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Defective Immune Regulation in Multiple Sclerosis: E3 Ubiquitin Ligase Cbl-b and TLR Tolerance

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Abstract

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) with unknown etiology. The variable response to therapy in MS suggests a need for personalized approaches based on genetic differences. Genome-wide association studies have linked *CBLB* genetic variants with MS. Cbl-b is an E3 ubiquitin ligase that regulates T cell activation and Cbl-b-deficient ($^{-/-}$) mice show T cell abnormalities described in MS patients. Our observation of an aberrant trafficking of Cbl-b$^{-/-}$ T cells led us to ask whether the MS drug, FTY720, postulated to modulate T cell trafficking, is less effective in Cbl-b$^{-/-}$ mice during EAE. We documented that FTY720 significantly inhibits EAE in Cbl-b$^{-/-}$ mice, suggesting that MS patients with potential Cbl-b abnormalities may still be excellent candidates for FTY720 treatment.

In addition to the autoimmune phenotype, Cbl-b$^{-/-}$ mice demonstrate enhanced anti-tumor immunity. Cbl-b$^{-/-}$ T cells are resistant to multiple immunoregulatory mechanisms such as Tregs. PD-L1/PD-1 is an important immunoregulatory pathway and a target in cancer immunotherapy. We have now discovered that Cbl-b$^{-/-}$ T cells and NK cells are resistant to PD-L1/PD-1. These results suggest that targeting Cbl-b in cancer immunotherapy provides an approach to simultaneously override numerous immunoregulatory checkpoints.

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Environmental factors such as the gut commensal bacteria have been linked to MS. Previously we have identified a human gut microbiome-derived lipodipeptide termed Lipid 654 (L654). L654 is present in the human systemic circulation, acts as a human TLR2 ligand and is detected at significantly lower levels in MS patients. We postulated that the gut microbiome may regulate systemic immunity by inducing homeostatic TLR tolerance through microbial products such as L654, and hypothesized that MS-associated low levels of L654 may reflect defective TLR tolerance and lead to TLR hyper-responsiveness in MS. Here we document the enhanced *ex vivo* TLR2 responses from $CD14^+$ monocytes of MS patients with active disease. We also report that Lipid 342, a breakdown product of L654, is also detected at significantly lower levels in MS patients. These results highlight a mechanism of how the gut microbiome may regulate human systemic immunity, and suggest that L342 may be a blood biomarker in MS.

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Defective Immune Regulation in Multiple Sclerosis:

E3 Ubiquitin Ligase Cbl-b and TLR Tolerance

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CHAPTER 1 Introduction

The concept of autoimmunity was first postulated by Paul Ehrlich at the beginning of the 20th century, in which he described it as "horror autotoxicus." The immune system is thought to have originally evolved to fight infections against foreign pathogens, such as viruses, bacteria and fungi. The ability of the immune system to distinguish self from non-self is dependent on highly intricate mechanisms of immune regulation. Proper functioning of immunoregulatory mechanisms allow for robust protective immune responses against pathogens while preserving the integrity of tissues and organs, and preventing autoimmunity.

Today, the mortality rates from infectious diseases have tremendously declined owing to the development of effective vaccines. However, the prevalence of autoimmune disease is on the rise especially in the developed countries at an alarming rate (1). Autoimmune diseases consist of a broad range of inflammatory diseases characterized by dysregulated immune responses against self-antigens that result in damages and destructions of tissues and organs. Since Paul Ehrlich's postulate of autoimmunity, our understanding of immune regulation and self-tolerance has advanced tremendously. However, very little is known about the factors that trigger the loss of self-tolerance and predispose an individual to developing autoimmune disease. Lack of knowledge of such triggers for autoimmune disease is an enormous hurdle for developing effective and specific therapeutic approaches for autoimmune disease.

Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease in which an individual's own immune system attacks the central nervous system, causing debilitating physical and cognitive disabilities (2). MS is highly prevalent and affects millions of people worldwide. In the United States, the prevalence is estimated to be 100 per 100,000 (0.1 %). The estimated female to male ratio in the U.S. is 3.5:1 today and it has been increasing over the last decades (3).

The symptoms of MS include fatigue, visual, sensory and motor impairment, cognitive dysfunction and physical disability (2). There are three forms of MS: relapsing-remitting MS (RR-MS), primary progressive MS (PP-MS) and secondary progressive MS (SP-MS). Majority of patients are diagnosed with MS when an acute episode affects one site, known as clinically isolated syndrome (CIS) (2). The appearance of the demyelination detected by the magnetic resonance imaging (MRI) in a new region leads to the diagnosis of RR-MS. The average onset of RR-MS is 25-30 years of age. Most of RR-MS patients demonstrate further progression of disease within 10-15 years, leading to the diagnosis of SP-MS. PP-MS is a rare progressive phenotype with late onset around 40 years of age (2).

Disease pathogenesis of MS

The disease pathogenesis of MS is thought to be initiated with increased migration of autoreactive T cells across the blood-brain barrier (BBB) (2). This is followed by reactivation of these T cells by activated APCs in the CNS, leading to inflammatory cascades in the CNS that result in axonal degeneration, astrogliosis, and formation of demyelinated lesions in the CNS parenchyma (2).

The early lesions in the CNS of patients with MS demonstrate the presence of macrophages, $CD8^+$ T cells, $CD4^+$ T cells, B cells and plasma cells (listed in the order of frequencies found in lesions) (4). $CD4^+$ T cell subsets that are implicated with MS are Th1 (T helper type 1) cells and Th17 (T helper type 17) cells. Th1 cells are characterized by expression of the Th1-specific transcription factor T-bet and the ability to produce IFN-γ. Th1 cell differentiation is promoted by IL-12 produced by activated APCs. Th17 cells are defined by expression of transcription factor RORγt and the production of IL-17A. Th17 cell differentiation is driven by IL-6, IL-1β, IL-23 and TGF-β in the cytokine milieu. As the CNS damages accumulate along with the ongoing inflammation, epitope spreading occurs in which additional waves of myelin-specific T cells are recruited the CNS. These myelin-reactive Th1 and Th17 T cells produce GM-CSF that promotes the recruitment of innate immune cells such as monocytes, macrophages and neutrophils into the CNS. These cells cause axonal damage and neuronal death by producing oxygen species such as NOS and through their phagocytic activity (4). $CD8^+$ T cells also contribute to the disease pathogenesis of MS by producing cytokines such as IL-17A and through their cytotoxic activities (4). Recently, the pathogenic role of B cells through production of pro-inflammatory cytokines such as IL-6 and antigen presentation has also been demonstrated in the progressive forms of MS, in particular (4). Various types of the CNS-resident cells, such as microglia, astrocytes and oligodendrocytes also participate in the inflammatory cascades by further amplifying the inflammation (4).

Experimental autoimmune encephalomyelitis (EAE)

The experimental autoimmune encephalomyelitis (EAE) is a robust model for studying the pathogenesis of MS. In EAE, the CNS inflammation mediated by $CD4^+$ T cells leads to demyelination of axons in the CNS, ultimately resulting in progressive hind-limb and front-limb paralysis. Different mouse strains are used in EAE, in which the SJL ($H\text{-}2^s$) strain demonstrates a relapsing-remitting course of paralysis, and the C57BL/6 strain demonstrates a chronic progressive disease course (5).

There are largely two distinct approaches for inducing EAE in mice. The active model of EAE is induced by immunization of mice with myelin proteins or peptides that are emulsified in complete Freund's adjuvant (CFA) (5). The active EAE model is useful when studying the induction phase of disease in which the priming of myelin-specific T cells occurs in the periphery, followed by their migration to the CNS. The adoptive transfer model of EAE is induced by the introduction or a "transfer" of an *in vitro* pre-activated myelin epitope-specific $CD4^+$ T cells to a naïve mouse (5). The adoptive transfer EAE is often used to study the effector phase of disease. The effector phase of EAE begins with the entry of autoreactive T cells into the CNS and followed by the inflammatory cascades and the resulting CNS pathology that mirror those observed in patients with MS (5).

Current therapeutic approaches in MS

A variety of anti-inflammatory and immunosuppressive disease-modifying drugs (DMD) have been developed to stop the ongoing inflammation and the CNS damage in MS. Most of the DMDs that have been approved by FDA for treatment of RR-MS reduce the number of lesions

and relapses in RR-MS patients. However, most of them cause unfavorable side effects, and fail to halt the progression of disease (6, 7).

More recently, modulation of the immune cell trafficking has become a significant focus in the therapeutic approach for MS (8). FTY720 (Fingolimod, Gilenya) is an orally administered drug for RR-MS. FTY720 is postulated to mediate its therapeutic effect in MS by causing degradation of the lymphocyte homing receptor $S1P_1$, which prevents the egress of T and B cells from lymph nodes, rendering them unable to migrate to the CNS (9). While FTY720 have has demonstrated therapeutic efficacy in RR-MS patients (6), a subset of MS patients do not respond to FTY720 including those with the progressive form of MS (6). With the concept of personalized medicine in mind, the relationship between therapeutic efficacy of FTY720 and *CBLB* gene in which its variants are associated with MS susceptibility, will be discussed in the Chapter 2.

Lastly, there is no blood biomarker that allows accurate diagnosis of MS (10). The diagnosis of MS currently requires invasive methods such as collection of CSF and analysis of oligoclonal bands, and extensive analysis of T1/T2 lesions in the brain through MRI (2). Chapter 4 will focus on a molecule derived from the human gut microbiome that our laboratory and collaborators have recently identified, and are currently characterizing its potential use as a blood biomarker for MS.

Genetic and environmental factors in MS

Genome wide association studies (GWAS) have revealed more than a hundred genetic variants that are associated with susceptibility to MS. The majority of the MS-associated variants are found in the immunological genes, highlights the indispensable role of the immune system in the pathogenesis of MS. HLA alleles were the first to be characterized as MS risk variants (2). In addition, recent GWAS revealed MS risk variants in *TNFSFR1*, *IL-7Rα, IL-2Rα*, and *CD6* genes (11-13). Moreover, multiple variants in the *CBLB* gene that encodes Cbl-b E3 ubiquitin ligase have also been associated with MS susceptibility (12-14). Despite the evidence for involvement of genetic factors in MS, a concordance rate for monozygotic twins is only 25-30% (15), suggesting the major contribution of environmental factors to MS susceptibility.

Epstein- Barr virus (EBV) infection, smoking, UV radiation, and vitamin D deficiency have been most frequently associated with MS (4). However, the data supporting the causative role of these environmental factors are insufficient and remain unresolved. Most recently, the role of human gut microbiome in the pathogenesis of MS has been vigorously studied. There are reports on alterations in the gut microbiome in patients with MS compared to healthy individuals (16-18). The relationship between the human gut microbiome and MS and potential mechanisms of how the microbiome regulate systemic immunity will be discussed in Chapter 4.

Mechanisms of immune regulation

$CD4^+$ T cells with the T cell receptor (TCR) specificity to myelin antigens are found in the periphery of both MS patients and healthy individuals. Immunoregulatory mechanisms that prevent the activation of these autoreactive T cells in healthy individuals are likely defective in

patients with MS. Immunoregulatory mechanisms involve central tolerance and peripheral tolerance. Central tolerance involves the thymic education of T cells involving positive and negative selection, in which T cells with TCRs that are high affinity toward self-antigens are eliminated. The importance of central tolerance is underscored by the systemic autoimmunity seen in patients with APECED (Autoimmune polyendocrynopathy-candidiasis-ectodermal dystrophy). These patients possess mutations in the transcription factor AIRE (Autoimmune regulator), which plays a critical role in the process of negative selection. However, mechanisms of central tolerance are insufficient to eliminate all autoreactive T cells, allowing some of them to escape into the periphery.

$CD4^+$ $CD25^+$ Foxp3 regulatory T cells (Tregs) play an essential role in the peripheral tolerance (19). Tregs express the canonical transcription factor Forkhead box P3 (Foxp3), and are a subset of $CD4^+$ T cells specialized in suppressing immune responses through various mechanisms. These suppressive mechanisms include secretion of immunoregulatory cytokines such as TGF-β, IL-10, and contact-dependent mechanisms such as granzyme and CTLA-4 (19). The essential role of Tregs in preventing autoimmunity is underscored by the fatal systemic autoimmune disease seen in patients with IPEX (Immunodysregulation polyendocrynopathy enteropathy X-linked syndrome) (19). These patients lack functional Tregs due to the loss-of-function mutations in the *Foxp3* gene. Diminished suppressive functions of Tregs have been reported in MS patients, in which the suppressive capacity of Tregs is compromised (20), and/or T cells of these patients are resistant to Treg-mediated suppression (21). Various other regulatory cell subsets such as type 1 regulatory cells (Tr-1 cells), myeloid-derived suppressor cells

(MDSCs), and alternatively activated M2 macrophages act together to maintain peripheral tolerance in mice and humans.

In addition to regulatory cells, peripheral tolerance involves cell-intrinsic mechanisms that mediate negative regulation of T cells. For example, the requirement for co-stimulatory signals through CD28 acts to prevent the activation of T cells in the absence of infection (22). Optimal T cell activation requires both TCR stimulation and CD28 co-stimulation. CD28 co-stimulation is provided by DCs that express B7 co-stimulatory molecules. DCs must become activated in response to PAMPs in the microenvironment to upregulate co-stimulatory molecule B7.1 (22).

There are a variety of co-inhibitory receptors that act to ensure the requirement for co-stimulation. Programmed Death 1 (PD-1) is one of such receptors upregulated on T cells upon TCR stimulation. Triggering of PD-1 by its ligands PD-L1 and PD-L2 expressed on APCs and tissue parenchymal cells results in inhibition of T cell activation (23). Cytotoxic T Lymphocyte-associated Antigen-4 (CTLA-4) is another co-inhibitory receptor expressed by activated T cells (22). CTLA-4 acts by 1) outcompeting CD28 for binding to B7 molecules, thereby reducing co-stimulatory signals, and 2) inducing negative signals in T cells that are inhibitory to T cell activation (22).

Cbl-b E3 ubiquitin ligase: a gatekeeper for lymphocyte activation

Casitas B lineage lymphoma-b (Cbl-b) is an E3 ubiquitin ligase is highly expressed in cells of hematopoietic origin. E3 ubiquitin ligases are enzymes specialized to confer specificity to the

ubiquitin-mediated post-transcriptional modification of proteins. In particular, Cbl-b is most highly expressed T and B cells (24, 25), and it has been established as a gatekeeper of T cell activation (24, 25).

Cbl-b deficient ($^{-/-}$) mice demonstrate spontaneous systemic autoimmunity reminiscent of SLE, characterized by increased titers of anti-dsDNA antibody and multi-organ lymphocyte infiltration in multiple peripheral organs (24). Cbl-b$^{-/-}$ mice also demonstrate exacerbated disease severity in the induced models of T cell-mediated diseases such as EAE and allergic asthma (25-27).

Mouse Cbl-b$^{-/-}$ T cells demonstrate hyper-reactivity through the lower threshold for activation, increased IL-2 production, and CD28 co-stimulation independence (24, 25). Cbl-b interacts with various TCR proximal molecules such as Vav1, Zap70 and PI3K. In particular, Cbl-b directly interacts with p85α regulatory subunit of PI3K, targeting it for in its modification in a proteolysis-independent manner leading to inhibition of the PI3K activation in T cells (28). Consequently, Cbl-b$^{-/-}$ T cells demonstrate hyperactivated PI3K-Akt (29). PI3K-Akt is one of many pathways that are activated downstream of TCR stimulation and CD28-co-stimulation. The activation of PI3K-Akt particularly downstream of CD28 promotes T cell survival and proliferation through the activation of NF-κB (nuclear factor κB), NFAT (nuclear factor of activated T cells), Bcl-xL, mTORC1 (mammalian target of rapamycin complex 1), and GLUT1 (glucose transporter 1), all of which act to mediate the robust metabolic changes of a T cell (22). Multiple abnormalities in T cell functions that are documented in Cbl-b$^{-/-}$ T cells may result from the hyperactivated PI3K-Akt (29-31).

The role of Cbl-b in MS

T cell abnormalities demonstrated in Cbl-b$^{-/-}$ mice mirror those documented in MS patients. Similarly to Cbl-b$^{-/-}$ T cells, T cells isolated from RR-MS patients have been shown to depend less on CD28 co-stimulatory signals (32, 33). Additionally, resistance to Tregs has been documented in Cbl-b$^{-/-}$ T cells and in CD4^{+} T cells isolated from RR-MS patients. During EAE, Cbl-b$^{-/-}$ mice develop more severe disease compared to WT mice. These findings highlight the value of Cbl-b$^{-/-}$ mice in studying the disease pathogenesis of MS. Moreover, diminished expression levels of Cbl-b have been found in peripheral blood mononuclear cells (PBMCs) (34) and naïve CD4^{+} T cells of RR-MS patients during relapse (35).

Most importantly, three variants in the *CBLB* gene are associated with MS susceptibility (12-14). One of these *CBLB* variants, rs12487066, results in decreased levels of Cbl-b expression in CD4^{+} T cells of RR-MS patients, and is associated with diminished response of these cells to the anti-proliferative effect of IFN-β *in vitro* (35). At present, it still remains unclear whether this variant rs12487066 compromises the therapeutic effecacy of IFN-β in RR-MS patients. This particular finding involving the MS-associated *CBLB* variant rs12487066 led us to investigate the functional outcome of Cbl-b deficiency on the therapeutic efficacy of FTY720 during EAE in the context of personalized medicine. This work will be discussed in Chapter 2.

Resistance to immunoregulation in Cbl-b$^{-/-}$ T cells

Certain types of pro-inflammatory cytokines influence the responsiveness of T cells to Treg-mediated suppression. One of such cytokines is IL-6 (36). In EAE, elevated levels of IL-6 and TNFα in the CNS have been shown to induce resistance of CD4^{+} T cells to Tregs (37).

Additionally, IL-6 has been shown to be elevated in MS patients (21, 38) and to mediate resistance to Tregs in $CD4^+$ T cells of MS patients with active disease (21). TNFα has been implicated in resistance to Tregs in patients with JIA (38).

We have previously demonstrated that Cbl-$b^{-/-}$ $CD4^+$ T cells are resistant to Treg-mediated suppression (39, 40). The mechanism underlying the resistance to Treg-mediated suppression in Cbl-$b^{-/-}$ T cells still remains unclear. Based on the observation that the enhanced activation status of PI3K-Akt pathway is shared among Cbl-$b^{-/-}$ T cells and other instances of resistance to Tregs documented by others, the hyperactivation of PI3K-Akt pathway has been proposed to be the underlying mechanism of resistance to Treg-mediated suppression (30, 31).

TGF-β is one of the mechanisms by which Tregs exert their immunosuppressive functions. TGF-β inhibits T cell proliferation and promotes the differentiation of naïve $CD4^+$ T cells to inducible Tregs (iTregs) (41). Importantly, Cbl-$b^{-/-}$ $CD4^+$ T cells are resistant to TGF-β-mediated immune regulation (29, 40-42). CTLA-4 is another mechanism employed by Tregs to inhibit T cell activation. It has been shown that Cbl-$b^{-/-}$ T cells are resistant to negative signals downstream of CTLA-4 *in vitro* (43). In Chapter 3, we will discuss the finding from our laboratory that documented resistance of Cbl-$b^{-/-}$ T cells to PD-L1/PD-1 mediated regulation.

Regulation of autoimmunity and anti-tumor immunity by Cbl-b

Since Cbl-b plays an indispensable role in multiple immunoregulatory mechanisms in T cells, the expression and function of Cbl-b must be tightly regulated. Naïve T cells express high levels of Cbl-b (44). Interestingly, co-stimulation through CD28 has been shown to result in degradation

of Cbl-b (43, 45). In contrast, signaling through CTLA-4 and PD-1 in T cells has been shown to induce the upregulation of Cbl-b expression in T cells (43, 46). Thus, activating signals (i.e. CD28 co-stimulation) are often associated with diminished function of Cbl-b, and inhibitory signals (i.e. CTLA-4 and PD-1) are correlated with enhanced expression of Cbl-b.

In the context of cancer, immunoregulatory mechanisms contribute to the dampening of immune responses against tumors. We postulate that the resistance of Cbl-b$^{-/-}$ T cells to immunoregulatory mechanisms such as Tregs, TGF-β, CTLA-4 and now PD-L1/PD-1 may be highly relevant to human cancer immunotherapy (discussed further in Chapter 3).

The immune system and the human gut microbiome

To introduce further complexities to the mechanisms of immunoregulation discussed above that act to maintain "self-tolerance," the immune system must also maintain a "symbiotic" relationship with hundreds of species of bacteria that reside in the gastrointestinal tract (GI), called the gut microbiome (47). In mice, the gut microbiome mediates both disease-promoting and disease-inhibiting role in CNS autoimmunity (EAE) by promoting the development and expansion of Th17 cells in the CNS (48), and by inducing the expansion of regulatory T cell subsets in the gut (49, 50). In humans, there is increasing evidence that suggests the interaction between the commensal bacteria in the GI and the immune system (17, 47, 51, 52). However, which factors from the human gut microbiome directly interact with the systemic immunity in humans still remains unknown.

TLR tolerance in MS

Our laboratory previously documented and characterized a unique bacterially derived lipodipeptide named Lipid 654 (L654) (51, 53). L654 acts as a human TLR2 ligand, and the presence of L654 was detected in the systemic circulation of all individuals studied (51, 53). Importantly, the serum levels of L654 were significantly lower in MS patients compared to healthy individuals (51). Based on these findings, we had postulated that bacterially derived products such as L654 act to prevent immune hyper-reactivity through TLR tolerance, and may influence susceptibility to MS. TLR tolerance is a state of tolerance induced upon repeated ligation of a TLR by its ligands, which results in dampening of the magnitude of the signaling downstream of the TLR (54). We postulated that MS patients may have a defect in TLR tolerance, and therefore may demonstrate TLR hyper-responsiveness. We have also identified and characterized a new breakdown product of L654 named L342, which was also found at significantly lower levels in the plasma of MS patients. This work involving TLR tolerance in MS and L342 as a potential biomarker for MS will be discussed in Chapter 4.

