CL, Neville KL, Miller SD. 2003. Microglia are activated to become competent antigen presenting and effector cells in the inflammatory environment of the Theiler's virus model of multiple sclerosis. *J Neuroimmunol* 144: 68-79
48. Carson MJ, Reilly CR, Sutcliffe JG, Lo D. 1998. Mature microglia resemble immature antigen-presenting cells. *Glia* 22: 72-85
49. Katz-Levy Y, Neville KL, Girvin AM, Vanderlugt CL, Pope JG, Tan LJ, Miller SD. 1999. Endogenous presentation of self myelin epitopes by CNS-resident APCs in Theiler's virus-infected mice. *J Clin Invest* 104: 599-610
50. Brisebois M, Zehntner SP, Estrada J, Owens T, Fournier S. 2006. A pathogenic role for CD8+ T cells in a spontaneous model of demyelinating disease. *J Immunol* 177: 2403-11
51. Hung HY. 2009. *Characterization of the CD4+CD25+ regulatory T cells in an animal model of spontaneous demyelination in the central nervous system*. McGill University, Montreal, Quebec, Canada
52. Estrada Guadarrama JA. 2010. *Mechanisms of demyelination and axonal damage in a CD8+ T cell-mediated model of spontaneous demyelinating disease*. McGill University, Montreal, Canada. 181 pp.
53. Cone RE, Chattopadhyay S, Sharafieh R, Lemire Y, O'Rourke J. 2009. The suppression of hypersensitivity by ocular-induced CD8(+) T cells requires compatibility in the Qa-1 haplotype. *Immunol Cell Biol* 87: 241-8
54. Hu D, Ikizawa K, Lu L, Sanchirico ME, Shinohara ML, Cantor H. 2004. Analysis of regulatory CD8 T cells in Qa-1-deficient mice. *Nat Immunol* 5: 516-23
55. Suzuki H, Kundig TM, Furlonger C, Wakeham A, Timms E, Matsuyama T, Schmits R, Simard JJ, Ohashi PS, Griesser H, et al. 1995. Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. *Science* 268: 1472-6
56. Saitoh O, Abiru N, Nakahara M, Nagayama Y. 2007. CD8+CD122+ T cells, a newly identified regulatory T subset, negatively regulate Graves' hyperthyroidism in a murine model. *Endocrinology* 148: 6040-6
57. Lee YH, Ishida Y, Rifa'i M, Shi Z, Isobe K, Suzuki H. 2008. Essential role of CD8+CD122+ regulatory T cells in the recovery from experimental autoimmune encephalomyelitis. *J Immunol* 180: 825-32

58. Ciubotariu R, Colovai AI, Pennesi G, Liu Z, Smith D, Berlocco P, Cortesini R, Suciu-Foca N. 1998. Specific suppression of human CD4+ Th cell responses to pig MHC antigens by CD8+CD28- regulatory T cells. *J Immunol* 161: 5193-202
59. Colovai AI, Liu Z, Ciubotariu R, Lederman S, Cortesini R, Suciu-Foca N. 2000. Induction of xenoreactive CD4+ T-cell anergy by suppressor CD8+CD28- T cells. *Transplantation* 69: 1304-10
60. Ben-David H, Sharabi A, Dayan M, Sela M, Mozes E. 2007. The role of CD8+CD28 regulatory cells in suppressing myasthenia gravis-associated responses by a dual altered peptide ligand. *Proc Natl Acad Sci U S A* 104: 17459-64
61. Paas-Rozner M, Sela M, Mozes E. 2001. The nature of the active suppression of responses associated with experimental autoimmune myasthenia gravis by a dual altered peptide ligand administered by different routes. *Proc Natl Acad Sci U S A* 98: 12642-7
62. Singh RP, La Cava A, Wong M, Ebling F, Hahn BH. 2007. CD8+ T cell-mediated suppression of autoimmunity in a murine lupus model of peptide-induced immune tolerance depends on Foxp3 expression. *J Immunol* 178: 7649-57
63. Seo SK, Choi JH, Kim YH, Kang WJ, Park HY, Suh JH, Choi BK, Vinay DS, Kwon BS. 2004. 4-1BB-mediated immunotherapy of rheumatoid arthritis. *Nat Med* 10: 1088-94
64. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151-64
65. Hori S, Nomura T, Sakaguchi S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057-61
66. Fontenot JD, Gavin MA, Rudensky AY. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4: 330-6
67. Murphy K, Travers P, Walport M, Janeway C. 2008. *Janeway's immunobiology*. New York: Garland Science. xxi, 887 p. pp.
68. Wu HY, Quintana FJ, da Cunha AP, Dake BT, Koeglsperger T, Starossom SC, Weiner HL. 2011. In vivo induction of Tr1 cells via mucosal dendritic cells and AHR signaling. *PLoS One* 6: e23618
69. Wing K, Sakaguchi S. 2010. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol* 11: 7-13
70. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27: 68-73
71. Williams LM, Rudensky AY. 2007. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* 8: 277-84