CHAPTER 2 Cbl-b-Deficient Mice Express Alterations in Trafficking-Related Molecules but Retain Sensitivity to the Multiple Sclerosis Therapeutic Agent, FTY720

ABSTRACT

The variable response to therapy in multiple sclerosis (MS) suggests a need for personalized approaches based on individual genetic differences. GWAS have linked *CBLB* gene polymorphisms with MS and recent evidence demonstrated that these polymorphisms can be associated with abnormalities in T cell function and response to interferon-β therapy. Cbl-b is an E3 ubiquitin ligase that regulates T cell activation and Cbl-b-deficient (Cbl-$b^{-/-}$) mice show T cell abnormalities described in MS patients. We now show that Cbl-$b^{-/-}$ T cells demonstrate significant lymph node trafficking abnormalities. We thus asked whether the MS-approved drug, FTY720, postulated to trap T cells in lymphoid tissues, is less effective in the context of Cbl-b dysfunction. We now report that FTY720 significantly inhibits EAE in Cbl-$b^{-/-}$ mice. Our results newly document a role for Cbl-b in T cell trafficking but suggest nevertheless that MS patients with Cbl-b abnormalities may still be excellent candidates for FTY720 treatment.

INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) primarily affecting individuals 20 to 50 years old. The cause of MS is unknown and the disease in incurable, though a number of therapeutic drugs are available. Unfortunately, all presently available therapeutic options are effective only in the relapsing remitting (RR) subset of patients, and for each treatment option a proportion of these patients remain resistant to the therapeutic effects. The variable response of MS patients to the different drugs available suggests that a personalized treatment approach based on individual clinical and genetic differences may be desirable. However, how genetic variations in MS patients influence treatment outcomes is unclear.

It is known that both genetic and environmental factors are involved in the etiology of MS. In identifying genetic factors potentially underlying the cause of MS, genome wide association studies (GWAS) have revealed a number of single nucleotide polymorphisms (SNPs) that are potentially relevant in the pathogenesis of MS. One of the SNPs demonstrated in some, but not all, of such MS GWAS studies involves the *CBLB* gene (12-14). The *CBLB* gene encodes for Cbl-b (Casitas B-lineage lymphoma-b), an E3 ubiquitin ligase that negatively regulates T cell activation (24, 25, 55). Cbl-b has been shown to be relevant in regulating T cell responses in models of human disease such as allergic airway inflammation (56) and Cbl-b has been shown to be essential for TGF-β receptor signaling through direct inhibition of SMAD7 (57). Importantly, Cbl-b deficiency in mice (Cbl-$b^{-/-}$ mice) leads to multi-organ cellular infiltration associated with T cell hyper-reactivity (24), co-stimulation independence in T cell activation (25), and T cell resistance to regulatory T cell (Treg)-mediated suppression (39, 40). These abnormalities in Cbl-

$b^{-/-}$ mice have also been documented in MS patients (21, 32, 33, 58, 59). Consistent with this, Cbl-$b^{-/-}$ mice have been described to show increased susceptibility to experimental autoimmune encephalomyelitis (EAE), the murine model of MS (25, 45). Recently, one of three described MS-associated *CBLB* SNPs was reported to alter T cell Cbl-b expression levels and T cell function in both MS patients and healthy individuals carrying this SNP (35). Importantly, this alteration in T cell function was found to interfere with the normal immune-regulatory function of type I IFN, a commonly used drug to treat MS (35). These findings suggest that this *CBLB* SNP could potentially be important in predicting therapeutic effectiveness of type I IFN in this subset of patients. Thus, there is a potentially significant functional role for Cbl-b in at least a subset of MS patients and this in turn suggests that Cbl-$b^{-/-}$ mice could prove useful both for studying pathogenic mechanisms in MS and for predicting personalized therapeutic approaches in this subset of MS patients.

The various therapeutic approaches available for the treatment of MS mediate their effects through different physiologic mechanisms. FTY720 (Fingolimod/Gilenya), an FDA-approved orally administered drug for relapsing remitting MS (RRMS), targets the sphingosine-1-phosphate receptors, $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ (60). Though still controversial, FTY720 theoretically mediates its therapeutic effect in MS by causing degradation of the lymphocyte homing receptor $S1P_1$ (61). This blocks the egress of T and B cells from lymph nodes resulting in lymph node trapping of these cells and an inability of the immune system to mount an attack on self-antigens in the CNS (9). As with all the treatment options in MS, FTY720 is effective only in a proportion of patients with RRMS (6), but which patients will fare better with which specific treatment option is not yet predictable. In the present study, our goal was to use Cbl-$b^{-/-}$

mice as a new model for analyzing the efficacy of FTY720 in the context of altered Cbl-b function. Moreover, the efficacy of FTY720 had been demonstrated in studies using EAE in wild-type (WT) mice (62-65), but had never been tested in mice such as Cbl-b$^{-/-}$ mice that have both an MS-relevant genetic alteration and hyperactive T cells.

We now report for the first time that Cbl-b plays a role in regulating T cell trafficking and expression of trafficking related molecules, thus extending our knowledge of the involvement of Cbl-b in the regulation of T cell function. However, despite this role of Cbl-b in regulating T cell trafficking, FTY720 treatment was highly effective in inhibiting EAE in Cbl-b$^{-/-}$ mice. Overall, our findings document a novel role for Cbl-b in regulating T cell trafficking, but suggest, nevertheless, that MS patients with Cbl-b abnormalities may still be excellent candidates for FTY720 treatment.

MATERIALS AND METHODS

Mice

Female C57BL/6 (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Cbl-b$^{-/-}$ mice on a C57BL/6 background were a gift from Dr. H. Gu (Columbia University, New York, NY). RAG-1$^{-/-}$ mice were purchased from the Jackson Laboratory and bred and maintained in our facility. All mice were maintained and bred under specific pathogen-free conditions in accordance with the guidelines of the Center for Laboratory Animal Care at the University of Connecticut Health Center (Farmington, CT).

Adoptive transfer of $CD4^+$ $CD25^-$ effector T cells to RAG-$1^{-/-}$ mice

$CD4^+$ $CD25^-$ effector T cells (Teffs) were isolated via magnetic bead purification (Miltenyi Biotec, Auburn, CA) from spleens of 6-8 weeks old female C57BL/6 WT and Cbl-$b^{-/-}$ mice. Viability of Teffs was determined via trypan blue exclusion prior to adoptive transfer. 0.9-1.4 x 10^6 Teffs were given intraperitoneally (i.p.) to 6-8 weeks old female RAG-$1^{-/-}$ mice. On day 4-11 post transfer, mesenteric lymph nodes (mLNs) and peripheral lymph nodes (pLNs; axillary, cervical, and inguinal LNs pooled) of the recipient RAG-$1^{-/-}$ mice were harvested and LN cells were stained with anti-CD4-APC (Biolegend, San Diego, CA), anti-CD44-Pacific Blue (Biolegend) and anti-CD62L-APC eFluor780 (eBioscience, San Diego, CA) antibodies and analyzed by flow cytometry. The data were analyzed using Flowjo software.

CFA/MOG_{35-55} immunization and $S1P_1$/CD69/CCR7 expression assay

6-8 weeks old female C57BL/6 WT and Cbl-$b^{-/-}$ mice were immunized subcutaneously (s.c.) in the footpads with 80 μg of myelin oligodendrocyte glycoprotein peptide (35–55) (MOG_{35-55}) emulsified with complete Freund's adjuvants (CFA) containing 150 μg of H37RA mycobacteria (Difco BD Diagnostics, Sparks, MD). On day 4-5 post immunization, draining popliteal LNs and non-draining mesenteric LNs were harvested and LN cells stained with rat anti-$S1P_1$ monoclonal antibody (R&D Systems, Minneapolis, MN), biotin-SP-congugated AffiniPure $F(ab')_2$ fragment donkey anti-rat IgG (H+L) (Jackson Immunoresearch, West Grove, PA), streptavidin-PE (Life Technologies, Norwalk, CT), anti-CD4-APC, anti-CD8α-FITC (Biolegend), anti-CD44-Pacific Blue, anti-CD69-PerCP-Cy.5.5 (Biolegend), and analyzed by flow cytometry. CCR7 staining was performed using human CCL19-Ig (generously provided from the Dr. Lefrançois lab,

University of Connecticut Health Center) and goat anti-human IgG-Alexa Fluor 488 (Life Technologies).

Lymphopenia assay after administration of the S1P lyase inhibitor, THI

6-8 weeks old female C57BL/6 WT and Cbl-b$^{-/-}$ mice were administered 200 μg of THI (2-acetyl-5-hydroxybutyl imidazole) (Cayman Chemical, Ann Arbor, MI) or vehicle (sterile water) via oral gavage. At 24 and 48 hours post treatment, these mice were bled, red blood cells lysed with ACK buffer, total PB leukocytes stained with anti-CD4-APC and anti-CD8α-FITC antibodies (Biolegend) and samples analyzed by flow cytometry.

Lymphopenia assay after administration of the S1P$_1$-selective agonist, SEW2871

6-8 weeks old female C57BL/6 WT and Cbl-b$^{-/-}$ mice were administered 20 mg/kg of SEW2871 (Cayman Chemical) or vehicle (50% DMSO/25% Tween-20, v/v) via oral gavage. These mice were bled at 14, 22, and 38 hours post treatment, red blood cells lysed with ACK buffer, total PB leukocytes stained with anti-CD4-APC and anti-CD8α-FITC antibodies (Biolegend), and samples analyzed by flow cytometry.

Induction of experimental autoimmune encephalomyelitis (EAE) and FTY720 treatment

6-8 weeks old female C57BL/6 WT and Cbl-b$^{-/-}$ mice were immunized s.c. in the back with 165 μg of MOG$_{35–55}$ emulsified with CFA containing 300 μg of H37RA mycobacteria (Difco BD). 150 ng of Pertussis toxin (PTX) (List Biological Laboratories, Campbell, CA) was administered i.p. on day 0 and 2. On the day of EAE induction (day 0), these mice were also administered either 0.375 mg/kg of FTY720 (Cayman Chemical) or vehicle (2% (2-hydroxypropyl)-β-

cyclodextrin) (Sigma-Aldrich, St. Louis, MO). FTY720 (0.375 mg/kg) or vehicle was given every five days after the immunization up to day 20. Mice were evaluated daily for signs of EAE for approximately 30 days. EAE was scored as: grade 1, tail paralysis; grade 2, weakness of hind limbs with an altered gait; grade 3, hind limb paralysis; grade 4, front limb paralysis; and grade 5, death.

Statistical Analysis

Unless noted otherwise, values reported in all analyses are expressed as the mean ± SEM. Differences between groups were analyzed using an unpaired two-tailed Student's *t*-test. For EAE, the Mann-Whitney test was used to determine statistical significance. Statistical significance was accepted at $p<0.05$.

RESULTS

Cbl-b$^{-/-}$ CD4^{+} T cells show impaired mLN accumulation after adoptive transfer into RAG-1$^{-/-}$ mice.

In prior unpublished studies, we had noted a decreased mesenteric lymph node (mLN) recovery of Cbl-b$^{-/-}$ compared to wild type C57BL/6 (WT) CD4^{+} T cells after adoptive transfer into RAG-1$^{-/-}$ mice. Despite this abnormal mLN accumulation, Cbl-b$^{-/-}$ CD4^{+} T cells are efficient mediators of disease in the adoptive transfer model of colitis (unpublished observations). These findings suggested a potential abnormality in Cbl-b$^{-/-}$ CD4^{+} T cell trafficking or lymph node (LN) accumulation. To examine this question, we analyzed mLN and peripheral lymph node (pLN)

recovery of WT and Cbl-b$^{-/-}$ CD4^{+} T cells at various time points after adoptive transfer into RAG-1$^{-/-}$ mice.

WT and Cbl-b$^{-/-}$ CD4^{+} CD25^{-} effector T cells (Teff) were transferred into C57BL/6 RAG-1$^{-/-}$ mice. On day 4 post transfer only very low numbers of cells could be recovered from the mLNs of the RAG-1$^{-/-}$ recipients. However, at this early time point, the number of transferred WT and Cbl-b$^{-/-}$ CD4^{+} T cells found in mLNs of the recipient RAG-1$^{-/-}$ mice was not statistically different (**Figure 2-1A**). In contrast, on day 8-11 post transfer, in confirmation of our preliminary results, both the absolute number and the frequency of Cbl-b$^{-/-}$ CD4^{+} T cells recovered in mLNs were significantly lower than WT cells (**Figure 2-1B, 2-1C**). In addition, the total mLN cell number recovered from mice receiving Cbl-b$^{-/-}$ Teff was also significantly lower than in those receiving WT cells (data not shown). On day 15-18 post transfer, comparable number of Cbl-b$^{-/-}$ CD4^{+} T cells and WT cells were found in the mLNs (**Figure 2-8**). However, at this later time point, RAG-1$^{-/-}$ recipients are developing severe colitis and the mLNs are draining a site of active colitic inflammation. Thus, the interpretation of the mLN T cell recovery in terms of T cell trafficking and accumulation becomes complex.

To understand whether the decreased recovery seen with Cbl-b$^{-/-}$ T cells at day 8-11 was specific for the mLNs, we also examined peripheral lymph node (pLN) recovery of WT and Cbl-b$^{-/-}$ CD4^{+} T cells at various time points after adoptive transfer into RAG-1$^{-/-}$ mice. The pLNs examined were the inguinal, the axillary, and the cervical lymph nodes. Because the recovery of CD4^{+} T cells from the mLNs at day 4 was extremely low, we did not study the pLNs at this time point. When the recovery of CD4^{+} T cells from the pLNs was examined at day

8-11, we found that, in contrast to the mLNs, recovery of Cbl-b$^{-/-}$ CD4^{+} T cells was actually higher than that of WT CD4^{+} T cells (**Figure 2-1B**). At day 15-18 post transfer, comparable numbers of Cbl-b$^{-/-}$ CD4^{+} T cells and WT cells were found in the pLNs (data not shown). These results suggest that the decreased accumulation seen with transferred Cbl-b$^{-/-}$ CD4^{+} T cells was specific for the mLNs.

Because spontaneous colitis develops in RAG-1$^{-/-}$ mice adoptively transferred with Teff, we postulated that the day 8-11 mLN-specific decrease in accumulation noted with Cbl-b$^{-/-}$ CD4^{+} T cells may be related to an increase in CD4^{+} T cell activation occurring in the mLNs but not the pLNs. To examine this possibility, we first analyzed CD44 expression on the transferred CD4^{+} T cells. We found that greater than 90% of both WT and Cbl-b$^{-/-}$ CD4^{+} T cells expressed high levels of CD44 in both mLNs and pLNs (data not shown). This high level of CD44 expression is likely a result of the systemic activation induced under lymphopenic conditions.

To further pursue the postulate that there is more activation occurring in the transferred CD4^{+} T cells in the mLNs as compared to the pLNs, we next measured CD62L expression on the transferred WT and Cbl-b$^{-/-}$ CD4^{+} T cells. At day 8-11 after adoptive transfer, the time point at which a significant decrease in recovery of Cbl-b$^{-/-}$ CD4^{+} T cells was seen, we found that both WT and Cbl-b$^{-/-}$ CD4^{+} T cells showed a lower percentage of CD62L expression in the mLNs compared to the pLNs (**Figure 2-1D**). These results suggest that, post-transfer for both WT and Cbl-b$^{-/-}$ CD4^{+} T cells, there is a higher frequency of activated cells in the mLNs compared to the pLNs at this time point. This difference in activation between the mLNs and the pLNs likely relates to the colitis which is beginning to develop at that time. These results, in turn, suggest

that the difference in accumulation of Cbl-b$^{-/-}$ versus WT CD4^{+} T cells in the mLNs is dependent on the colitis-related, foreign antigen-induced activation occurring in the mLNs. Specifically, we postulated that the observed difference between the mLN accumulation of Cbl-b$^{-/-}$ versus WT CD4^{+} T cells is dependent on the transferred T cells being highly activated and furthermore, that this activation affects Cbl-b$^{-/-}$ T cells differently than WT T cells. These transferred CD4^{+} T cells express molecules, including S1P$_{1,}$ CD69, and CCR7, which are all likely relevant to the mechanism proposed for the effect of FTY720 in MS. Thus, we next analyzed the effect of Cbl-deficiency on these molecules.

Cbl-b$^{-/-}$ CD4^{+} T cells show enhanced S1P$_1$ expression and decreased CD69 expression in draining LNs after CFA/MOG$_{35-55}$ immunization.

To further characterize the decreased LN accumulation of Cbl-b$^{-/-}$ T cells, we next analyzed the expression of lymph node ingress/egress-related molecules on activated WT and Cbl-b$^{-/-}$ T cells. We began by characterizing the expression of the sphingosine-1-phosphate receptor 1 (S1P$_1$). Although S1P$_1$ is documented to be a major regulator of T cell egress from lymph nodes and peripheral tissues (66, 67), there have been no prior reports of the relationship between S1P$_1$ expression and Cbl-b, or of the S1P$_1$ expression status in mice with Cbl-b deficiency. Moreover, characterizing T cell S1P$_1$ expression was particularly relevant in light of our goal of using Cbl-b$^{-/-}$ mice as a new model for analyzing the MS-treatment efficacy of the S1P$_1$-modulating drug FTY720 (Fingolimod) in the context of altered Cbl-b function.

Given our finding of decreased LN accumulation of Cbl-b$^{-/-}$ CD4^{+} T cells in the context of activation and our goal of establishing a model for studying therapeutic responses relevant to

MS, we induced T cell activation by utilizing an immunization protocol similar to EAE. Cbl-b$^{-/-}$ and WT mice were immunized subcutaneously in the footpads with myelin oligodendrocyte glycoprotein peptide (35–55) (MOG_{35-55}) emulsified with complete Freund's adjuvant (CFA). On day 4 and 5 post immunization, draining popliteal LNs (dLNs) and mLNs (as non-draining controls) were harvested and T cell expression of $S1P_1$ was analyzed.

In dLNs, the frequency of Cbl-b$^{-/-}$ CD4$^+$ T cells expressing $S1P_1$ was significantly increased compared to WT CD4$^+$ T cells (**Figure 2-2A**). In addition, Cbl-b$^{-/-}$ CD8$^+$ T cells also showed the trend of increased frequency of $S1P_1^+$ cells compared to WT, although this did not reach statistical significance (**Figure 2-2A**). Mean fluorescence intensity (MFI) analysis also indicated a statistically significant increase in $S1P_1$ MFI of Cbl-b$^{-/-}$ CD4$^+$ T cells compared to WT cells in dLNs. For CD8$^+$ T cells, there was no statistical difference in $S1P_1$ MFI between WT and Cbl-b$^{-/-}$ mice (**Figure 2-9A**). In contrast, in the mLNs (i.e., non-draining), the frequency of $S1P_1^+$ Cbl-b$^{-/-}$ CD4$^+$ and CD8$^+$ T cells did not differ from that of WT (**Figure 2-2B**). MFI analysis also showed no difference in $S1P_1$ expression between WT and Cbl-b$^{-/-}$ CD4$^+$ and CD8$^+$ T cells in the mLNs (**Figure 2-9B**). These results represent the first demonstration of increased $S1P_1$ expression in Cbl-b$^{-/-}$ T cells and indicate that this increase correlates with the enhanced T cell activation status seen in the dLNs.

To further characterize the increased $S1P_1$ expression in activated Cbl-b$^{-/-}$ T cells, we analyzed the relationship between $S1P_1$ expression and CD44 expression. We noted that the frequency of CD44^{hi} CD4$^+$ and CD8$^+$ T cells were comparable between Cbl-b$^{-/-}$ and WT T cells in the dLNs. Surprisingly, we found that the increased $S1P_1$ expression seen in Cbl-b$^{-/-}$ CD4$^+$ T cells versus

WT $CD4^+$ T cells in the dLNs was restricted to the $CD44^{lo}$ populations, with the $CD44^{hi}$ populations showing no difference in $S1P_1$ expression between Cbl-$b^{-/-}$ T cells versus WT T cells (**Figure 2-10**). This may suggest that the enhanced $S1P_1$ expression on Cbl-$b^{-/-}$ T cells occurs very early in the activation process and might account for an early egress from the LNs by Cbl-$b^{-/-}$ $CD4^+$ T cells.

Expression of CD69 and CCR7 on Cbl-$b^{-/-}$ T cells after CFA/MOG_{35-55} immunization.

It has been documented that T cell expression of CD69 plays a role in the down-regulation of T cell $S1P_1$ expression (68, 69). We next analyzed the CD69 expression on WT and Cbl-$b^{-/-}$ T cells after immunization with CFA/MOG_{35-55}. We found that the frequency of Cbl-$b^{-/-}$ $CD4^+$ T cells expressing CD69 was significantly decreased compared to WT $CD4^+$ T cells in the dLNs. Surprisingly, this was not the case with the dLN $CD8^+$ T cells (**Figure 2-3A**). MFI analysis revealed no statistical difference in CD69 expression levels between WT and Cbl-$b^{-/-}$ $CD4^+$ T cells (**Figure 2-11A**), suggesting that the frequency of $CD4^+$ T cells that express CD69, rather than the CD69 expression levels, is decreased in dLNs of Cbl-$b^{-/-}$ mice. In the non-draining mLNs, the frequency of $CD69^+$ Cbl-$b^{-/-}$ $CD4^+$ T cells, rather than being decreased, was significantly increased compared to WT $CD4^+$ T cells (**Figure 2-3B**). The frequency of $CD69^+$ Cbl-$b^{-/-}$ $CD8^+$ T cells in the mLNs was again comparable to that of WT $CD8^+$ T cells (**Figure 2-3B**). Despite the increased frequency of Cbl-$b^{-/-}$ $CD4^+$ T cells expressing CD69 in mLNs, MFI analysis did not show any difference in CD69 expression between Cbl-$b^{-/-}$ and WT $CD4^+$ and $CD8^+$ T cells in mLNs (**Figure 2-11B**). Since it is known that CD69 expression down-regulates the expression of $S1P_1$, the decreased CD69 expression in Cbl-$b^{-/-}$ $CD4^+$ T cells in the dLNs may play a role in the increase in $S1P_1$ expression seen in these cells. Both the decreased frequency of

CD69-expressing Cbl-b$^{-/-}$ CD4^{+} T cells in the dLNs, along with the increased S1P$_1$ expression on these cells, may potentially contribute to their abnormal LN accumulation *in vivo*.

In parallel to the CD44^{lo}-restricted increased S1P$_1$ expression in Cbl-b$^{-/-}$ CD4^{+} T cells, the decreased frequency of Cbl-b$^{-/-}$ CD4^{+} T cells expressing CD69 versus that of WT in the dLNs was again restricted to the CD44^{lo} populations, with the CD44^{hi} populations showing no difference in the frequency of CD69^{+} CD4^{+} T cells (**Figure 2-12**). This may suggest that the further downregulation of CD69-expressing Cbl-b$^{-/-}$ CD4+ T cells, along with enhanced expression of S1P$_1$ on these cells, occurs very early in the activation process and might account for an early egress from the LNs by Cbl-b$^{-/-}$ CD4^{+} T cells.

In addition to CD69, CCR7 has been shown to promote T cell entry and retention in lymph nodes (70, 71). We next examined the status of CCR7 expression in WT and Cbl-b$^{-/-}$ T cells in dLNs and non-draining mLNs after immunization with CFA/MOG$_{35-55.}$ We found no significant difference in frequency of CCR7 expression between the WT and Cbl-b$^{-/-}$ T cells for both CD4^{+} and CD8^{+} T cells (data not shown). However, because approximately 80% of both WT and Cbl-b$^{-/-}$ T cells expressed CCR7, we analyzed CCR7 expression using mean fluorescence intensity (MFI). As seen in Figure 4A and B, there was no significant difference in CCR7 MFI between WT and Cbl-b$^{-/-}$ T cells in both dLNs and non-draining mLNs. Our results suggest that the abnormal LN accumulation noted for Cbl-b$^{-/-}$ CD4^{+} T cells is not related to alterations in CCR7 expression.

Normal response of Cbl-b$^{-/-}$ mice to T cell lymphopenia induced by S1P lyase inhibitor, THI.

Our finding of the significantly increased frequency of Cbl-b$^{-/-}$ CD4^{+} T cells expressing S1P$_1$ compared to WT CD4^{+} T cells in the dLNs, prompted us to more directly understand the impact of Cbl-b deficiency on T cell S1P$_1$ physiology. We next compared S1P$_1$ functional status in WT versus Cbl-b$^{-/-}$ T cells using two approaches. In the first approach, we tested the induction of T cell lymphopenia mediated through the enhancement of sphingosine-1-phosphate (S1P), the endogenous ligand of S1P$_1$. For this, we utilized the S1P lyase inhibitor, THI (2-acetyl-4-tetrahydroxybutyl imidazole) (72). S1P lyase normally degrades S1P and is highly expressed in the lymph nodes and other tissues compared to the blood, resulting in a S1P gradient between the tissues and the blood. This S1P gradient actively promotes the egress of lymphocytes from the LNs to the blood/lymph. The inhibition of S1P lyase by THI disrupts this S1P gradient. This results in internalization and loss of S1P$_1$ expression on lymphocytes with entrapment of these cells in LNs and a significant decrease in blood lymphocytes (72). Thus, we measured the response to THI by analyzing the decrease in the frequency of CD4^{+} and CD8^{+} T cells in the blood of the THI-treated mice.

Prior to THI treatment, the frequencies of CD4^{+} and CD8^{+} T cells in the blood did not differ between WT and Cbl-b$^{-/-}$ mice (data not shown). Of note, the absolute numbers of CD4^{+} and CD8^{+} T cells in the blood of Cbl-b$^{-/-}$ mice were higher than the absolute numbers of CD4^{+} and CD8^{+} T cells in the blood of WT mice prior to THI treatment (mean WT CD4^{+} T cells = 3.53 x 10^5/ml; Cbl-b$^{-/-}$ CD4^{+} T cells = 5.23 x 10^5/ml; WT CD8^{+} T cells = 1.98 x 10^5/ml; Cbl-b$^{-/-}$ CD8^{+} T cells = 3.00 x 10^5/ml). Twenty-four hours after treatment with THI, the frequency of blood CD4^{+} and CD8^{+} T cells of WT and Cbl-b$^{-/-}$ mice decreased significantly but equally (**Figure 2-5A and 2-5B**). Forty-eight hours after THI treatment, the frequency of blood CD4^{+} and CD8^{+} T

cells returned significantly towards pre-treatment levels and this return was also equal between WT and Cbl-b$^{-/-}$ mice (**Figure 2-5A and 2-5B**). Thus, we found that the T cell lymphopenia resulting from administration of the S1P lyase inhibitor, THI, was comparable between Cbl-b$^{-/-}$ and WT mice. This suggests that the functional status of $S1P_1$ in Cbl-b$^{-/-}$ T cells is normal under non-inflammatory, naïve condition.

Comparable response of Cbl-b$^{-/-}$ and WT mice to T cell lymphopenia induced by a selective $S1P_1$ agonist, SEW2871.

In the second functional approach, we tested the induction of T cell lymphopenia mediated through the administration of an exogenous $S1P_1$-selective agonist, SEW2871 (73). SEW2871 directly binds to $S1P_1$ on T cells, resulting in $S1P_1$ internalization and subsequent entrapment of T cells in LNs (73). While Cbl-b$^{-/-}$ mice showed more CD4$^+$ and CD8$^+$ T cell lymphopenia compared to WT mice in response to SEW2871 at 14 hours post treatment, we found comparable responses in WT and Cbl-b$^{-/-}$ mice at 22 and 38 hours (**Figure 2-6A and 2-6B**). The reasons for the enhanced early response to SEW2871 by Cbl-b$^{-/-}$ mice T cells in a non-inflammatory, naïve condition is unclear. However, taking into account the later time points, at which time there is no difference between the SEW2871-induced T cell lymphopenia in WT and Cbl-b$^{-/-}$ mice, overall our results with THI and SEW2871 suggest no significant alteration in the $S1P_1$ functional status of Cbl-b$^{-/-}$ T cells in a non-activated milieu.

Exacerbated EAE yet high efficacy of FTY720 in Cbl-b$^{-/-}$ mice during EAE.

Our results to this point suggested that under activating conditions, Cbl-b$^{-/-}$ CD4$^+$ T cells demonstrate enhanced expression of $S1P_1$ and decreased expression of CD69 and that these

characteristics potentially result in enhanced LN egress. By extension, these results also suggested that MS patients with *CBLB* SNPs might have abnormalities in T cell trafficking and that these could impinge on the efficacy of treatment with FTY720. To address this possibility, we next asked whether FTY720 would be less effective in treating EAE in Cbl-b$^{-/-}$ versus mice WT mice. Theoretically, this approach would, for the first time test the efficacy of FTY720 in the context of Cbl-b dysfunction and T cell hyper-reactivity – both characteristics associated with MS.

We induced EAE in WT and Cbl-b$^{-/-}$ mice using CFA and MOG_{35-55}, and on the same day began treating these mice with either 0.375 mg/kg of FTY720 or the vehicle control (VC). Based on previous published studies in which FTY720 was used in treating EAE in WT mice, we chose an intermediate dosage of FTY720 but administered it only every 5 days rather than daily as in most of the earlier studies (63, 65, 74). In the vehicle-treated mice, we found an enhancement of EAE in Cbl-b$^{-/-}$ mice compared to WT mice (Mann-Whitney test: ***$p<0.0004$, Figure 7) as previously reported by other investigators (25, 45). Despite our use of a restricted FTY720 treatment regimen (i.e., administered only every 5th day), we noted significant inhibition of EAE by FTY720 in WT mice compared to VC-treated WT mice (****$p<0.0001$) (**Figure 2-7**). Most importantly however, despite the enhanced baseline EAE and the evidence for both decreased CD69 and enhanced $S1P_1$ expression in activated T cells in Cbl-b$^{-/-}$ mice, we also found significant inhibition of EAE by FTY720 in Cbl-b$^{-/-}$ mice compared to VC-treated Cbl-b$^{-/-}$ mice (****$p<0.0001$) (**Figure 2-7**). These results suggest that FTY720 can inhibit EAE in the context of both Cbl-b deficiency and hyperactive T cell responses.

DISCUSSION

A number of therapeutic options are now available for patients with MS. Unfortunately, almost all of these agents are associated with severe side effects and are limited in their efficacy to only a proportion of patients with RRMS. This has spurred interest in a "personalized medicine" approach involving individualization of treatment regimens for MS patients based not only on clinical sub-types of MS but also on an individual's genetic profile. In identifying genetic factors potentially involved in the pathogenesis of MS, GWAS have revealed a number of potentially MS-relevant genetic polymorphisms or SNPs. While understanding the role of these SNPs in the disease pathogenesis is a primary research goal, another goal has emerged, namely to identify whether these SNPs can associate with (and potentially predict) differential responses to the various therapeutic approaches.

One gene identified as being associated with MS is *CBLB*. GWAS have identified an association of MS with three different polymorphisms in the *CBLB* gene (12-14). As recently discussed, while not all GWAS have identified these MS-associated *CBLB* gene SNPs, the different outcomes of the various GWAS may be related to differing ethnic origins and geographical locations of the populations analyzed (75). Interestingly, decreased Cbl-b mRNA and decreased Cbl-b protein expression in PBMCs of patients with RRMS have also previously been described (34). The *CBLB* gene encodes for the E3 ubiquitin ligase Cbl-b. Cbl-b is known to be involved in regulating a number of cellular processes but has been most characterized as a regulator of T cell activation. In Cbl-b$^{-/-}$ mice, T cells have been shown to be hyperactive (24, 25), co-stimulation-independent (25), have a lower activation threshold (24, 25), and to be resistant to Tregs (39, 40), TGF-β (41), and the induction of anergy (76). Interestingly, many of these abnormalities have

also been documented in MS patients (21, 32, 33, 58, 59). In addition, Cbl-b$^{-/-}$ mice have been shown to have increased susceptibility to EAE (25, 45).

Sturner *et al*. recently studied T cell functional abnormalities associated with the *CBLB* SNP – MS-risk allele, rs12487066 (35). This specific *CBLB* SNP was studied because it had previously been identified in a GWAS of individuals with MS recruited from the U.K. and the US. These authors believed their patient cohort, derived from Northern Germany, would be similar to the U.K./U.S. cohort. In this study, it was reported that in RRMS patients this risk allele altered both T cell Cbl-b expression levels and T cell function, and specifically interfered with the normal regulatory function of type I IFN on T cells (35). Thus, this recent study for the first time associated a specific *CBLB* SNP with both an alteration in immunological function and an abnormal response to an MS treatment option.

Although Cbl-b$^{-/-}$ mice and MS patients demonstrate many similar T cell abnormalities, Cbl-b$^{-/-}$ mice have not been specifically used as a model for studying MS until now. Given the immunological similarities between Cbl-b$^{-/-}$ mice and a subset of patients with MS, these mice may provide a better model than wild type C57BL/6 mice for studying many aspects of the pathophysiology of MS. Moreover in the present study we postulated that Cbl-b$^{-/-}$ mice, because of their T cell hyper-reactivity, may prove to be a relevant new tool for studying personalized treatment approaches in MS, and specifically for a subset of MS patients with polymorphisms in the *CBLB* gene.

FTY720 (Fingolimod/Gilenya), an FDA-approved orally available drug for treating RRMS, is postulated to target the sphingosine-1-phosphate receptors, $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ (60). Though controversial, FTY720 theoretically mediates its therapeutic effect in MS by causing the degradation of the lymphocyte homing receptor $S1P_1$ (61), which then blocks the egress of T and B cells from lymph nodes resulting in an inability of the immune system to mount an attack on self-antigens in the CNS (9). As with all present treatment choices for MS, FTY720 is not effective in all of RRMS patients (6). Importantly, pre-clinical studies demonstrating the efficacy of this drug were carried out in C57BL/6 mice (62-65), and thus not in mice with either known genetic alterations related to MS or in mice with MS-like hyper-reactive cellular immune responses.

Our specific interest in studying the potential inter-relationship between abnormalities in Cbl-b and FTY720 initially arose from two sources. First, it is known that Cbl-b normally regulates the PI3K-Akt pathway (28) and thus affects many targets downstream of this pathway including the $S1P_1$-regulating transcription factor KLF2 (77, 78). Second, we had preliminary results indicating that T cells from Cbl-$b^{-/-}$ mice demonstrated impaired LN accumulation when adoptively transferred into RAG-$1^{-/-}$ mice, suggesting a potential abnormality in T cell $S1P_1$ function in Cbl-$b^{-/-}$ mice. We thus postulated that Cbl-$b^{-/-}$ mice, and by extension MS patients carrying *CBLB* gene SNPs, would be refractory to the EAE/MS-inhibiting effect of FTY720.

We now report for the first time that Cbl-$b^{-/-}$ T cells demonstrate: 1) abnormal accumulation in the mLNs upon adoptive transfer into RAG-$1^{-/-}$ mice; 2) enhanced $S1P_1$ expression in dLN $CD4^+$ T cells after immunization *in vivo* and; 3) decreased CD69 expression in dLN $CD4^+$ T cells after

immunization *in vivo*. Thus, in addition to the well-documented list of altered T cell functions resulting from deletion of Cbl-b, we now add altered expression of trafficking-related molecules and altered trafficking after adoptive transfer. Importantly, these newly defined abnormalities are associated with activated Cbl-b$^{-/-}$ T cells. In non-activated scenarios, Cbl-b$^{-/-}$ T cells show no difference from WT T cells in expression of trafficking-related molecules and no functional differences in response to exogenous agents that modulate $S1P_1$-dependent T cell trafficking.

In T cells, activation of the PI3K-Akt pathway downstream of the TCR and co-stimulatory receptors such as CD28 has been shown to negatively regulate $S1P_1$ expression by inactivating the Foxo1-KLF2 axis (79, 80). Cbl-b normally negatively regulates the PI3K-Akt pathway (28, 29, 81) and the deletion of Cbl-b is known to enhance the activation of this pathway (29, 81). This enhanced activation of the PI3K-Akt pathway in Cbl-b$^{-/-}$ mice leads to increased inactivation of Foxo1 (29), which theoretically inhibits KLF2 activity and should result in decreased expression of $S1P_1$. Our finding of enhanced $S1P_1$ expression in activated Cbl-b$^{-/-}$ CD4$^+$ T cells suggests the involvement of a more complex mechanism, or at least more complex kinetics, involved in the regulation of $S1P_1$ in Cbl-b$^{-/-}$ CD4$^+$ T cells. Given that T cell expression of CD69 is known to result in the decreased expression of $S1P_1$ (68), our findings of enhanced expression of $S1P_1$ by Cbl-b$^{-/-}$ CD4$^+$ T cells may be related to the decreased expression of CD69 by these cells after immunization.

Because of our findings of abnormal mLN accumulation after adoptive transfer, enhanced expression of $S1P_1$ and decreased expression of CD69 by Cbl-b$^{-/-}$ CD4$^+$ T cells under activating conditions, we postulated that a characteristic of activated Cbl-b$^{-/-}$ CD4$^+$ T cells might be

enhanced lymph node egress. By extension, these results suggested that MS patients with *CBLB* SNPs might also have abnormalities in $CD4^+$ T cell trafficking and that these could impinge on the efficacy of treatment with FTY720. As such, our primary goal was to utilize Cbl-$b^{-/-}$ mice as a new model for studying the efficacy of FTY720 in this subset of MS patients.

In these studies, we opted to be on the low end of published effective doses of FTY720 in EAE by administering a middle range dose, but utilized an every fifth day, rather than the usual, every-day dosing schedule. In inducing EAE in vehicle-treated WT and Cbl-$b^{-/-}$ mice, we found the expected enhancement of EAE in Cbl-$b^{-/-}$ mice compared to WT mice. However, despite enhanced EAE in vehicle-treated Cbl-$b^{-/-}$ mice, as well as enhanced $S1P_1$ and decreased CD69 expression in activated Cbl-$b^{-/-}$ $CD4^+$ T cells, FTY720 treatment nevertheless resulted in a significant inhibition of EAE in both WT mice and Cbl-$b^{-/-}$ mice.

It is not clear at this time why Cbl-$b^{-/-}$ mice are sensitive to FTY720 despite enhanced $S1P_1$ and decreased CD69 T cell expression under activating conditions. It is possible that the degree of alteration in $S1P_1$ and CD69 expression is not sufficient to overcome the immune-regulatory effects mediated by FTY720. In this regard, it is of interest that Thangada *et al.* reported that knock-in mice expressing an internalization-defective $S1P_1$ were still sensitive to the lymphopenic effects of FTY720 (82). Importantly, there are also suggestions that FTY720 may inhibit EAE/MS through mechanisms other than lymph node trapping of T cells. These may include effects on endothelial cells, the blood brain barrier, or resident cells of the CNS (83). While this will be a topic of future study in our laboratory, our finding of note in the present study is the efficacy of FTY720 in inhibiting EAE in the context of Cbl-b dysfunction.

In sum, in these studies we have identified novel characteristics of T cell trafficking related to Cbl-b deficiency and these characteristics are potentially also relevant to this subset of MS patients. Moreover, we have documented for the first time the efficacy of FTY720 in inhibiting EAE in the context of both Cbl-b deficiency and MS-like T cell hyper-reactivity. Consistent with the report of Sturner *et al*. (35), our present results suggests that Cbl-b$^{-/-}$ mice may prove to be a valuable new model both for studying pathogenic mechanisms in MS and for testing personalized treatment approaches in a subset of MS patients. Finally, our results suggest for the first time that those MS patients with aberrations in Cbl-b function are still likely to be excellent candidates for treatment with FTY720.

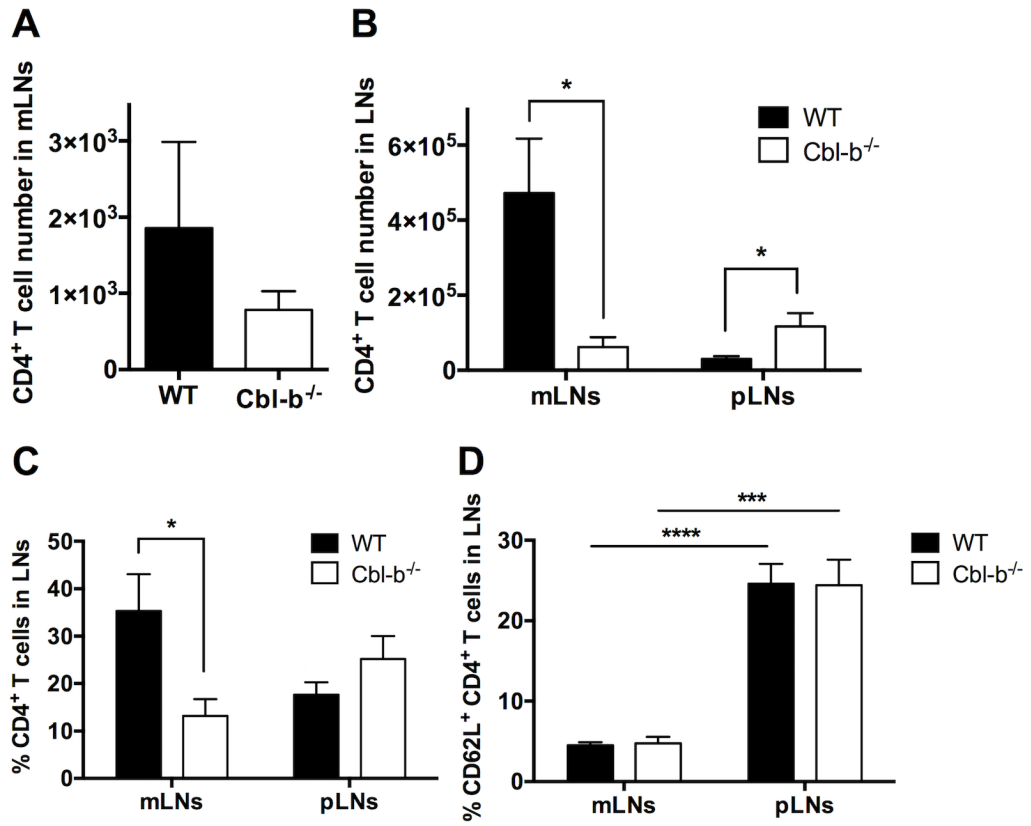


Figure 2-1. Decreased accumulation of Cbl-b$^{-/-}$ CD4^{+} T cells in mLNs of RAG-1$^{-/-}$ mice after adoptive transfer. 0.9-1.4 x 10^{6} WT or Cbl-b$^{-/-}$ CD4^{+} CD25^{-} T cells were injected i.p. into RAG-1$^{-/-}$ mice and mLNs and pLNs were harvested on day 4 and day 8-11 for analysis. (A and B) Absolute number of transferred WT or Cbl-b$^{-/-}$ CD4^{+} T cells in LNs of recipient RAG-1$^{-/-}$ mice on day 4 (A), and day 8-11 (B) post transfer. (C) Frequency of transferred WT or Cbl-b$^{-/-}$ CD4^{+} T cells in LNs of recipients on day 8-11 post transfer. (D) Frequency of WT and Cbl-b$^{-/-}$ CD62L^{+} CD4^{+} T cells in mLNs and pLNs of recipients on day 8-11 post transfer. *p<0.05, ***p=0.0001, ****p<0.0001 by Student's *t*-test. n=7-9/group for A, B, and C. n=6/group for D. The data shown are from three (A), five (B and C) and two (D) independent experiments.

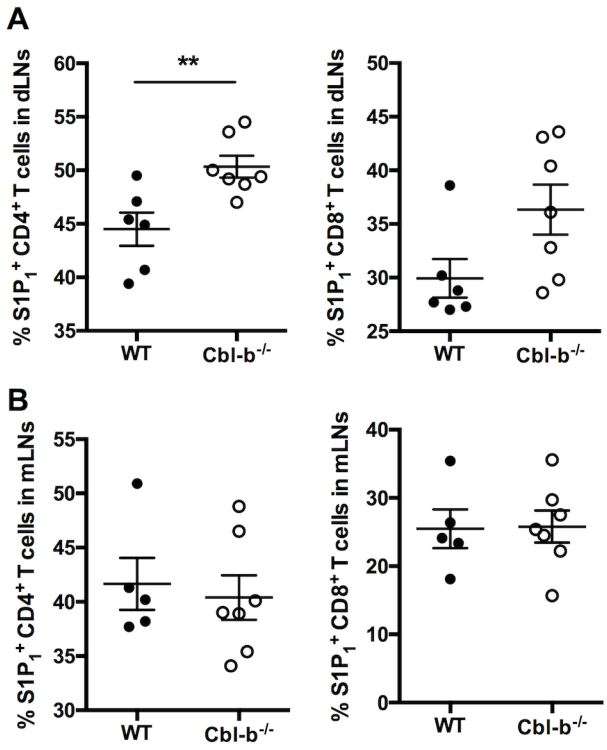


Figure 2-2. Increased frequency of $S1P_1^+$ $CD4^+$ T cells in draining LNs of Cbl-$b^{-/-}$ mice after CFA/MOG_{35-55} immunization. WT and Cbl-$b^{-/-}$ mice were immunized s.c. in the footpads with 150 μg of CFA and 80 μg of MOG_{35-55} and the popliteal LNs were harvested 4-5 days later. (A) Percentage of $CD4^+$ T cells (left) and $CD8^+$ T cells (right) that are $S1P_1^+$ in the popliteal LNs of WT and Cbl-$b^{-/-}$ mice. (B) Percentage of $CD4^+$ T cells (left) and $CD8^+$ T cells (right) that are $S1P_1^+$ in the mLNs of WT and Cbl-$b^{-/-}$ mice. **$p<0.01$ by Student's *t*-test. The data shown are from three independent experiments.