72. Paust S, Cantor H. 2005. Regulatory T cells and autoimmune disease. *Immunol Rev* 204: 195-207
73. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. 2001. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol* 31: 1122-31
74. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. 2001. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med* 193: 1303-10
75. Ng WF, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD, Isaacs JD, Lechler RI. 2001. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* 98: 2736-44
76. Lee SM, Gao B, Fang D. 2008. FoxP3 maintains Treg unresponsiveness by selectively inhibiting the promoter DNA-binding activity of AP-1. *Blood* 111: 3599-606
77. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T, Nomura T. 2006. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212: 8-27
78. Levings MK, Sangregorio R, Roncarolo MG. 2001. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 193: 1295-302
79. Walker LS, Chodos A, Eggena M, Dooms H, Abbas AK. 2003. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med* 198: 249-58
80. Klein L, Khazaie K, von Boehmer H. 2003. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc Natl Acad Sci U S A* 100: 8886-91
81. Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, Zheng XX, Strom TB, Bluestone JA. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 171: 3348-52
82. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, Flores M, Li N, Schweighoffer E, Greenberg S, Tybulewicz V, Vignali D, Clynes R. 2008. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol* 180: 5916-26
83. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, Hopner S, Centonze D, Bernardi G, Dell'Acqua ML, Rossini PM, Battistini L, Rotzschke O, Falk K. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110: 1225-32
84. Sarris M, Andersen KG, Randow F, Mayr L, Betz AG. 2008. Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity* 28: 402-13
85. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 190: 995-1004

86. Joetham A, Takeda K, Taube C, Miyahara N, Matsubara S, Koya T, Rha YH, Dakhama A, Gelfand EW. 2007. Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta. *J Immunol* 178: 1433-42
87. Li MO, Wan YY, Flavell RA. 2007. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* 26: 579-91
88. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450: 566-9
89. Thornton AM, Shevach EM. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188: 287-96
90. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, Shimizu J, Sakaguchi S. 1998. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 10: 1969-80
91. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* 8: 1353-62
92. Tran DQ, Glass DD, Uzel G, Darnell DA, Spalding C, Holland SM, Shevach EM. 2009. Analysis of adhesion molecules, target cells, and role of IL-2 in human FOXP3+ regulatory T cell suppressor function. *J Immunol* 182: 2929-38
93. Shevach EM. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30: 636-45
94. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21: 589-601
95. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. 2005. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 174: 1783-6
96. Garin MI, Chu CC, Golshayan D, Cernuda-Morollon E, Wait R, Lechler RI. 2007. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* 109: 2058-65
97. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, Baker J, Jeffery LE, Kaur S, Briggs Z, Hou TZ, Futter CE, Anderson G, Walker LS, Sansom DM. 2011. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 332: 600-3
98. May KF, Jr., Chang X, Zhang H, Lute KD, Zhou P, Kocak E, Zheng P, Liu Y. 2007. B7-deficient autoreactive T cells are highly susceptible to suppression by CD4(+)CD25(+) regulatory T cells. *J Immunol* 178: 1542-52

99. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12: 431-40
100. Tai X, Cowan M, Feigenbaum L, Singer A. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* 6: 152-62
101. Bour-Jordan H, Salomon BL, Thompson HL, Szot GL, Bernhard MR, Bluestone JA. 2004. Costimulation controls diabetes by altering the balance of pathogenic and regulatory T cells. *J Clin Invest* 114: 979-87
102. Montagnoli C, Bacci A, Bozza S, Gaziano R, Mosci P, Sharpe AH, Romani L. 2002. B7/CD28-dependent CD4+CD25+ regulatory T cells are essential components of the memory-protective immunity to Candida albicans. *J Immunol* 169: 6298-308
103. Liston A, Rudensky AY. 2007. Thymic development and peripheral homeostasis of regulatory T cells. *Curr Opin Immunol* 19: 176-85
104. Golovina TN, Mikheeva T, Suhoski MM, Aqui NA, Tai VC, Shan X, Liu R, Balcarcel RR, Fisher N, Levine BL, Carroll RG, Warner N, Blazar BR, June CH, Riley JL. 2008. CD28 costimulation is essential for human T regulatory expansion and function. *J Immunol* 181: 2855-68
105. Chen ML, Pittet MJ, Gorelik L, Flavell RA, Weissleder R, von Boehmer H, Khazaie K. 2005. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A* 102: 419-24
106. Suvas S, Kumaraguru U, Pack CD, Lee S, Rouse BT. 2003. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *J Exp Med* 198: 889-901
107. Boettler T, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum HE, von Weizsacker F, Thimme R. 2005. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* 79: 7860-7
108. Fournier S, Rathmell JC, Goodnow CC, Allison JP. 1997. T cell-mediated elimination of B7.2 transgenic B cells. *Immunity* 6: 327-39
109. Quah BJ, Warren HS, Parish CR. 2007. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc* 2: 2049-56
110. Stephens LA, Mottet C, Mason D, Powrie F. 2001. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol* 31: 1247-54
111. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. 2001. CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* 167: 1245-53
112. Leibson PJ, Pease LR. 2004. Having it both ways: MHC recognition in cis and trans. *Nat Immunol* 5: 237-8
113. Cheung TC, Oborne LM, Steinberg MW, Macauley MG, Fukuyama S, Sanjo H, D'Souza C, Norris PS, Pfeffer K, Murphy KM, Kronenberg M,

Spear PG, Ware CF. 2009. T cell intrinsic heterodimeric complexes between HVEM and BTLA determine receptivity to the surrounding microenvironment. *J Immunol* 183: 7286-96

114. Held W, Mariuzza RA. 2011. Cis-trans interactions of cell surface receptors: biological roles and structural basis. *Cell Mol Life Sci* 68: 3469-78
115. Noel PJ, Boise LH, Green JM, Thompson CB. 1996. CD28 costimulation prevents cell death during primary T cell activation. *J Immunol* 157: 636-42
116. Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. 2001. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J Immunol* 166: 3008-18