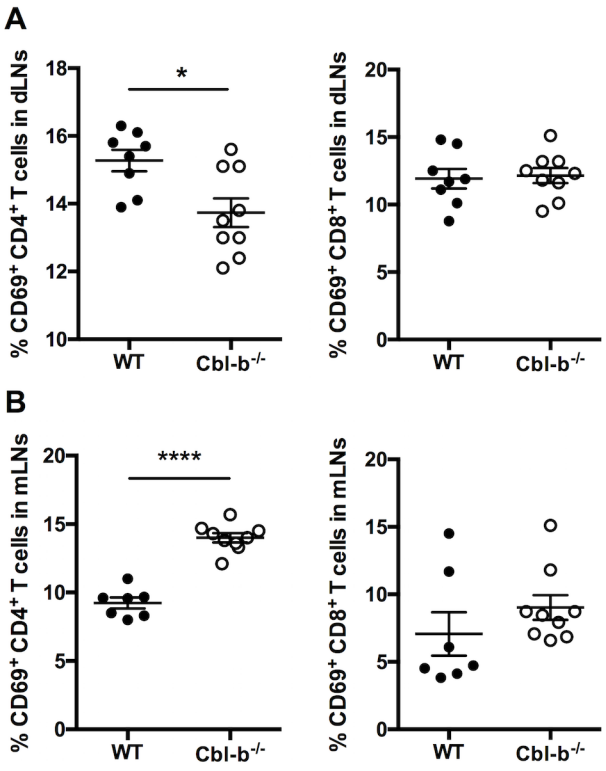


Figure 2-3. Decreased frequency of CD69$^+$ CD4$^+$ T cells in draining LNs of Cbl-b$^{-/-}$ mice after CFA/MOG$_{35-55}$ immunization. WT and Cbl-b$^{-/-}$ mice were immunized as in Figure 2 and the popliteal LNs were harvested 4-5 days later. (A) Percentage of CD4$^+$ T cells (left) and CD8$^+$ T cells (right) that are CD69$^+$ in the popliteal LNs of WT and Cbl-b$^{-/-}$ mice. (B) Percentage of CD4$^+$ T cells (left) and CD8$^+$ T cells (right) that are CD69$^+$ in the mLNs of WT and Cbl-b$^{-/-}$ mice. *p<0.05, ****p<0.0001 by Student's *t*-test. The data shown are from three independent experiments.

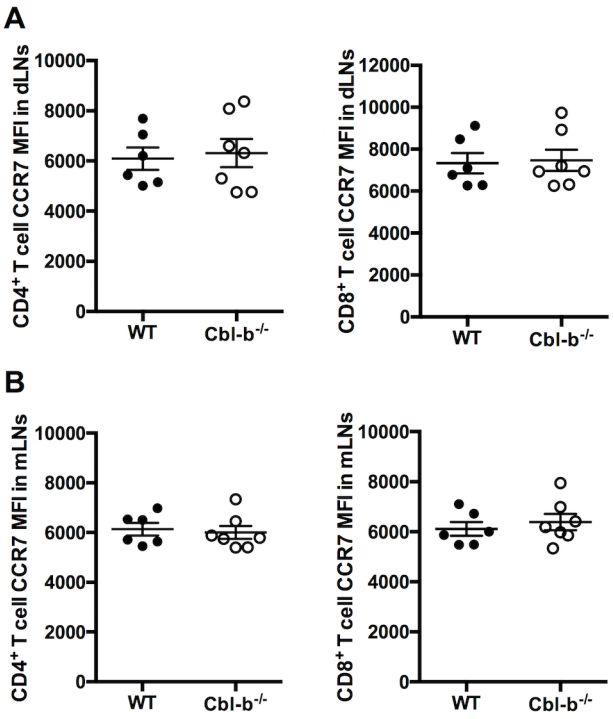


Figure 2-4. CCR7 expression is unaltered in Cbl-b$^{-/-}$ T cells after CFA/MOG$_{35-55}$ immunization. WT and Cbl-b$^{-/-}$ mice were immunized, the popliteal LNs harvested as in Figure 2, and stained with human CCL19-Ig and anti-human IgG-Alexa Fluor 488. (A) MFI of CCR7 expression on CD4^{+} T cells (left) and on CD8^{+} T cells (right) in draining popliteal LNs of WT and Cbl-b$^{-/-}$ mice. (B) MFI of CCR7 expression on CD4^{+} T cells (left) and on CD8^{+} T cells (right) in the mLNs of WT and Cbl-b$^{-/-}$ mice. Results were analyzed using Student's *t*-test. The data shown are from three independent experiments.

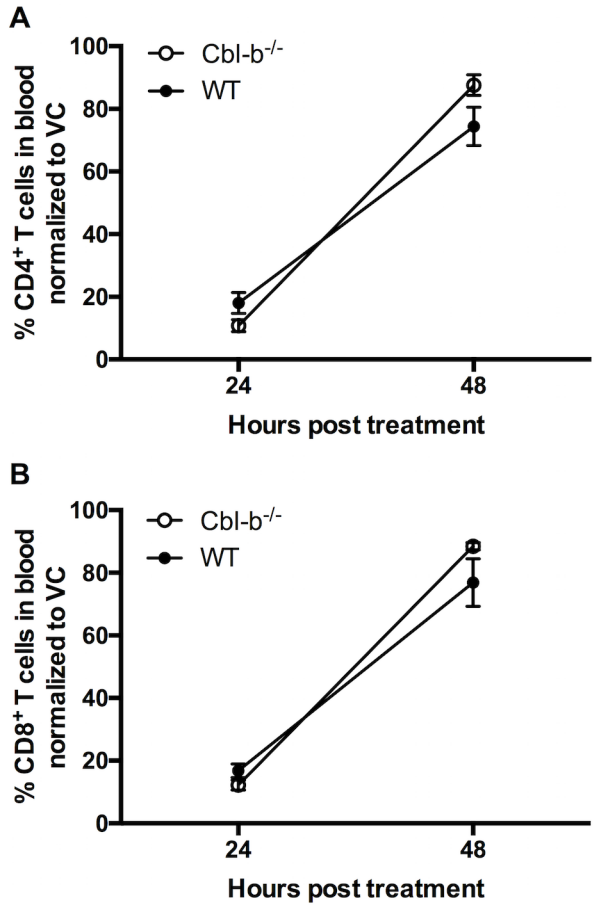


Figure 2-5. Equal lymphopenic response of WT and Cbl-b$^{-/-}$ mice induced by the S1P lyase inhibitor, THI. WT and Cbl-b$^{-/-}$ mice were administered 10 mg/kg of THI or vehicle (VC) via gavage, and percent of CD4^{+} and CD8^{+} T cells in the total blood leukocytes was determined by flow cytometry. (A) Percentage of blood CD4^{+} T cells and (B) Percentage of blood CD8^{+} T cells of WT or Cbl-b$^{-/-}$ mice, 24 and 48 hrs after administration of VC or THI. Percentages represent mean percentage of T cells in THI-treated mice / mean percentage of T cells in VC-treated mice x100 at each time point. Results were analyzed using Student's *t*-test. n=3-4/group. The data shown are from two independent experiments.

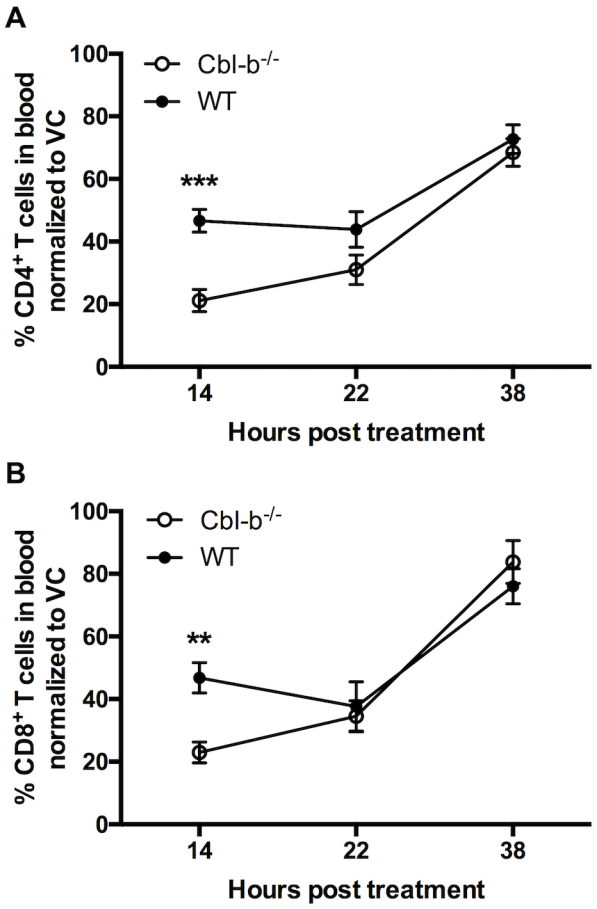


Figure 2-6. Equal lymphopenic response of WT and Cbl-b$^{-/-}$ mice induced by the selective S1P$_1$ agonist, SEW2871. WT and Cbl-b$^{-/-}$ mice were administered 20 mg/kg of SEW2871 or vehicle (VC) via gavage, and percentage of CD4$^+$ and CD8$^+$ T cells in the total blood leukocytes was determined by flow cytometry. (A) Percentage of blood CD4$^+$ T cells and (B) Percentage of blood CD8$^+$ T cells of WT or Cbl-b$^{-/-}$ mice treated with VC or SEW2871 at 14, 22 and 38 hrs after treatment. Percentages represent mean percentage of T cells in SEW2871-treated mice / mean percentage of T cells in VC-treated mice x100 at each time point. **p<0.01, ***p<0.001 by Student's *t*-test. n=4-7/group. The data shown are from five independent experiments.

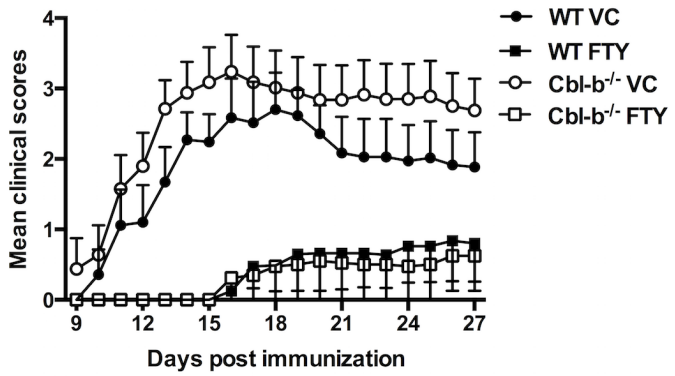


Figure 2-7. Efficacy of FTY720 in inhibiting EAE in Cbl-b$^{-/-}$ mice. EAE was induced in WT and Cbl-b$^{-/-}$ mice by s.c. immunization with 165 μg of MOG_{35-55} and 300 μg of CFA. 150 ng of Pertussis toxin was also given i.p. on day 0 and 2. On the day of EAE-induction, the mice were administered either 0.375 mg/kg of FTY720 (FTY) or vehicle (VC) via gavage. FTY720 or vehicle was then administered every five days until day 20. Clinical scores: 1 = tail flaccidity, 2 = abnormal gait, 3 = hind leg paralysis, 4 = front leg paralysis, 5 = death. The disease severity differed significantly between the VC-treated WT group vs. the VC-treated Cbl-b$^{-/-}$ group, ***p=0.0004. In addition, the disease severity differed significantly between the VC-treated WT group vs. the FTY720-treated WT group, ****p<0.0001, and also between the VC-treated Cbl-b$^{-/-}$ group vs. the FTY720-treated Cbl-b$^{-/-}$ group, ****p<0.0001 by Mann-Whitney test. The data shown are from two independent experiments (n = 7-8/treatment group in total).

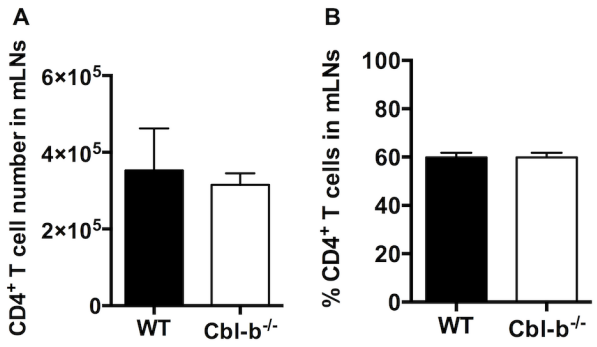


Figure 2-8. Accumulation of Cbl-b$^{-/-}$ CD4^{+} T cells is comparable to WT cells in mLNs of RAG-1$^{-/-}$ mice 18 days after adoptive transfer. 0.9-1.4 x 10^6 WT or Cbl-b$^{-/-}$ CD4^{+} CD25^{-} T cells were injected i.p. into RAG-1$^{-/-}$ mice and mLNs were harvested on day 18 for analysis. (A) Absolute number of transferred WT or Cbl-b$^{-/-}$ CD4^{+} T cells in mLNs of recipient RAG-1$^{-/-}$ mice on day 18. (B) Frequency of transferred WT or Cbl-b$^{-/-}$ CD4^{+} T cells in mLNs of recipients on day 18 post transfer. n = 2/group.

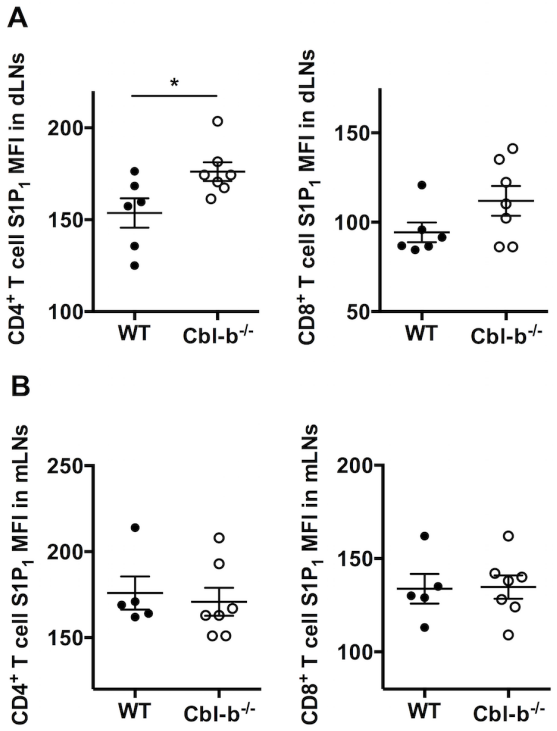


Figure 2-9. MFI analysis of $S1P_1$ expression in WT and Cbl-$b^{-/-}$ T cells after CFA/MOG_{35-55} immunization. WT and Cbl-$b^{-/-}$ mice were immunized s.c. in the footpads with 150 μg of CFA and 80 μg of MOG_{35-55} and the popliteal LNs were harvested 4-5 days later. (A) MFI of $S1P_1$ staining in $CD4^+$ T cells (left) and $CD8^+$ T cells (right) in the draining popliteal LNs of WT and Cbl-$b^{-/-}$ mice. (B) MFI of $S1P_1$ staining in $CD4^+$ T cells (left) and $CD8^+$ T cells (right) in the mLNs of WT and Cbl-$b^{-/-}$ mice. *$p<0.05$ by Student's *t*-test. The data shown are from three independent experiments.

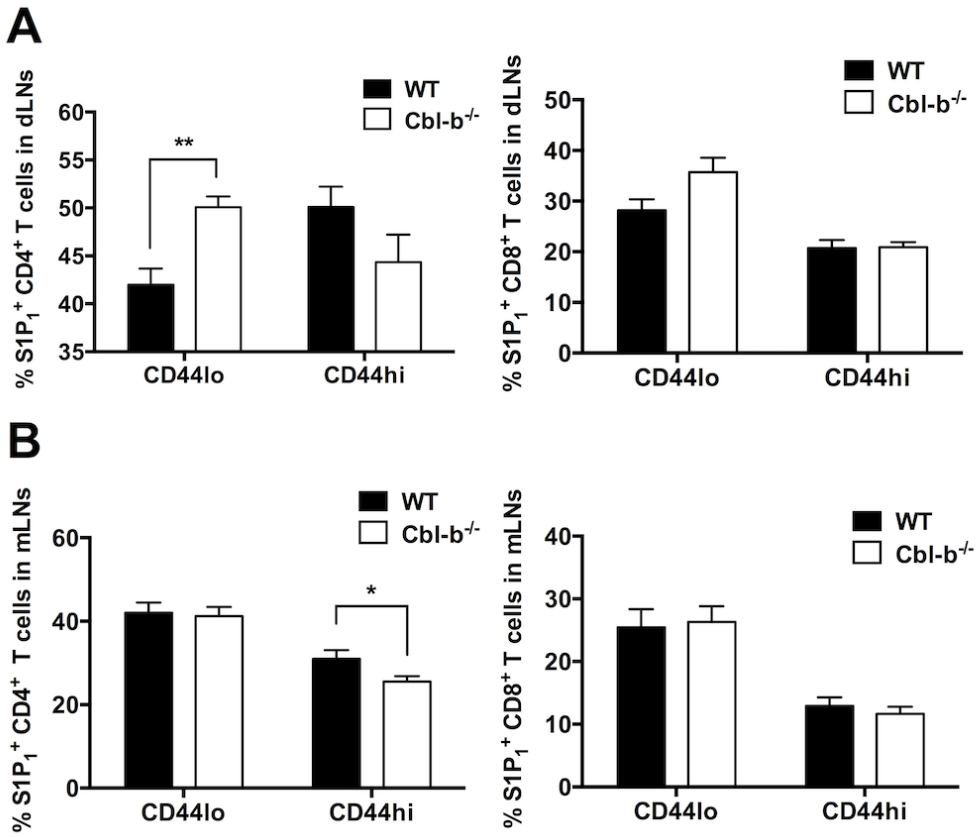


Figure 2-10. $S1P_1$ expression in $CD44^{lo}$ and $CD44^{hi}$ populations of WT and Cbl-$b^{-/-}$ T cells in draining popliteal LNs after CFA/MOG_{35-55} immunization. WT and Cbl-$b^{-/-}$ mice were immunized s.c. in the footpads with 150 μg of CFA and 80 μg of MOG_{35-55} and the popliteal LNs were harvested 4-5 days later. (A) Percentage of $CD44^{lo}$ and $CD44^{hi}$ $CD4^+$ T cells (left) and $CD8^+$ T cells (right) that express $S1P_1$ in draining popliteal LNs of WT and Cbl-$b^{-/-}$ mice. (B) Percentage of $CD44^{lo}$ and $CD44^{hi}$ $CD4^+$ T cells (left) and $CD8^+$ T cells (right) that express $S1P_1$ in non-draining mLNs of WT and Cbl-$b^{-/-}$ mice. *$p<0.05$, **$p<0.01$ by Student's *t*-test. The data shown are from three independent experiments.

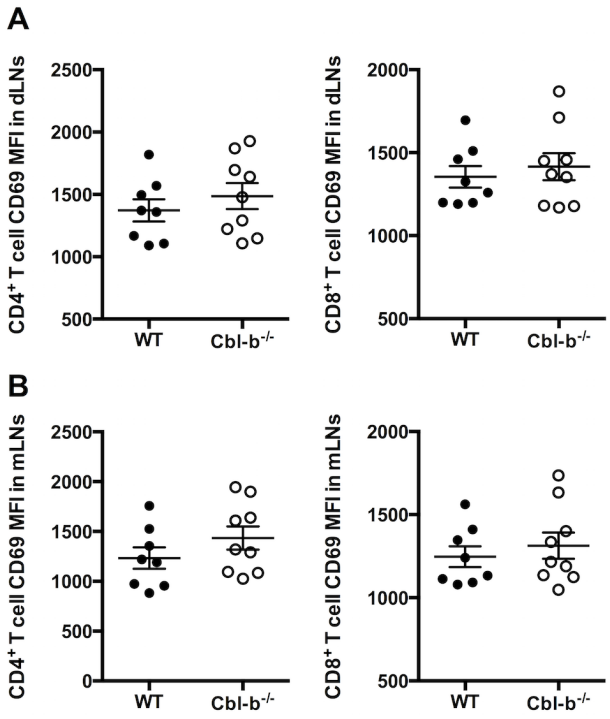


Figure 2-11. MFI analysis of CD69 expression in WT and Cbl-b$^{-/-}$ T cells after CFA/MOG$_{35-55}$ immunization. WT and Cbl-b$^{-/-}$ mice were immunized s.c. in the footpads with 150 μg of CFA and 80 μg of MOG$_{35-55}$ and the popliteal LNs were harvested 4-5 days later. (A) MFI of CD69 staining in CD4^{+} T cells (left) and CD8^{+} T cells (right) in the popliteal LNs of WT and Cbl-b$^{-/-}$ mice. (B) MFI of CD69 staining in CD4^{+} T cells (left) and CD8^{+} T cells (right) in the mLNs of WT and Cbl-b$^{-/-}$ mice. Results were analyzed using Student's *t*-test. The data shown are from three independent experiments.

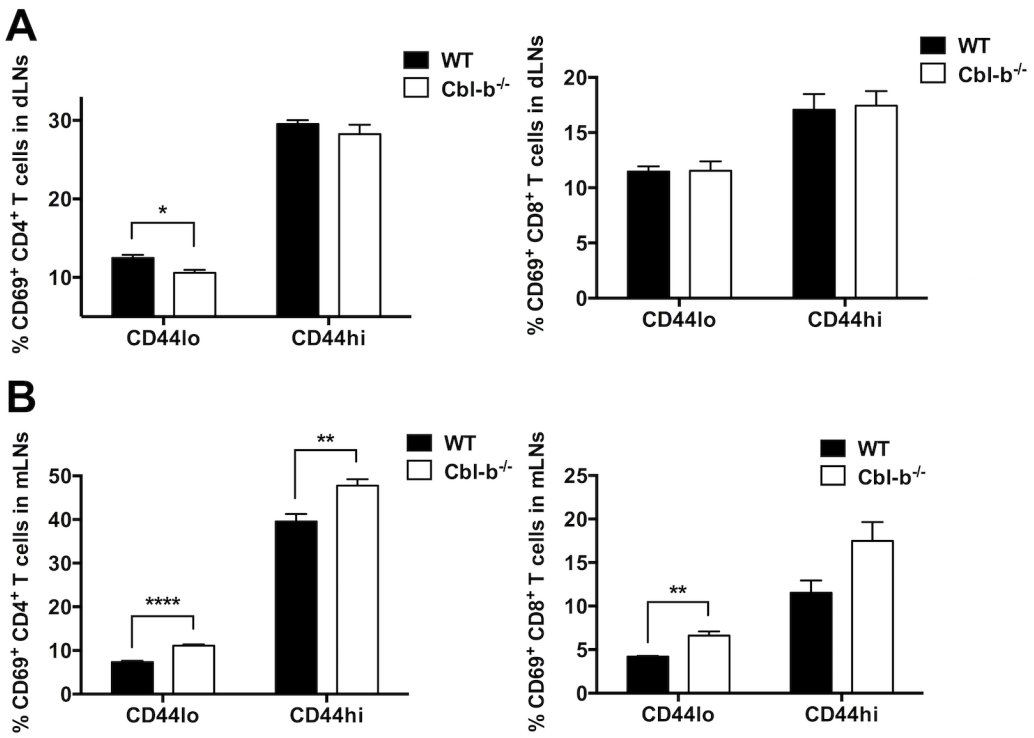


Figure 2-12. CD69 expression in CD44^{lo} and CD44^{hi} populations of WT and Cbl-b$^{-/-}$ T cells in draining popliteal LNs after CFA/MOG$_{35-55}$ immunization. WT and Cbl-b$^{-/-}$ mice were immunized s.c. in the footpads with 150 μg of CFA and 80 μg of MOG$_{35-55}$ and the popliteal LNs were harvested 4-5 days later. (A) Percentage of CD44^{lo} and CD44^{hi} CD4^{+} T cells (left) and CD8^{+} T cells (right) that express CD69 in draining popliteal LNs of WT and Cbl-b$^{-/-}$ mice. (B) Percentage of CD44^{lo} and CD44^{hi} CD4^{+} T cells (left) and CD8^{+} T cells (right) that express CD69 in non-draining mLNs of WT and Cbl-b$^{-/-}$ mice. *p<0.05, **p<0.01, ****p<0.0001 by Student's *t*-test. The data shown are from three independent experiments.

CHAPTER 3 Cbl-b Deficiency Mediates Resistance to Programmed Death-Ligand 1/Programmed Death-1 Regulation

ABSTRACT

Cbl-b is an E3 ubiquitin ligase that negatively regulates T cell activation. Cbl-b$^{-/-}$ T cells are hyper-reactive and co-stimulation independent, and Cbl-b$^{-/-}$ mice demonstrate robust T cell and NK cell-mediated anti-tumor immunity. As a result of these murine studies, Cbl-b is considered a potential target for therapeutic manipulation in human cancer immunotherapy. The PD-L1/PD-1 pathway of immune regulation is presently an important therapeutic focus in tumor immunotherapy and although Cbl-b$^{-/-}$ mice have been shown to be resistant to several immuno-regulatory mechanisms, the sensitivity of Cbl-b$^{-/-}$ mice to PD-L1-mediated suppression has not been reported.

We now document that Cbl-b$^{-/-}$ T cells and NK cells are resistant to PD-L1/PD-1-mediated suppression. Using a PD-L1 Ig fusion protein, this resistance is shown for both *in vitro* proliferative responses and IFN-γ production and is not associated with decreased PD-1 expression on Cbl-b$^{-/-}$ cells. In co-culture studies, Cbl-b$^{-/-}$ CD8^{+}, but not CD4^{+} T cells diminish the PD-L1 Ig-mediated suppression of bystander naïve WT CD8^{+} T cells. Using an *in vivo* model of B16 melanoma in which numerous liver metastases develop in WT mice in a PD-1 dependent manner, Cbl-b$^{-/-}$ mice develop significantly fewer liver metastases without the administration of anti-PD-1 antibody. Overall, our findings identify a new mode of immuno-regulatory resistance associated with Cbl-b deficiency and suggest that resistance to PD-L1/PD-1-mediated suppression is a novel mechanism by which Cbl-b deficiency leads to enhanced anti-

tumor immunity. Our results suggest that targeting Cbl-b in cancer immunotherapy offers the opportunity to simultaneously override numerous relevant “checkpoints” including sensitivity to regulatory T cells, suppression by TGF-β, and immune-regulation by both CTLA-4, and as we now report, by the PD-L1/PD-1 pathway.

INTRODUCTION

Cbl-b (Casitas B-lineage lymphoma-b) is an E3 ubiquitin ligase that regulates many aspects of cell activation. Cbl-b functions in T cells to regulate the activation of several proximal T cell receptor signaling molecules including PI3K (24, 25, 28, 55). Cbl-b deficiency in mice (Cbl-b$^{-/-}$ mice) leads to spontaneous autoimmunity characterized by multi-organ cellular infiltration (24) as well as increased susceptibility to induced models of autoimmunity such as experimental autoimmune encephalomyelitis (EAE) (25, 27, 45). T cells from Cbl-b$^{-/-}$ mice demonstrate hyper-reactivity (24) and co-stimulation independence (25). Moreover we and others have shown that Cbl-b$^{-/-}$ T cells are resistant to suppression by Tregs (39, 40, 84) and TGF-β (39, 41, 42). The potential relevance of alterations in Cbl-b in autoimmunity is also supported by the finding that single nucleotide polymorphisms in the *CBLB* gene are associated with human autoimmune diseases such as Systemic Lupus Erythematosus (85) and Multiple Sclerosis (MS) (13). More recently, Cbl-b$^{-/-}$ mice have also become a focus for the study of T cell-mediated anti-tumor immunity, and our laboratory and others have reported that Cbl-b$^{-/-}$ mice are resistant to the outgrowth of spontaneous and transplantable tumors (41, 42, 84). In addition to T cell mediated effects, it has recently been reported that Cbl-b$^{-/-}$ mice have enhanced NK cell-mediated tumor immunity (86). As a result of these studies, Cbl-b is considered a target for therapeutic manipulation in cancer immunotherapy.

The PD-L1/PD-1 pathway is recognized as an important mechanism of immune regulation in mice and humans (23, 87). Moreover, targeting this pathway for inhibition has generated much interest as a novel therapeutic approach for enhancing tumor immunity in certain human malignancies (88-90). A number of mechanisms have been proposed for the normal PD-

L1/PD-1-mediated regulation of T cells (91-93) and this includes the upregulation of Cbl-b in T cells in response to PD-L1/PD-1 signaling (46). This upregulation of Cbl-b is postulated to be required for TCR down-modulation and subsequent inhibition of T cell activation by PD-L1/PD-1 signaling (46). While these studies suggest the potential involvement of Cbl-b in the normal PD-L1/PD-1 inhibition of T cell responses, this has not been directly examined in the context of Cbl-b deficiency.

In the present study, we analyzed PD-L1/PD-1-mediated immune regulation utilizing Cbl-b$^{-/-}$ mice. We document for the first time that Cbl-b deficiency in mice results in functional resistance of T cells and NK cells to PD-L1/PD-1-mediated regulation. Our results thus add to Cbl-b's role in immune regulation and identify a new mechanism by which Cbl-b deficiency can lead to enhanced anti-tumor immunity.

MATERIAL AND METHODS

Mice

Female C57BL/6 (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Cbl-b$^{-/-}$ mice on a C57BL/6 background were a gift from Dr. H. Gu (Columbia University, New York, NY). Female C57BL/6 congenic mice (CD45.1^{+}) were also purchased from the Jackson Laboratory. All mice were maintained and bred under specific pathogen-free conditions in accordance with the guidelines of the UConn Health Institutional Animal Care and Use Committee (IACUC) and the Center for Comparative Medicine at UConn Health. The UConn Health Institutional Animal Care and Use Committee has approved the protocol (protocol 101448-0919) used in these studies.

***In vitro* suppression of T cell proliferation with the recombinant PD-L1 Ig**

Splenic naïve $CD8^+$ $CD44^{low}$ cells isolated via positive selection by magnetic bead purification (Miltenyi Biotec, Auburn, CA) from WT and Cbl-$b^{-/-}$ mice were labeled with 2.5 μM CFSE (Molecular Probe, Eugene, OR) and stimulated with 2 μg/ml of plate-bound anti-CD3 ab and 0.4 μg/ml of soluble anti-CD28 ab in the presence of 9-10 μg/ml of plate-bound control Ig or PD-L1 Ig for 72 hours in 10% FCS complete RPMI 1640 in round-bottom 96-wells at 5 x 10^5 cells/ml. Splenic naive $CD4^+$ $CD44^{low}$ cells isolated via negative selection by magnetic bead purification (Miltenyi Biotec) from WT and Cbl-$b^{-/-}$ mice were labeled with CFSE and stimulated with 2.5 μg/ml of plate-bound anti-CD3 ab, 1 μg/ml of soluble anti-CD28 ab in the presence of 10 μg/ml of plate-bound control Ig or PD-L1 Ig for 48 hours in 10% FCS complete RPMI 1640 in round-bottom 96-wells at 2.5 x 10^5 cells/ml. These cells in both cultures were stained with Live/Dead Near IR (Live/Dead) (Molecular Probe), anti-CD8α or anti-CD4 (Biolegend) and analyzed by flow cytometry using Becton Dickinson LSRII for CFSE dilution. Absolute numbers of cells in these cultures were quantified using MACSQuant Analyzer 10 (Miltenyi Biotec). For PD-1 expression, unstimulated splenocytes from WT and Cbl-$b^{-/-}$ mice and $CD8^+$ or $CD4^+$ T cells stimulated in above conditions without Igs were surface-stained with Live/Dead, anti-CD8α or anti-CD4, anti-CD44 and anti-PD-1 (Biolegend) and analyzed by flow cytometry.

***In vitro* suppression of T cell IFN-γ production by the recombinant PD-L1 Ig**

Splenic naïve $CD8^+$ $CD44^{low}$ T cells isolated via negative selection by magnetic bead purification (Miltenyi Biotec) from WT and Cbl-$b^{-/-}$ mice were stimulated with 2 μg/ml of plate-bound anti-CD3 ab and 0.4 μg/ml of soluble anti-CD28 ab in the presence of 9-10 μg/ml of

plate-bound control Ig or PD-L1 Ig for 72 hours in 10% FCS complete RPMI 1640 in round-bottom 96-wells at 5 x 10^5 cells/ml. Splenic naïve $CD4^+$ $CD44^{low}$ T cells isolated via negative selection by magnetic bead purification (Miltenyi Biotec) from WT and Cbl-$b^{-/-}$ mice were stimulated with 2.5 μg/ml of plate-bound anti-CD3 ab and 1 μg/ml of soluble anti-CD28 ab in the presence of 10 μg/ml of plate-bound control Ig or PD-L1 Ig for 72 hours in 10% FCS complete RPMI 1640 in round-bottom 96-wells at 2.5 x 10^5 cells/ml. $CD4^+$ T cells were then re-stimulated overnight with 2.5 μg/ml of plate-bound anti-CD3 ab and 1 μg/ml of soluble anti-CD28 ab without any Igs. 5 μg/ml of Brefeldin A (Sigma-Aldrich, St. Louis, MO) was added to both cultures in the last 4 hours. Cells were surface-stained with Live/Dead, anti-CD8α or anti-CD4 then fixed and permeabilized using BD Cytoperm/Cytofix kit (BD Biosciences, San Jose, CA), intracellularly stained with anti-IFN-γ (BD Biosciences) and analyzed by flow cytometry.

***In vitro* suppression of NK cell IFN-γ production by the recombinant PD-L1 Ig**

Splenic NK cells isolated via negative selection by magnetic bead purification (STEMCELL Technologies, Vancouver, Canada) from WT and Cbl-$b^{-/-}$ mice were cultured for 7 days in the presence of 1000 U/ml recombinant human IL-2 (National Institutes of Health, Bethesda, MD) in 10% FCS complete RPMI 1640 (supplemented with 50 μM 2-mercaptoethanol, L-glutamine, penicillin/streptomycin, non-essential amino acids and sodium pyruvate) in 24-wells at 2.5 x 10^5 cells/ml. The purity of NK cells on day 7 was determined by surface-staining with Live/Dead, anti-CD3, anti-NK1.1 and anti-CD49b (eBioscience, San Diego, CA) by flow cytometry and the cell number per well for the stimulation was normalized based on the NK cell purity ($CD3^-$ $NK1.1^+$ $CD49b^+$). These NK cells were stimulated with 15-20 μg/ml of plate-bound anti-NK1.1 ab (Biolegend) in the presence of 12.5-15 μg/ml of plate-bound control Ig or PD-L1 Ig and 5

μg/ml Brefeldin A for 5 hours in round-bottom 96-wells at 5 x 10^5 cells/ml. These stimulated NK cells and unstimulated NK cells (from 7-day IL-2 culture) were surface-stained with Live/Dead, fixed and permeabilized as described above and intracellularly stained with anti-IFN-γ or anti-PD-1 then analyzed by flow cytometry.

Co-culture of WT and Cbl-b$^{-/-}$ T cells

Splenic naïve CD8$^+$ CD44^{low} T cells isolated via negative selection by magnetic bead purification (Miltenyi Biotec) from WT congenic mice (CD45.1$^+$) and Cbl-b$^{-/-}$ mice were labeled with CFSE and stimulated with 2 μg/ml of plate-bound anti-CD3 ab, 0.4 μg/ml of soluble anti-CD28 ab in the presence of 9 μg/ml of plate-bound control Ig or PD-L1 Ig for 72 hours in 10% FCS complete RPMI 1640 in round-bottom 96-well plates at 5 x 10^5 cells/ml. Single cultures were set up in which WT congenic naïve CD8$^+$ T cells and Cbl-b$^{-/-}$ CD8$^+$ T cells were stimulated separately. Co-cultures were set up in which these two naïve CD8$^+$ T cell populations were cultured and stimulated together. Splenic naïve CD4$^+$ CD44^{low} T cells isolated via negative selection by magnetic bead purification (Miltenyi Biotec) from WT congenic mice and Cbl-b$^{-/-}$ mice were labeled with CFSE and stimulated with 2.5 μg/ml of plate-bound anti-CD3 ab, 1 μg/ml of soluble anti-CD28 ab in the presence of 15 μg/ml of plate-bound control Ig or PD-L1 Ig for 48 hours in 10% FCS complete RPMI 1640 in round-bottom 96-well plates at 2.5 x 10^5 cells/ml. Single cultures were set up in which WT congenic naïve CD4$^+$ T cells and Cbl-b$^{-/-}$ naïve CD4$^+$ T cells were stimulated separately. Co-cultures were set up in which these two naïve CD4$^+$ T cell populations were cultured and stimulated together. Cells in these cultures were stained with Live/Dead, anti-CD8α, or anti-CD4, anti-CD45.1 and anti-CD45.2 (Biolegend) and analyzed by flow cytometry using Becton Dickinson LSRII for CFSE dilution.

Liver metastasis model of B16 melanoma and anti-PD-1 antibody treatment

B16 (B16F10) cells were grown to approximately 75% confluency in 10% FCS complete RPMI 1640 (supplemented with L-glutamine, penicillin/streptomycin, non-essential amino acids and sodium pyruvate). 1 x 10^6 B16 (B16F10) cells were intra-splenically injected into WT and Cbl-b$^{-/-}$ mice. For the experiment with anti-PD-1 ab treatment, only WT mice were administered 100 μg/mouse of either anti-PD-1 monoclonal ab (BioXCell, West Lebanon, NH) or isotype control (BioXCell) intraperitoneally on the day of tumor injection and every other day. On days 7-9, the mice were perfused with saline and the liver and spleen were harvested from each mouse. The images of each spleen and the front and the back side of each liver tissue were taken using Panasonic Lumix DMC-FS7 camera (Panasonic, Osaka, Japan). Using these images the percentages of tumor foci area in total liver area were quantified for each mouse using ImageJ software (National Institutes of Health).

Statistical analysis

Data were analyzed by unpaired two-tailed Student's *t*-test or one-way ANOVA using GraphPad Prism Version 6.0 (GraphPad Software, La Jolla CA). Statistical significance were accepted at $p<0.05$.

RESULTS

Cbl-b$^{-/-}$ T cells are resistant to PD-L1 Ig-mediated suppression of proliferation.

It has previously been demonstrated that *in vitro* T cell proliferation and cytokine production can be suppressed by the immobilized recombinant PD-L1 fusion protein (PD-L1 Ig) (87, 94-96). To begin to test the sensitivity of Cbl-b$^{-/-}$ T cells to PD-L1-mediated suppression, we tested the ability of plate-bound PD-L1 Ig to suppress the *in vitro* proliferation of WT and Cbl-b$^{-/-}$ CD8^{+} T cells. Mouse splenic naïve CD44^{low} CD8^{+} T cells were purified from WT and Cbl-b$^{-/-}$ mice and labeled with CFSE. These populations were then stimulated *in vitro* with plate-bound anti-CD3 ab and soluble anti-CD28 ab in the presence of either plate-bound PD-L1 Ig or plate-bound control human IgG_1 (control Ig). After three days, cells were assayed for dilution of CFSE by flow cytometry. We found that TCR-stimulated *in vitro* proliferative responses of naïve WT CD8^{+} T cells were significantly suppressed by PD-L1 Ig (mean % CFSE-diluted cells: control Ig: 63.7%; PD-L1 Ig: 15.03%; p=0.0234) (**Figure 3-1A and 3-1B**). In contrast, the proliferation of naïve Cbl-b$^{-/-}$ CD8^{+} T cells was not suppressed by PD-L1 Ig (mean % CFSE-diluted cells: control Ig: 85.2%, mean PD-L1 Ig: 82.5%; p=0.204) **(Figure 3-1A and 3-1B)**. Thus, the mean percent suppression of CFSE-diluted WT CD8^{+} T cells by PD-L1 Ig was 79.6%, and that of CFSE-diluted Cbl-b$^{-/-}$ CD8^{+} T cells was 3.13% (p=0.0057) (**Figure 3-1B**). These differences in percent PD-L1 Ig-mediated suppression of CD8^{+} T cells were also reflected in absolute numbers of CFSE-diluted cells (data not shown).

To determine if the difference in PD-L1 Ig-mediated suppression was associated with differences in cell death, we compared the number of dead cells present in the control Ig wells with the number of dead cells in the PD-L1 Ig wells at the termination of the cultures. We found

that PD-L1 Ig-mediated suppression of WT $CD8^+$ T cells was associated with an increase in dead cells by 46%. In contrast, PD-L1 Ig-mediated suppression of Cbl-$b^{-/-}$ $CD8^+$ T cells was associated with an increase in dead cells by 13.7% (**Figure 3-1C**). Overall, our results suggest a resistance of Cbl-$b^{-/-}$ $CD8^+$ T cells to PD-L1 Ig-mediated suppression of proliferation.

Next we asked whether Cbl-$b^{-/-}$ $CD4^+$ T cells were also resistant to PD-L1 Ig-mediated suppression of *in vitro* proliferation. Mouse splenic naïve $CD44^{low}$ $CD4^+$ T cells isolated from WT and Cbl-$b^{-/-}$ mice were labeled with CFSE and subjected to the similar stimulation cultures described above for $CD8^+$ T cells. After two days, cells were assayed for dilution of CFSE by flow cytometry. Similar to $CD8^+$ T cells, the *in vitro* proliferative responses of WT $CD4^+$ T cells were significantly inhibited by PD-L1 Ig (mean % CFSE-diluted cells: control Ig: 68.4%; PD-L1 Ig: 14.1%; p=0.0005) **(Figure 3-2A and 3-2B)**. In contrast, the proliferative responses of Cbl-$b^{-/-}$ $CD4^+$ T cells were significantly less suppressed in the presence of PD-L1 Ig (mean % CFSE-diluted cells: control Ig: 82.0 %; PD-L1 Ig: 66.9%; p=0.0114) **(Figure 3-2A and 3-2B)**. While a small and statistically significant degree of PD-L1 Ig-mediated suppression was noted in Cbl-$b^{-/-}$ $CD4^+$ T cells (mean percent suppression of Cbl-$b^{-/-}$ $CD4^+$ T cells: 18.4%), this level of suppression was markedly less than that seen in WT $CD4^+$ T cells (mean percent suppression: 79.8%) (**Figure 3-2B**). These differences in percent PD-L1 Ig-mediated suppression of $CD4^+$ T cells were also reflected in absolute numbers of CFSE-diluted cells (data not shown).

To determine if differences in PD-L1-mediated suppression between naïve WT versus Cbl-$b^{-/-}$ $CD4^+$ cells were associated with differences in cell death, we compared the number of dead cells present in the control Ig wells with the number of dead cells in PD-L1 Ig wells at the

termination of cultures. We found that PD-L1-mediated suppression of WT $CD4^+$ T cells was associated with an increase in dead cells by 30%. In contrast, PD-L1-mediated suppression of Cbl-$b^{-/-}$ $CD4^+$ T cells was not associated with a significant change in the number of dead cells (**Figure 3-2C**). Overall, as with Cbl-$b^{-/-}$ $CD8^+$ T cells, our results suggest a resistance of Cbl-$b^{-/-}$ $CD4^+$ T cells to PD-L1 Ig-mediated suppression of proliferation.

The expression of PD-1 is not decreased in Cbl-$b^{-/-}$ $CD8^+$ and $CD4^+$ T cells.

To determine whether the resistance of Cbl-$b^{-/-}$ T cells to PD-L1 Ig-mediated suppression is a result of decreased PD-1 expression, we compared the expression of PD-1 in WT versus Cbl-$b^{-/-}$ $CD8^+$ and $CD4^+$ T cells before and after *in vitro* TCR stimulation. In $CD8^+$ T cells, the frequency of PD-1^+ cells was comparable between unstimulated WT and Cbl-$b^{-/-}$ $CD8^+$ T cells and this was true for total $CD8^+$ T cells, $CD44^{low}$ and $CD44^{high}$ $CD8^+$ populations (**Figure 3-3A**). After three days of *in vitro* stimulation, approximately 95% of $CD8^+$ T cells were positive for PD-1 expression. Therefore, we examined the MFI of PD-1 expression in these activated cells and found that the PD-1 MFI was comparable between Cbl-$b^{-/-}$ and WT $CD8^+$ T cells **(Figure 3-3B)**. Unstimulated Cbl-$b^{-/-}$ $CD4^+$ T cells, when compared to unstimulated WT $CD4^+$ T cells, demonstrated an increased frequency of PD-1^+ cells. This was true for total $CD4^+$ T cells, $CD44^{low}$ and $CD44^{high}$ $CD4^+$ populations (**Figure 3-3C)**. After two days of *in vitro* stimulation, the PD-1 MFI was significantly higher in Cbl-$b^{-/-}$ $CD4^+$ T cells compared to WT cells **(Figure 3-3D)**. These results suggest that the resistance of Cbl-$b^{-/-}$ T cells to PD-L1 Ig-mediated suppression is not a result of decreased PD-1 expression.

PD-L1 Ig-mediated suppression of IFN-γ is significantly greater for WT than for Cbl-b$^{-/-}$ T cells.

We next asked whether Cbl-b$^{-/-}$ T cells were resistant not only to PD-L1 Ig-mediated suppression of proliferation, but also resistant to suppression of IFN-γ production *in vitro*. Splenic naïve CD44^{low} CD8^{+} T cells isolated from WT and Cbl-b$^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 abs in the presence of plate-bound PD-L1 Ig or control Ig. After three days of culture, these cells were re-stimulated overnight with anti-CD3 and anti-CD28 ab, stained for intracellular IFN-γ, and analyzed by flow cytometry.

The frequency of IFN-γ producing WT CD8^{+} T cells was significantly suppressed by PD-L1 Ig **(**mean IFN-γ positive cells: control Ig: 12.8%; PD-L1 Ig: 0.3%; p=0.0221) **(Figure 3-4A and 3-4B)**. In contrast, the frequency of IFN-γ producing Cbl-b$^{-/-}$ CD8^{+} T cells was not statistically suppressed by PD-L1 Ig (mean IFN-γ positive cells: control Ig: 18.1%, mean PD-L1 Ig: 14.8%; p=0.1374) **(Figure 3-4A and 3-4B)**. Thus, the level of suppression was significantly lower in Cbl-b$^{-/-}$ CD8^{+} T cells than that seen in WT CD8^{+} T cells (mean percent suppression of IFN-γ producing cells: WT CD8^{+}: 96.3%; Cbl-b$^{-/-}$ CD8^{+}: 18.1%; p=0.0001) (**Figure 3-4C**).

We next stimulated WT and Cbl-b$^{-/-}$ splenic naïve CD44^{low} CD4^{+} T cells with anti-CD3 and anti-CD28 abs in the presence of plate-bound PD-L1 Ig or control Ig. As with WT CD8^{+} T cells, the frequency of IFN-γ producing WT CD4^{+} T cells was significantly suppressed by PD-L1 Ig **(**mean IFN-γ positive cells: control Ig: 7.7%; PD-L1 Ig: 2.4%; p=0.0177) (**Figure 3-4D and 3-4E)**. The frequency of IFN-γ producing Cbl-b$^{-/-}$ CD4^{+} T cells was also suppressed by PD-L1 Ig (mean IFN-γ positive cells: control Ig: 28.4%; PD-L1 Ig: 22.6%; p=0.0146) (**Figure 3-4D**

and 3-4E), but the level of suppression was significantly less than that seen with WT $CD4^+$ T cells (mean percent suppression of IFN-γ producing cells: WT $CD4^+$: 68.3%; Cbl-$b^{-/-}$ $CD4^+$: 20.1%; $p<0.0001$) **(Figure 3-4F)**. Thus, consistent with the proliferation results, Cbl-$b^{-/-}$ $CD8^+$ and $CD4^+$ T cell IFN-γ production is significantly less suppressed by PD-L1 Ig than is WT $CD8^+$ and $CD4^+$ T cell IFN-γ production.

Cbl-$b^{-/-}$ NK cells are resistant to PD-L1-mediated suppression of IFN-γ production.

The role of NK cells has been documented in anti-tumor immunity in mice and humans and these cells have been shown to contribute to the robust anti-tumor immunity of Cbl-$b^{-/-}$ mice (86). Similar to T cells, NK cells can also express PD-1 and the engagement of PD-1 on NK cells inhibits their activation and cytotoxic function (97). We next asked whether Cbl-$b^{-/-}$ NK cells were also resistant to PD-L1/PD-1 suppression.

WT and Cbl-$b^{-/-}$ splenic NK cells were isolated and initially cultured in the presence of IL-2 as previously described (98). The mean purity or percentages of NK cells after 7 days of culture with IL-2 was WT = 97.57% and Cbl-$b^{-/-}$ = 96.3% when gated on $CD3^-$ $NK1.1^+$ cells. After 7 days of culture, WT NK cells contained an average of 1.7% $CD3^+$ T cells and Cbl-$b^{-/-}$ NK cells contained an average of 0.5% $CD3^+$ T cells (data not shown). The NK cells were then stimulated *in vitro* with plate-bound anti-NK1.1 ab in the presence of either plate-bound PD-L1 Ig or control Ig and IFN-γ production measured by intracellular staining and flow cytometry. Additionally, the expression of PD-1 on the unstimulated NK cells was examined by flow cytometry. The frequency of IFN-γ producing WT NK cells after stimulation with plate-bound anti-NK1.1 ab was significantly suppressed by PD-L1 Ig ($p=0.0022$) **(Figure 3-5A and 3-5B)**. In

contrast, the frequency of IFN-γ-producing Cbl-b$^{-/-}$ NK cells was not suppressed by PD-L1 Ig (**Figure 3-5A and 3-5B**). Overall, the mean percentage of PD-L1 Ig-mediated suppression of IFN-γ producing WT NK cells was 11.5% while the mean percent suppression of IFN-γ producing Cbl-b$^{-/-}$ NK cells was −3.4% (p=0.0006) (**Figure 3-5C**). Although it did not reach statistical significance, PD-L1 Ig reduced the IFN-γ expression of WT NK cells based on the MFI, while that of Cbl-b$^{-/-}$ NK cells was not reduced by PD-L1 Ig (**Figure 5D**). Finally, PD-1 expression by Cbl-b$^{-/-}$ NK cells was slightly, but not statistically lower than PD-1 expression on WT NK cells (p=0.304) (**Figure 3-5E)**. These results suggest that, in addition to Cbl-b$^{-/-}$ T cells, Cbl-b$^{-/-}$ NK cells are also significantly less sensitive to suppression by PD-L1 Ig.

Does co-culturing with Cbl-b$^{-/-}$ T cells result in bystander WT T cell resistance to PD-L1-mediated suppression?

To begin to investigate the potential functional relevance of Cbl-b$^{-/-}$'s resistance to PD-L1 suppression, we focused on reports documenting that enhanced levels of certain common γ chain-signaling cytokines may have a role in overcoming PD-L1-mediated suppression (99). It has been documented that activated Cbl-b$^{-/-}$ T cells secrete increased levels of IL-2 compared to WT T cells (24, 25). Therefore, we next explored whether this might result in the induction of bystander PD-L1 resistance in WT T cells. We cultured naïve Cbl-b$^{-/-}$ T cells or naïve WT T cells either separately or together and then asked whether factors secreted by activated Cbl-b$^{-/-}$ T cells would induce PD-L1 resistance in bystander (co-cultured) WT T cells.

In the CD8^{+} T cell cultures, there was a clear, though not statistically significant (p=0.08) effect of naive Cbl-b$^{-/-}$ CD8^{+} T cells in diminishing the PD-L1 Ig-mediated suppression of

bystander naïve WT $CD8^+$ T cells (**Figure 3-6A**). The mean PD-L1 Ig-mediated suppression of WT $CD8^+$ T cells in single culture was 79.6% and this suppression decreased to a mean of 42.9% when WT $CD8^+$ T cells were co-cultured with Cbl-$b^{-/-}$ $CD8^+$ T cells (**Figure 3-6A**). These results suggest that Cbl-$b^{-/-}$ $CD8^+$ T cells can induce a partial resistance of bystander WT $CD8^+$ T cells to PD-L1 Ig-mediated suppression. In contrast, the level of PD-L1 Ig-mediated suppression of WT $CD4^+$ T cells remained unchanged regardless of whether WT $CD4^+$ T cells were cultured alone or co-cultured together with Cbl-$b^{-/-}$ $CD4^+$ T cells (**Figure 6B**). In terms of potential functional relevance, these results suggest that the adoptive transfer of $CD8^+$ T cells in which the expression of the *CBLB* gene has been silenced may be accompanied by a loss of sensitivity to PD-L1-mediated suppression among bystander endogenous T cells.

Cbl-$b^{-/-}$ mice develop significantly fewer metastases in a PD-L1/PD-1 dependent model of B16 melanoma liver metastasis.

As Cbl-$b^{-/-}$ T cells were resistant to PD-L1 Ig-mediated regulation *in vitro*, we sought to test this in an *in vivo* B16 melanoma tumor model in which inhibiting the PD-L1/PD-1 pathway has been shown to prevent the outgrowth of liver metastases (100). In this model, splenic injection of B16 melanoma in WT mice results in significant metastatic foci in the liver. However, if anti-PD-1 antibody is administered, liver metastases are significantly reduced (100). In addition, Iwai *et al.* documented that PD-$1^{-/-}$ mice develop significantly fewer liver metastases compared to WT mice in this model (100). Thus, PD-L1/PD-1-mediated immune regulation appears to normally allow spread of the melanoma to the liver, while inhibiting or deleting this normal PD-1/PD-L1-mediated immune regulation prevents the spread of the tumor to the liver. This model allows us to ask whether Cbl-$b^{-/-}$ mice can eliminate the B16 liver metastases on their own, i.e., in the

absence of anti-PD-1 antibody, and thus to assess the functional relevance of the *in vitro* resistance of Cbl-b$^{-/-}$ T cells to PD-L1/PD-1 in an *in vivo* tumor setting.

In using this model, Cbl-b$^{-/-}$ mice could potentially eliminate liver metastases on the basis of their resistance to TGF-β or Tregs (39-42, 84). However, if Cbl-b$^{-/-}$ mice were <u>*unable*</u> to eliminate the liver metastases in the absence of anti-PD-1 antibody, this would suggest that Cbl-b$^{-/-}$ mice are *not* resistant to PD-L1/PD-1 suppression *in vivo*. In contrast, demonstrating that Cbl-b$^{-/-}$ mice can indeed eliminate the liver metastases in the absence of anti-PD-1 antibody would minimally suggest a lack of dominant sensitivity to PD-L1/PD-1 suppression *in vivo* and thus be consistent with our *in vitro* findings.

We first confirmed using WT mice that the model was sensitive to anti-PD-1 antibody treatment. WT mice were injected intra-splenically with B16 melanoma and treated with either anti-PD-1 ab or an isotype control. As reported by Iwai *et al* (100), WT mice treated with anti-PD-1 ab developed significantly fewer liver metastases than mice treated with isotype control (**Figure 3-7A and 3-7C**). No difference in the "primary" spleen tumor masses was noted between mice treated with anti-PD-1 ab or isotype (data not shown). Next, WT and Cbl-b$^{-/-}$ mice were injected intra-splenically with B16 melanoma and the presence of liver metastases was analyzed 7-9 days after the tumor injection. No anti-PD-1 ab or isotype treatment was given to these mice. Strikingly, we found that Cbl-b$^{-/-}$ mice developed significantly fewer liver metastases compared to WT mice (**Figure 3-7B and 7D**). In contrast, the primary spleen tumor masses were comparable between WT and Cbl-b$^{-/-}$ mice in all of the experiments (**Figure 3-7D**), suggesting that Cbl-b$^{-/-}$ mice are not generally capable of eliminating the B16 melanoma based on their

resistance to TGF-β or Tregs (39-42, 84). Our finding that Cbl-b$^{-/-}$ mice develop significantly fewer liver metastases compared to WT mice without the administration of anti-PD-1 antibody minimally suggests a lack of dominant sensitivity to PD-L1/PD-1 suppression *in vivo* and thus is consistent with our *in vitro* findings of resistance to PD-L1/PD-1-mediated suppression.

DISCUSSION

Cbl-b is an E3 ubiquitin ligase that negatively regulates T cell activation (24, 25, 28, 55). Cbl-b$^{-/-}$ mice demonstrate spontaneous autoimmunity and robust anti-tumor immunity. Recently, enhancement of anti-fungal immunity has also been documented in Cbl-b$^{-/-}$ mice (101-103). The enhanced anti-tumor responses of Cbl-b$^{-/-}$ mice have been documented in tumor models of EL4 (41), EG.7 (42), TC-1 (55, 84), UVB-induced skin tumor (84) and ATM-deficiency-induced T cell lymphoma (42). The T cell-intrinsic role of the enhanced anti-tumor immunity of Cbl-b$^{-/-}$ mice has been demonstrated in adoptive T cell transfer experiments using Cbl-b$^{-/-}$ or *Cblb*-silenced CD8^{+} T cells (42, 84, 104-106). In an attempt to elucidate the mechanisms underlying the robust anti-tumor activity of Cbl-b$^{-/-}$ T cells, we and others have shown that Cbl-b$^{-/-}$ T cells are resistant to suppression by Tregs (39, 40, 84) and TGF-β (41, 42). Because of the enhanced anti-tumor responses of Cbl-b$^{-/-}$ mice, Cbl-b has become a focus for understanding mechanisms of anti-tumor immunity and a target for manipulation in anti-tumor immunotherapeutic approaches. In this regard, siRNA-mediated knockdown of *CBLB* is currently being investigated as a therapeutic approach to enhance anti-tumor responses in cancer patients (104, 106, 107). Similar to the findings in mouse Cbl-b$^{-/-}$ CD8^{+} T cells, *CBLB*-silenced human CD8^{+} T cells demonstrate enhanced IFN-γ production (104, 106), reduced sensitivity to TGF-β (106), and independence from co-stimulation (106) and exogenous IL-2 in activation and proliferation

(104). These findings in human *CBLB*-silenced T cells suggest their potential for mediating robust anti-tumor activity *in vivo* and underlie the high level of interest in utilizing *CBLB* silencing in treating human cancer patients.

PD-1 belongs to the CD28/B7 family of co-stimulatory molecules and is expressed on activated $CD8^+$ and $CD4^+$ T cells, NK and NKT cells, B cells, activated monocytes and some dendritic cells (108). Triggering of PD-1 in T cells by its ligands PD-L1 and PD-L2 has been shown to inhibit activation and dampen subsequent proliferation and cytokine production (23, 87, 94-96). The expression of PD-L1 on tumors has been shown to increase the invasiveness of the tumor in mice and blockade of PD-L1/PD-1 interaction has been shown to halt tumor progression in mouse models (100, 109). Furthermore, the recent demonstration of the efficacy of the PD-L1/PD-1 blockade for the treatment of certain human cancers underscores the importance of inhibiting this pathway for effective anti-tumor immunity (88-90). Despite the documented ability of Cbl-$b^{-/-}$ mice to mount enhanced anti-tumor responses, to date there has been no investigation of the relationship of Cbl-b deficiency and the PD-L1/PD-1 pathway. In the present study, we now report a relationship with potential translational significance between Cbl-b deficiency and a resistance to PD-L1/PD-1-mediated immune regulation. This concept is supported by the following specific results in our study.

We find that *in vitro* proliferative responses of Cbl-$b^{-/-}$ $CD8^+$ and $CD4^+$ T cells are significantly less suppressed by a recombinant PD-L1 fusion protein (PD-L1 Ig) than are those of WT T cells. In addition to the proliferative responses, *in vitro* IFN-γ production of Cbl-$b^{-/-}$ $CD8^+$ and $CD4^+$ T cells is also significantly less suppressed by PD-L1 Ig compared to WT T

cells. The PD-1/PD-L1 resistance of Cbl-b$^{-/-}$ T cells is not a result of a difference in PD-1 expression in Cbl-b$^{-/-}$ CD8^{+} and CD4^{+} T cells. Recently, Cbl-b$^{-/-}$ mice have been reported to have NK cells with enhanced anti-tumor activity (86). This enhancement of anti-tumor activity in Cb-b$^{-/-}$ NK cells was reported to be associated with their resistance to TAM (Tyro3, Axl, and Mer) receptor-mediated inhibition (86). Similar to T cells, NK cells can express PD-1 and the engagement of PD-1 on NK cells inhibits their activation and cytotoxic function (97). We asked whether the enhanced anti-tumor activity of Cbl-b$^{-/-}$ NK cells might also be related to a resistance to PD-L1/PD-1-mediated suppression. We now find that in contrast to WT NK cells, Cbl-b$^{-/-}$ NK cells are not suppressed in *in vitro* IFN-γ production by PD-L1. Thus, we have described an additional mechanism by which Cbl-b$^{-/-}$ NK cells may be capable of mediating enhanced anti-tumor activity.

To begin to identify the potential functional relevance of resistance to PD-L1-Ig mediated suppression in Cbl-b$^{-/-}$ T cells, we asked whether Cbl-b$^{-/-}$ T cells could induce resistance to PD-L1-mediated suppression in bystander WT T cells. Although not statistically significant, we find an induction of bystander resistance in naïve WT CD8^{+} T cells when cultured with naïve Cbl-b$^{-/-}$ CD8^{+} T cells. There is presently a significant focus on developing approaches to silence *CBLB* in CD8^{+} T cells for subsequent use in enhancing anti-tumor immunity (104, 106, 110). Our co-culture results suggest that the adoptive transfer of CD8^{+} T cells in which *CBLB* has been silenced may result in a significant enhancement of T cell anti-tumor responses through both PD-L1 resistance in the *CBLB*-silenced T cells and induction of bystander PD-L1 resistance in the endogenous T cell populations.

Finally, we examined the *in vivo* relevance of the resistance of Cbl-b$^{-/-}$ T cells to PD-L1/PD-1 utilizing a previously established tumor model in which the spread of intra-splenically injected B16 melanoma requires functionally intact PD-L1/PD-1 regulation (100). The ideal system for testing the PD-1/Cbl-b interaction *in vivo* would require a model in which PD-L1/PD-1 suppression can be analyzed without other confounding suppressive influences. Current models only allow us to interrogate systems in which PD-L1/PD-1 has been shown to play a crucial role, while not directly ruling out the influence of other pathways. As such, we chose a model system in which blockade of PD-1 is known to be effective in eradicating liver metastases.

Strikingly, we found that, in the absence of anti-PD-1 treatment, Cbl-b$^{-/-}$ mice develop far fewer metastatic tumor foci in the liver compared to WT mice. It should be noted that simultaneously, we found that the "primary" spleen tumor masses were comparable between WT and Cbl-b$^{-/-}$ mice. This suggests that Cbl-b$^{-/-}$ mice are not broadly capable of eliminating the B16 melanoma in all clinical settings, for example as a result of their resistance to TGF-β or Tregs (39-42, 84). Moreover, this apparent lack of relevance of PD-L1/PD-1 regulation in splenic tumor outgrowth is consistent with the finding of Iwai *et al.,* who reported that when B16 melanoma is administered subcutaneously, PD-1$^{-/-}$ mice demonstrate tumor growth which is comparable to that seen in WT mice (100, 109). In sum, our finding that Cbl-b$^{-/-}$ mice develop significantly fewer liver metastases compared to WT mice suggests, minimally, a lack of dominant sensitivity of Cbl-b$^{-/-}$ mice to PD-L1/PD-1 suppression *in vivo* and thus is consistent with our *in vitro* findings of resistance to PD-L1/PD-1-mediated suppression.

There are several potential mechanisms by which Cbl-b$^{-/-}$ T cells may resist suppression by PD-L1/PD-1. Karwacz *et al.* have previously reported that PD-1/PD-L1 interaction following TCR/CD28 stimulation induces both the upregulation of Cbl-b and TCR down-modulation in CD8^{+} T cells (46). Cbl-b$^{-/-}$ CD8^{+} and CD4^{+} T cells have previously been shown to be defective in TCR down-modulation during T cell priming (111, 112). Based on these findings, Karwacz *et al.* have proposed that during PD-L1/PD-1 signaling, Cbl-b is required for the TCR down-modulation leading to the inhibition of T cell responses (46). However, the requirement for Cbl-b in the PD-L1/PD-1-mediated inhibition of T cell responses was never directly demonstrated in their study through the use of Cbl-b$^{-/-}$ T cells. Moreover, Karwacz *et al*. did not observe TCR down-modulation in response to PD-L1/PD-1 signaling in CD4^{+} T cells and only focused on CD8^{+} T cells in their study. However, as noted above, it has been reported that both Cbl-b$^{-/-}$ CD8^{+} and CD4^{+} T cells are defective in TCR down-modulation in response to in-vitro stimulation (111, 112). This suggests that the resistance to PD-L1-mediated suppression we now report for both Cbl-b$^{-/-}$ CD8^{+} and CD4^{+} T cells may involve mechanisms other than a resistance to PD-L1-induced TCR down-modulation.

Perhaps related to this concept, it has been reported that PD-L1/PD-1 signaling may act downstream by interacting with inhibitory phosphatases such as SHP-1 and SHP-2 (93, 95, 96). In this regard, Xiao *et al.* recently demonstrated that after TCR stimulation, SHP-1 dephosphorylates Cbl-b to prevent its degradation (44). Thus, in the context of both TCR and PD-1 signaling in T cells, SHP-1 may also act to prevent degradation of Cbl-b, allowing Cbl-b to optimally inhibit its targets such as PI3K through its E3 ubiquitin ligase activity. PI3K has been described as one of the proximal TCR signaling molecules affected by PD-L1/PD-1 inhibitory

signals along with Zap70 and PKC-θ (91, 92). Furthermore, Xiao *et al.* also documented that CD28 co-stimulatory signals block the interaction between SHP-1 and Cbl-b, allowing the degradation of Cbl-b. CD28 co-stimulation has previously been described to allow both mouse and human T cells to overcome suppression by PD-L1/PD-1 (99, 113). The disrupted interaction between SHP-1 and Cbl-b may be one of the underlying mechanisms by which CD28 co-stimulation diminishes PD-L1/PD-1 inhibitory signals (113).

Finally, Cbl-b$^{-/-}$ T cells demonstrate increased production of IL-2 (24, 25). Exogenous IL-2 and certain other γ chain signaling cytokines have been demonstrated to enable mouse and human T cells to overcome PD-L1/PD-1 inhibitory signals (99, 113). Our co-culture studies suggest that enhanced cytokine secretion by Cbl-b$^{-/-}$ CD8^{+} T cells may induce resistance to PD-L1/PD-1-mediated suppression in bystander WT CD8^{+} T cells. This also suggests that enhanced cytokine secretion may be playing a mechanistic role, in an autocrine fashion, in the PD-L1/PD-1 resistance in Cbl-b$^{-/-}$ CD8^{+} T cells. Our results indicate however, that both of these characteristics may not be the case for CD4^{+} T cells. At present it is unclear why bystander resistance is noted in CD8^{+} but not in CD4^{+} co-cultures. Future studies in our laboratory will explore this dichotomy and further identify the specific mechanisms by which Cbl-b plays a role in mediating the PD-L1/PD-1 inhibitory signals.

In sum, we have documented for the first time that Cbl-b deficiency in mice results in functional resistance of T cells and NK cells to PD-L1/PD-1-mediated immune regulation. Cbl-b$^{-/-}$ mice develop only a mild autoimmune phenotype consisting of anti-dsDNA antibodies and variably, some degree of multi-organ lymphocyte infiltration. These findings occur only after

approximately 9 months of age. As in all treatments that involve checkpoint inhibition in human cancer therapy, the use of T cells in which Cbl-b is inactivated will require a close watch for the development of autoimmune diseases Nevertheless, our results suggest that targeting Cbl-b in cancer immunotherapy offers the opportunity to simultaneously override numerous relevant "checkpoints" including sensitivity to regulatory T cells, suppression by TGF-β, and immune-regulation not only by CTLA-4 (because of the CD28-independence of Cbl-b$^{-/-}$ T cells) but also, as we now report, immune-regulation by the PD-L1/PD-1 pathway.

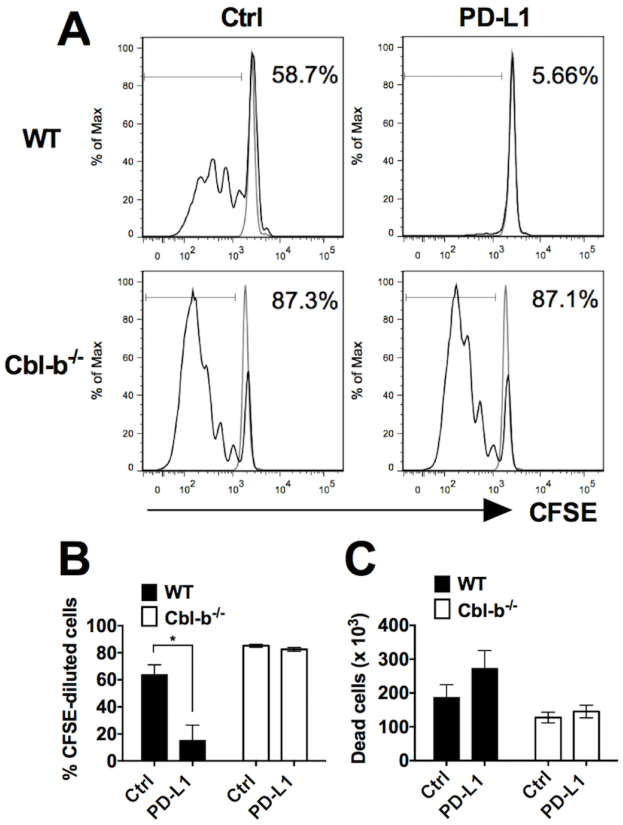


Figure 3-1. Cbl-b$^{-/-}$ CD8^{+} T cells are resistant to PD-L1 Ig-mediated suppression of proliferation. Purified splenic naive Cbl-b$^{-/-}$ and WT CD8^{+} T cells were CFSE-labeled and stimulated for 3 days with plate-bound anti-CD3 ab and soluble anti-CD28 ab in the presence of either plate-bound control Ig or PD-L1 Ig. After 3 days, the percentage of CFSE-diluted of cells was assayed by flow cytometry. (A) Representative FACS plots showing the percentages of CFSE-diluted cells in cultures of CD8^{+} T cells stimulated in the presence of control Ig or PD-L1 Ig (gated on live single CD8^{+} cells). Unstimulated cells are shown in gray. (B) Percentages of CFSE-diluted cells in cultures of CD8^{+} T cells stimulated in the presence of control Ig or PD-L1 Ig. (C) Number of dead cells in cultures of CD8^{+} T cells stimulated in the presence of control Ig or PD-L1 Ig. n=3; Mean ± SEM depicted. Student's *t*-test: *p<0.05.

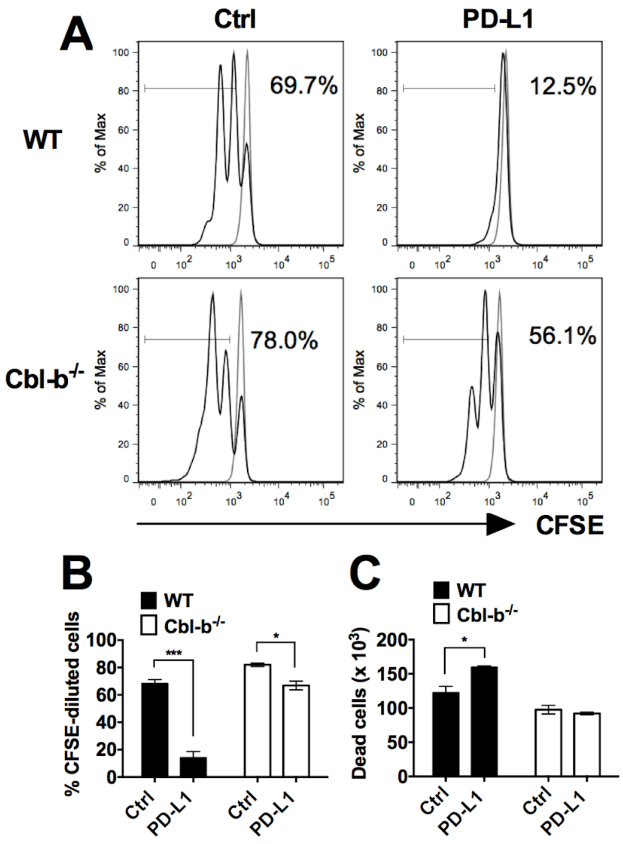


Figure 3-2. Cbl-b$^{-/-}$ CD4^{+} T cells are resistant to PD-L1 Ig-mediated suppression of proliferation. Purified splenic naïve Cbl-b$^{-/-}$ and WT CD4^{+} T cells were CFSE-labeled and stimulated for 2 days with plate-bound anti-CD3 ab and soluble anti-CD28 ab in the presence of either plate-bound control Ig or PD-L1 Ig. After 2 days, the percentage of CFSE-diluted cells was assayed by flow cytometry. (A) Representative FACS plots showing the percentages of CFSE-diluted cells in cultures of CD4^{+} T cells stimulated in the presence of control Ig or PD-L1 Ig (gated on live single CD4^{+} cells). Unstimulated cells are shown in gray. (B) Percentages of CFSE-diluted cells in cultures of CD4^{+} T cells stimulated in the presence of control Ig or PD-L1 Ig. (C) Numbers of dead cells in cultures of CD4^{+} T cells stimulated in the presence of control Ig or PD-L1 Ig. n=3; Mean ± SEM depicted. Student's *t*-test: *p<0.05, ***p<0.001.

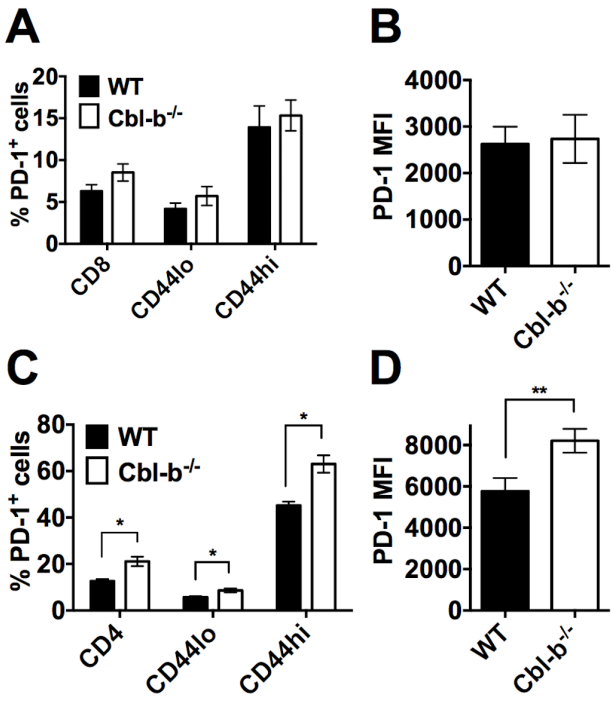


Figure 3-3. The expression of PD-1 is not decreased in Cbl-b$^{-/-}$ CD8^{+} and CD4^{+} T cells. For *ex vivo* studies, splenocytes were derived from Cbl-b$^{-/-}$ and WT mice and labeled with anti-CD8, anti-CD4, anti-PD-1, and anti-CD44 abs immediately *ex vivo*. Alternatively, purified CD4^{+} and CD8^{+} T cells were labeled with anti-PD-1 ab after 2-3 days in culture with plate-bound anti-CD3 ab and soluble anti-CD28 ab. These cells were subsequently assayed by flow cytometry. (A) Frequencies of total CD8^{+}, or CD8^{+} CD44^{low}, or CD8^{+} CD44^{high} T cells that were PD-1^{+} in unstimulated splenocytes (gated on live, single, CD8^{+}, CD44^{low} or CD44^{hi} cells). (B) MFI of PD-1 expression in *in vitro* stimulated CD8^{+} T cells (gated on live, single, CD8^{+} cells). (C) Frequencies of total CD4^{+}, or CD4^{+} CD44^{low}, or CD4^{+} CD44^{high} T cells that were PD-1^{+} in unstimulated splenocytes (gated on live, single, CD4^{+}, CD44^{low} or CD44^{hi} cells). (D) MFI of PD-1 expression in *in vitro* stimulated CD4^{+} T cells (gated on live, single, CD4^{+} cells). n=3; Mean ± SEM depicted. Student's *t*-test: *p<0.05, **p<0.01.

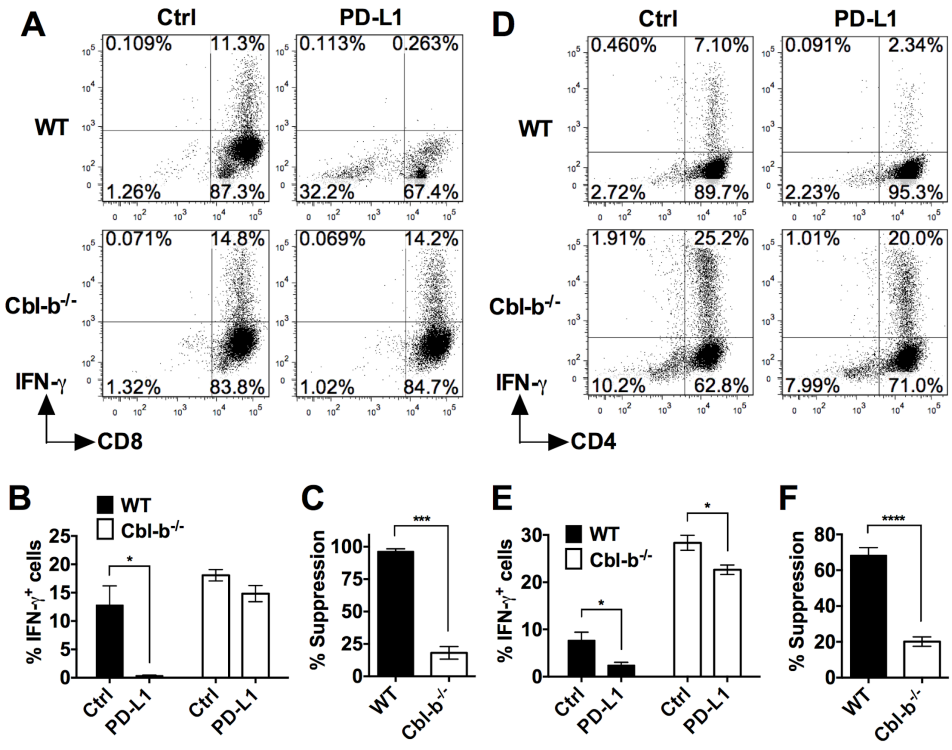


Figure 3-4. PD-L1 Ig-mediated suppression of IFN-γ is significantly greater for WT than for Cbl-b$^{-/-}$ T cells. Purified naïve splenic Cbl-b$^{-/-}$ and WT CD8^{+} CD44^{low} and CD4^{+} CD44^{low} T cells were stimulated for 3 days with plate-bound anti-CD3 ab and soluble anti-CD28 ab in the presence of either plate-bound control Ig or PD-L1 Ig. For both CD8^{+} and CD4^{+} T cell cultures, brefeldin A was added in the last 4 hours of culture and cells were intracellularly stained with anti-IFN-γ ab and analyzed by flow cytometry. (A) Representative FACS plots showing the percentages of IFN-γ^{+} cells in cultures of CD8^{+} T cells stimulated in the presence of control Ig or PD-L1 Ig (gated on live single CD8^{+} cells). (B) Mean frequencies of IFN-γ-producing CD8^{+} T cells stimulated in the presence of either control Ig or PD-L1 Ig. (C) Mean percentage suppression of CD8^{+} T cell IFN-γ production by PD-L1 Ig as shown in (B). (D) Representative FACS plots showing the percentages of IFN-γ^{+} cells in cultures of CD4^{+} T cells stimulated in the presence of control Ig or PD-L1 Ig (gated on live single CD4^{+} cells). (E) Mean frequencies of IFN-γ-producing CD4^{+} T cells stimulated in the presence of control Ig or PD-L1 Ig. (F) Mean

percentage suppression of $CD4^+$ T cell IFN-γ production by PD-L1 Ig as shown in (E). Mean ± SEM depicted. n = 3 for (B) and (C); n=5-6 for (E) and (F). Student's *t*-test: *p<0.05, ***p<0.001, ****p<0.0001.

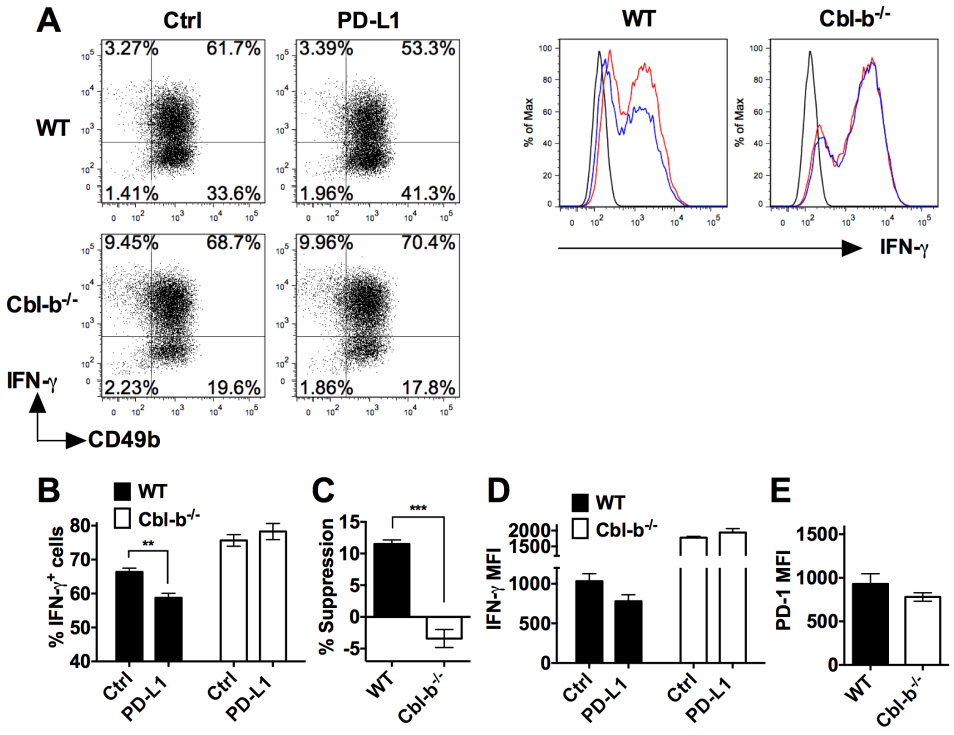


Figure 3-5. Cbl-b$^{-/-}$ NK cells are resistant to PD-L1 Ig-mediated suppression of IFN-γ production. Splenic NK cells were isolated from Cbl-b$^{-/-}$ and WT mice and cultured for 7 days in the presence of IL-2. These NK cells were harvested from the 7-day cultures and stimulated with plate-bound anti-NK1.1 ab in the presence of either plate-bound control Ig or plate-bound PD-L1 Ig and brefeldin A for 5 hours. The NK cells were then intracellularly stained with anti-IFN-γ ab and analyzed by flow cytometry. Additionally, unstimulated NK cells were stained with anti-PD-1 ab after the 7-day IL-2 culture and analyzed by flow cytometry. (A) Representative dot blots and corresponding histograms on 7-day cultured NK cells stained for intracellular IFN-γ. Cells were gated on live single WT or Cbl-b$^{-/-}$ CD3^{-} (dot blots) or CD3^{-} CD49b^{+} cells (histograms) that had been stimulated in the presence of control Ig or PD-L1 Ig. In histograms: black line represents isotype staining, red line represents control Ig, and blue line represents PD-L1 Ig. (B) Mean frequencies of IFN-γ-producing WT or Cbl-b$^{-/-}$ NK cells stimulated in the presence of control Ig or PD-L1 Ig (gated on live single CD3^{-} CD49b^{+} cells).

(C) Mean percentage suppression of IFN-γ producing NK cells after stimulation in the presence of PD-L1 Ig as shown in (A). (D) Mean MFI of IFN-γ staining in 7-day cultured WT or Cbl-b$^{-/-}$ NK cells stimulated in the presence of control Ig or PD-L1 Ig (gated on live single CD3^{-} CD49b^{+} cells). (E) Mean MFI by flow cytometry of PD-1 expression in unstimulated WT and Cbl-b$^{-/-}$ NK cells that were harvested from the 7-day IL-2 culture (gated on live single CD3^{-} NK1.1^{+} CD49b^{+} cells). Mean ± SEM depicted. n = 3. Student's *t*-test: **p<0.01, ***p<0.001.

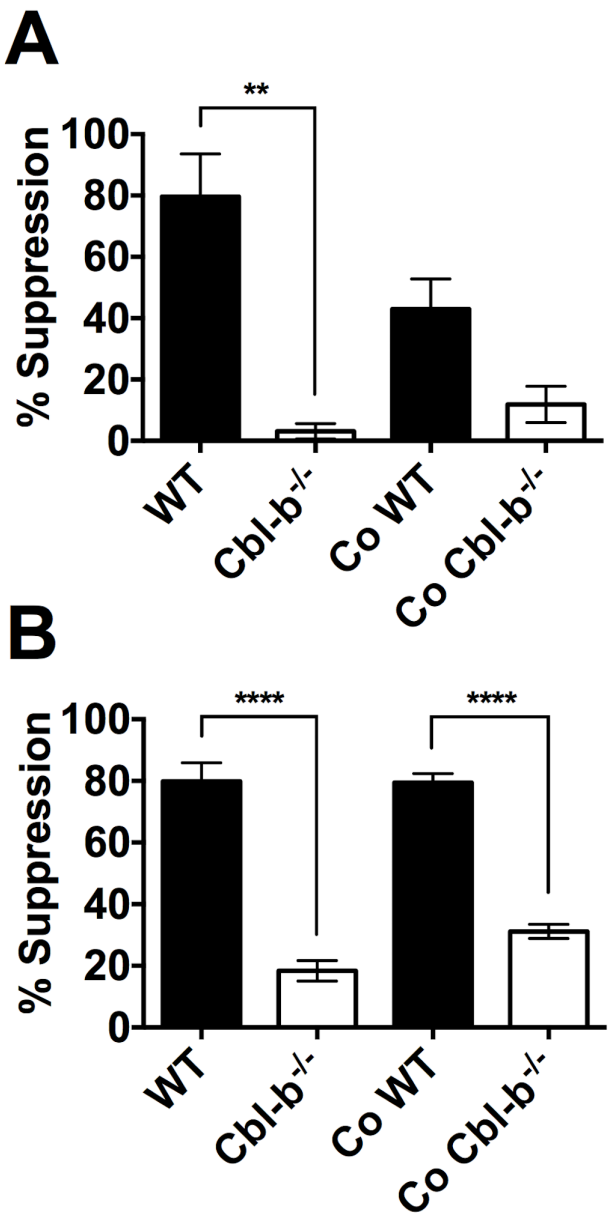


Figure 3-6. PD-L1 Ig-suppression in co-cultures of Cbl-b$^{-/-}$ and WT T cells.

Naïve CD8^{+} CD44^{low} T cells and naïve CD4^{+} CD44^{low} T cells were isolated from spleens of WT C57BL/6, CD45.1^{+} congenic mice and Cbl-b$^{-/-}$ mice (CD45.2^{+}). WT and Cbl-b$^{-/-}$ T cells were CFSE-labeled and stimulated for 3 (CD8^{+}) or 2 (CD4^{+}) days either separately (WT and Cbl-b$^{-/-}$ T cells in separate cultures) or together ("Co"= WT and Cbl-b$^{-/-}$ T cells cultured together) with plate-bound anti-CD3 ab and soluble anti-CD28 ab in the presence of either plate-bound control

Ig or PD-L1 Ig. At the end of culture, the percentage of CFSE-diluted cells was assayed by flow cytometry. (A) Mean percentage suppression by PD-L1 Ig of single and co-cultures of naïve $CD8^+$ $CD44^{low}$ T cells stimulated in the presence of control Ig or PD-L1 Ig. Co-WT indicates % suppression of WT $CD8^+$ T cells by PD-L1 in the co-culture of WT and Cbl-$b^{-/-}$ $CD8^+$ T cells. Co-Cbl-$b^{-/-}$ indicates % suppression of Cbl-$b^{-/-}$ $CD8^+$ T cells by PD-L1 Ig in the same co-culture. (B) Mean percentage suppression by PD-L1 Ig of single and co-cultures of naïve $CD4^+$ $CD44^{low}$ T cells stimulated in the presence of control Ig or PD-L1 Ig. Co-WT indicates % suppression of WT $CD4^+$ T cells by PD-L1 in the co-culture of WT and Cbl-$b^{-/-}$ $CD4^+$ T cells. Co-Cbl-$b^{-/-}$ indicates % suppression of Cbl-$b^{-/-}$ CD4+ T cells by PD-L1 Ig in the same co-culture. n=3; Mean ± SEM depicted. One-way ANOVA: **$p<0.01$, ****$p<0.0001$.

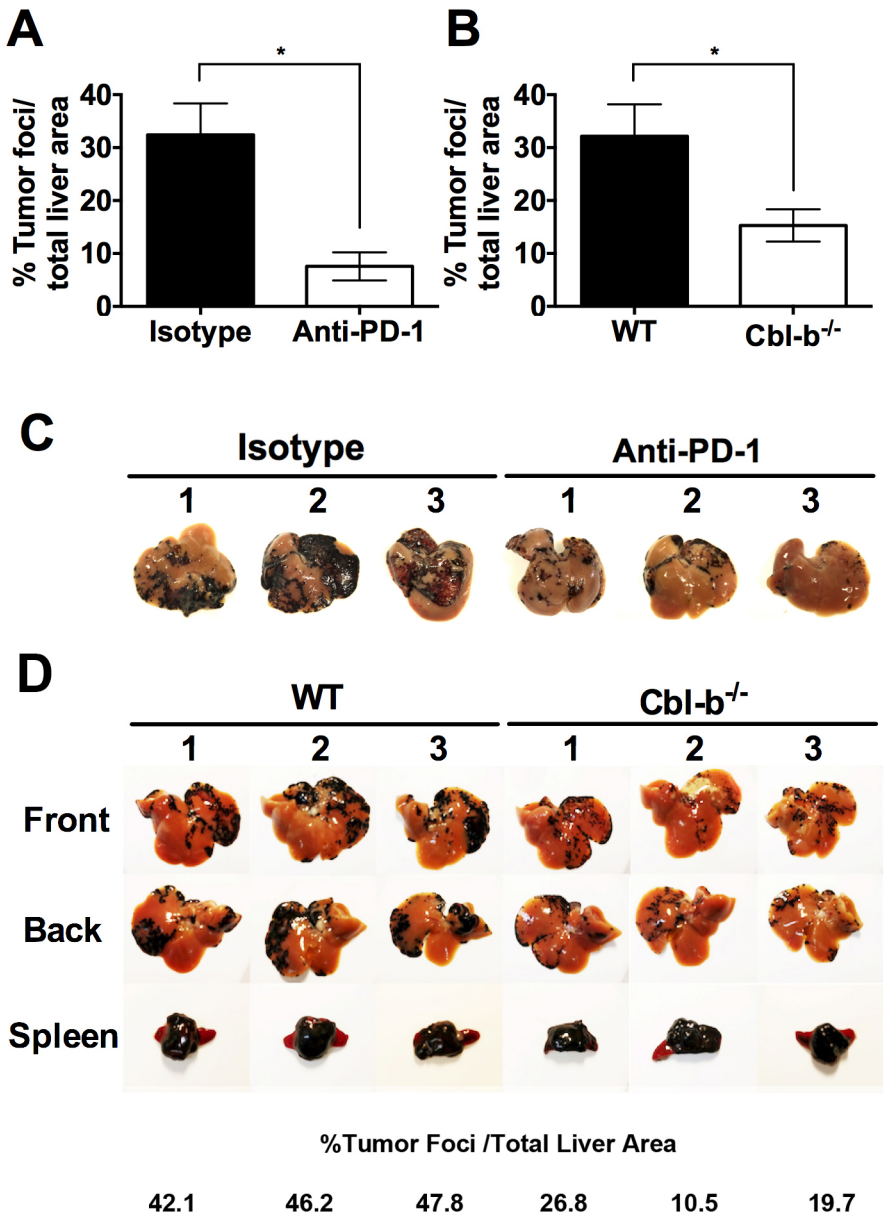


Figure 3-7. Cbl-b$^{-/-}$ mice develop significantly fewer liver metastases in a PD-L1/PD-1 dependent model of B16 melanoma liver metastasis.

(A) WT mice received an intra-splenic injection of B16 melanoma cells and then were treated with either 100 μg of isotype control ab or anti-PD-1 ab i.p. on the day of tumor injection and every other day until the mice were sacrificed. The mice were sacrificed on day 8, perfused with

saline, the liver and the spleen harvested, and the percentages of tumor foci area / total liver area were quantified using ImageJ software. (B) WT and Cbl-b$^{-/-}$ mice received an intra-splenic injection of B16 melanoma cells as in (A), but no isotype or anti-PD-1 ab was given. The mice were sacrificed on day 7-9, perfused with saline, the liver and the spleen harvested, and the percentages of tumor foci area / total liver area were quantified using ImageJ software. **(**C**)** Images of the livers from the experiment shown in (A). (D) Representative images of liver and spleen from (B) with percentages of tumor foci area/total liver area for the livers presented. Mean ± SEM depicted. n = 3 for (A), n = 12-14 for (B). Student's *t*-test: *p = 0.019 for (A); *p = 0.026 for (B).

CHAPTER 4 Enhanced Toll-Like Receptor 2 Responses in Multiple Sclerosis

ABSTRACT

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) with unknown etiology. The role of human gut microbiota in the pathogenesis of MS has become a significant focus of research. Previously, we have identified a Lipid 654 (L654), which is produced by the commensal *Bacteroidetes* species in the human gastrointestinal tract (GI) and acts as a human Toll-like receptor 2 (TLR2) ligand and is present in the human systemic circulation. Most importantly, we previously documented that the serum levels of L654 are significantly lower in MS patients compared to healthy controls. Based on the recent reports on the role of human gut microbiota in regulating the systemic immunity through the induction of tolerance, and our findings of lower L654 levels in MS, we postulated that MS patients may be defective in TLR tolerance and may demonstrate TLR hyper-responsiveness.

We now report that peripheral blood $CD14^+$ monocytes of a subset of MS patients with active disease demonstrate enhanced *ex vivo* TLR2 responses. Furthermore, we have now report that Lipid 342, which is a breakdown product of the previously characterized bacterially derived lipodipeptide L654, is present in the human systemic circulation, acts as a human TLR2 ligand, and is detected at significantly lower levels in the plasma of MS patients. These results identify a novel underlying pathogenic mechanism of MS, and highlight a potential clinically important relationship between the gut commensal microbiota and human systemic autoimmunity. Finally, L342 may represent an environmental factor that may be may be utilized as a novel blood biomarker for MS.

INTRODUCTION

Multiple Sclerosis is an autoimmune disease manifested in the central nervous system (CNS). MS is more common in women than in men and it affects approximately 0.1% of the population in the United States, mostly between the ages of 20-40 years. Currently, the majority of therapeutic agents approved for treating MS broadly suppress the functions of the immune system, and they often cause serious adverse side effects. Furthermore, there is currently no blood biomarker that allows accurate MS diagnosis. MS is thought to be mediated by an attack of self-reactive T cells on CNS myelin antigens, however the mechanisms responsible for both the onset and progression of MS still remain to be elucidated.

The etiology of MS still remains unknown, however both genetic and environmental factors have been proposed to contribute to the pathogenesis of MS. Several genome-wide association studies (GWAS) have been published revealing 97 variants in the immune genes that are associated with MS susceptibility (11-13, 114). However, the concordance rate for monozygotic twins with MS is only 25-30% (15, 115), suggesting the major contribution of environmental factors in influencing MS susceptibility. The role of human gut commensal microbiota in the pathogenesis of MS is currently being rigorously investigated. The gut commensal microbiota has been shown to influence CNS autoimmunity in an experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. Germ-free mice develop significantly attenuated disease during EAE while the introduction of commensal microbiota into the mice renders them susceptible to the disease development (116). The disease pathogenesis of EAE/MS is largely mediated by a subset of $CD4^+$ T cells called Th17 (T helper type 17) cells (4). Th17 cells are characterized by the expression of transcription factor RORγt and the production

of interleukin 17A (IL-17A) (117). Segmented-filamentous bacteria (SFB) are necessary and sufficient for the homeostatic development and expansion of Th17 cells in the gut (118), and also for the accumulation of Th17 cells in the CNS during EAE (48). In addition, the gut microbiome demonstrates an immunomodulatory effect in which treatment with a polysaccharide A (PSA) from *Bacteroidetes fragilis* has been shown to result in amelioration of EAE by promoting the expansion of regulatory T cell subsets in the gut (49, 50). These studies in mice connecting the gut commensal bacteria to the immune system laid the groundwork for the human studies with focus on the roles of human gut microbiota in regulating the human immune system.

There is accumulating evidence for the role of human gut microbiota in regulating human systemic autoimmune and inflammatory diseases. Recently, there are reports in allergic asthma (119) and type 1 diabetes (52) highlighting the essential role of the human gut microbiome in setting the threshold of immune responses and in regulating immune hyper-reactivity. Most recently, Jangi *et al* documented the increased abundance of *Methoanobrevibacter* and *Akkermansia* species and decreased levels of *Butyricinmonas* in the fecal samples of MS patients compared to those of healthy controls (17), which positively and negatively correlated with robust transcriptional changes in the MS-associated immune genes in MS patients, respectively (17). While these studies suggest the relationship between the human gut microbiome to the systemic immunity, the identity of bacterially derived factors that directly influence the systemic immunity in humans still remains unclear.

Our laboratory recently identified a unique bacterially derived serine-containing lipodipeptide, Lipid 654 (L654), that is produced by a number of commensal *Bacteroidetes*

species that reside in the gastrointestinal tract (GI) and functions as a ligand for human and mouse Toll-like receptor 2 (TLR2) (53). Using multiple-reaction-monitoring (MRM) mass spectrometric approaches, we reported that Lipid 654 is detectable and quantifiable in the serum of all individuals studied (51). Most importantly, the levels of L654 in the sera of MS patients were significantly lower compared to the levels found in the sera of healthy controls (51).

Based on these previous findings with L654, we previously postulated that microbial products such as L654 that are derived from the human gut microbiota enter the systemic circulation, and chronically stimulate the immune system and induce a state of relative TLR tolerance, which sets the threshold for immune activation and prevents overt immune hyper-reactivity. TLR tolerance is a state of tolerance induced upon repeated ligation of a TLR by its ligands, which results in dampening of the magnitude of the signaling downstream of the TLR, as well as in the upregulation of intracellular TLR signaling associated molecules with regulatory functions (54). In demonstrating the *in vivo* immunomodulatory effect of TLR tolerance, we recently documented that the mice that were given repeated injections of low dose TLR2 ligands showed significantly reduced disease severity during adoptively transferred EAE (120).

Based on the lower levels of L654 in sera of MS patients (51), the therapeutic effect of TLR2 tolerance during EAE (120), and the recent reports on the human gut microbiome regulating systemic immunity (17, 52, 119), we postulated that MS patients may be defective in TLR tolerance. Moreover, we further posited that the pathological outcome of potentially defective TLR tolerance may be TLR hyper-responsiveness in these patients.

Here we report for the first time that the peripheral blood (PB) $CD14^+$ monocytes isolated from a subset of MS patients demonstrate an enhanced response to canonical TLR2 ligands (Pam3CysK4 (P3C) and Pam2CysK4 (P2C)) when compared to healthy controls. Furthermore, this enhancement in the TLR2 response was only seen in a subset of MS patients with active disease.

Recently, we identified and characterized a new breakdown product of L654, termed Lipid 342 (L342). Similarly to L654, L342 was detectable in the plasma of all individuals tested using technical approaches that were simpler than those required to detect plasma L654. Most importantly, we report for the first time that the levels of L342 in the plasma of MS patients, as with the levels of L654, are significantly lower than those in the plasma of healthy controls. Moreover, L342 is a TLR2 agonist as it stimulated hIL-8 production from HEK293 cells transfected with human TLR2 and human CD14.

In these studies we now find 1) TLR2 hyper-responsiveness in a subset of MS patients, with potential correlation with disease activity, and 2) lower levels of L342 in the plasma of MS patients compared to healthy individuals. These results suggest that the enhancement of TLR2 responses may be a novel underlying pathogenic mechanism of MS, and highlight a potential clinically important relationship between the gut commensal microbiota and human systemic autoimmunity. Finally, L342 may represent an environmental factor that may be involved in the pathogenesis of MS, and may be utilized as a novel blood biomarker for MS.

MATERIAL AND METHODS

Patients

All studies were performed using protocols approved by the Institutional Review Board (IRB) at the University of Connecticut Health Center (UCHC). Healthy controls were recruited from volunteer donors at UCHC. Patients with MS were recruited both from the MS clinic at UCHC as well as from other physicians in the state of Connecticut. Most of the time, samples were drawn from patients who had not eaten for at least 2 hours.

Blood collection and plasma isolation

Approximately 30 ml of blood was collected from each patient in three BD Vacutainer® lavender top 10 ml K_2-EDTA-coated blood collection tubes. Plasma was isolated from one of the vials by centrifugation at 1,750 RCF in Becton Dickinson tabletop centrifuge with the brake off for 15 min at RT. The plasma layer was transferred to a clean glass vial using a glass pipette, and the plasma sample was stored at -80 C.

Monocyte and PBMC isolation from whole blood

After the blood collection, $CD14^+$ $CD16^-$ monocytes from each patient were isolated using the EasySep™ Direct Human Monocyte Isolation Kit (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's instructions. For PBMC isolation, the whole blood was diluted 1:1 with sterile PBS ($Ca^{++}Mg^{++}$ free) containing 2% of FCS (2% FCS PBS). The diluted blood was slowly dispensed on top of 15-ml of Lymphoprep™ density gradient medium (STEMCELL Technologies, Vancouver, Canada) placed in a bottom portion of a 50-ml SepMate™ tube (STEMCELL Technologies), then centrifuged at 1,200 RCF for 10 min at RT. Then, the

mononuclear cell layer was transferred to sterile 50 ml conical tubes, washed twice with 50 ml of sterile 2% FCS PBS then washed once with 15-ml of sterile 10% FCS complete RPMI 1640 (supplemented with L-glutamine, Pen/Strep, Gentamicin and HEPES), each wash followed by centrifugation at 864 RCF at 7 C.

$CD14^+$ monocyte stimulation and TLR2 expression analysis

After $CD14^+$ $CD16^-$ monocytes were isolated from the whole blood, cells were blocked with human FcR Blocking Reagent (Miltenyi Biotec, Auburn, CA) and stained with Live/Dead Near IR (Molecular Probes, Eugene, OR) anti-human CD14-APC (Tonbo Biosciences, San Diego, CA) and anti-human TLR2-PE (eBiosciences/Affymetrix Inc., Santa Clara, CA) or isotypes and analyzed by flow cytometry. For *in vitro* stimulation, $CD14^+$ monocytes were plated at 2 x 10^4/well and stimulated with 2 or 10 μg/ml of Mitogenic Pentapeptide Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Ser-Asn-Ala-OH (MMP) (Pam3CysK4, P3C) from Bachem Americas Inc. (H-9460.0005) or 20 or 10 ng/ml of *E. coli* lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO) for 4 hours. Human TNFα was measured in the supernatants using the Ready-SET-Go!® ELISA kit (eBiosciences/Affymetrix Inc.).

Immunological phenotyping and ex vivo stimulation of PBMCs with TLR ligands

Peripheral blood mononuclear cells (PBMCs) were cultured at 2 x 10^5 cells/well in 10% FCS complete RPMI 1640 with either no stimulus or with 2 or 10 μg/ml of Pam2CysK4 (P2C) (InvivoGen, San Diego, CA) or 10 or 50 ng/ml of *E. coli* LPS for 4 hours with 5 μg/ml of Brefeldin A (Sigma-Aldrich) for intracellular cytokine staining. The supernatant was collected and the levels of human TNFα were quantified using the human TNFα ELISA kit. For surface

staining, unstimulated cells were Fc blocked with human FcR block reagent then surface-stained with Live/Dead Near IR, anti-human CD14-APC, anti-human CD16-PE-Cy7 (BD Biosciences), anti-CD19-FITC (Biolegend, San Diego, CA), andanti-human TLR2-PE (Biolegend). For intracellular cytokine staining, cells were taken up and incubated with human FcR block then surface-stained with Live/Dead Near IR, anti-human CD14-APC, anti-human CD16-PE-Cy7, and anti-CD19-FITC. These cells were then fixed and permeabilized using BD Cytoperm Cytofix kit (BD Biosciences), blocked with 5% human serum then intracellularly stained with anti-human TNFα-PE (Biolegend). All the stained ells were analyzed using BD LSRII flow cytometers (BD Biosciences).

Lipid 342 extraction from plasma samples

Glass tubes were supplemented with 6-OH C18:0 (0.01 ug/µl in chloroform, 30 µl transferred into each tube). Plasma samples were thawed and 500 µl of each sample was transferred to a glass tube. The samples were acidified with 250 µl of acetic acid, extracted three times with 1 ml of chloroform and the samples pooled in 5 ml conical vials. The chloroform was dried under nitrogen and derivatized to form pentafluorobenzyl ester, TMS ether derivatives. Each sample was dissolved in 30 µl of acetonitrile and 10 µl of diisopropylethylamine. PFBB (33% in acetonitrile, 10 µl) was added to each vial, capped and heated for 20 min at 50 C. The samples were then dried under nitrogen and treated with 40 µl of BSTFA (bistrimethylsilyl trifluoracetamide) overnight at room temperature. The samples were then transferred to autosampler vials for gas chromatography mass spectrometry (GC-MS) analysis.

Measuring the plasma levels of Lipid 342 with gas chromatography mass spectrometry (GC-MS)

The samples were run on the Agilent 5975C GC-MS system (Agilent Technologies, Santa Clara, CA) with helium as carrier gas (maintained at 1 ml/min constant flow). One ul of each sample was injected and the column heated from 100 C to 290 C. The injection block was maintained at 290 C and the transfer tube was maintained at 300 C. The column was heated from 100 to 290 C at 15 C per minute and maintained at 290 C for three minutes. The mass spectrometer was run in the negative ion chemical ionization mode with a gas pressure of 40. The TMS ether negative molecular ion of Lipid 342 was monitored at m/z 414 and TMS ether of 6-OH C18:0 was monitored at m/z 371. Selected ion peaks were electronically integrated and the levels of L342 calculated from a standard curve run in parallel.

In vitro testing of Lipid 342 in HEK-TLR2/CD14 cell line

Human embryonic kidney cells (HEK293) transfected with human TLR2 and stably expressing CD14 (HEK-Blue™ hTLR2) were purchased from InvivoGen. Cells were cultured in 2.5% FCS Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 4.5 g/liter L-glucose, penicillin/streptomycin, sodium pyruvate, non-essential amino acids (NEAA), L-glutamine, normocin and HEK-Blue selection antibiotics. A day prior to the stimulation, these cells were cultured in serum free HL-1 media (Lonza, Wakersville, MD) containing penicillin/streptomycin, sodium pyruvate, non-essential amino acids (NEAA), L-glutamine, normocin and HEK-Blue selection antibiotics and 2-mercaptoethanol. Lipid 342 (L342) derived from the total lipids of *Porphymonas gingivalis* were prepared by Dr. Frank Nichols at the Department of Oral Health and Diagnostic Sciences at UCHC. Synthetic forms of L342 were prepared by Dr. Christopher

Dietz and Dr. Michael Smith at the Department of Chemistry at UConn Storrs. On the day of stimulation, all of the L342 samples were resuspended in the serum free HL-1 media and sonicated prior to their use. The cells were stimulated without or with 2.5 μg/ml of sonicated L342 or 10 pg/ml of Pam2Cys3K4 for 24 hours. The supernatant was collected and the levels of hIL-8 secretion were measured by using BD OptEIA hIL-8 ELISA set (BD Biosciences).

RESULTS

Peripheral blood CD14$^+$ monocytes from MS patients with active disease demonstrate enhanced TLR2 responses to Pam3CysK4

Based on the findings of 1) lower serum levels of L654 in MS patients compared to in healthy controls (51), 2) the therapeutic efficacy of TLR2 tolerance *in vivo* in adoptive transfer EAE (120), and 3) recent reports on the role of human gut microbiota in regulating systemic immunity (17, 52, 119), we postulated that MS patients may have defective homeostatic TLR tolerance *in vivo* and therefore may demonstrate TLR hyper-responsiveness compared to healthy individuals. In order to address this, we initially examined the *ex vivo* TLR2 responses of the peripheral blood (PB) CD14$^+$ cells from MS patients and healthy controls. We isolated PB CD14$^+$ monocytes from MS patients and healthy controls, stimulated them *in vitro* with the TLR2 ligand Pam3CysK4 (P3C), and measured the levels of TNFα production by ELISA. Since TLR2 has been shown to cross-tolerize to TLR4 (121), we also examined the *ex vivo* TLR4 responses in response to lipopolysaccharide (LPS).

At baseline without any stimulation, CD14$^+$ cells from both healthy controls and MS patients made almost 0 pg/ml of TNFα. In response to P3C stimulation, while the levels of TNFα

in the supernatant of $CD14^+$ cells from healthy controls mostly clustered together well below 100 pg/ml of TNFα, we found that there were much more variable levels of TNFα detected from the supernatant of $CD14^+$ cells from MS patients (ranging between 50 to 700 pg/ml) (**Figure 4-1A**). However, the difference between the levels of TNFα detected in the two cohorts did not reach statistical significance at baseline or either doses of P3C. In order to determine whether there is any correlation between TLR2 responses and disease activity of MS patients, we subdivided MS patients into two categories; relapsing/inactive (RR-MS and disease is currently stable) or progressive/active (PP-MS or SP-MS and having currently active disease) based on their self-disclosed disease activity at the time of the blood drawing. Importantly, we found a statistically significant increase in $CD14^+$ TNFα production by the progressive/active subset of MS patients in comparison to the levels of either healthy controls or the relapsing/inactive subset (**Figure 4-1C**). These results suggest that there is a subset of MS patients made up of those with progressive MS or active disease that demonstrate an increased TLR2 response.

For LPS/TLR4 responses, PB $CD14^+$ monocytes from MS patients demonstrated a trend of increased levels of TNFα in response to 20 ng/ml of LPS (**Figure 4-1B**). In contrast to TLR2 responses, however, stratifying MS patients based on disease activity did not reveal any statistical difference between healthy controls and MS patients (**Figure 4-1D**). Next, we examined the baseline levels of TLR2 expression in $CD14^+$ cells of MS patients compared to those of healthy controls by flow cytometry. Based on the mean fluorescence intensity (MFI) of TLR2 staining by flow cytometry, unstimulated PB $CD14^+$ cells from both healthy controls and MS patients demonstrated comparable levels of TLR2 expression (**Figure 4-1E**). These results suggest that while TLR2 expression in $CD14^+$ monocytes is comparable between MS patients

and healthy controls, the PB $CD14^+$ monocytes of MS patients with progressive and active disease status demonstrate an enhanced TLR2 response compared to those of healthy controls or MS patients with inactive disease status. Finally, while TLR4 responses to LPS appear to be somewhat increased in MS patients compared to healthy individuals, there are no statistically significant differences in these LPS responses between the two cohorts.

Whole PBMCs from MS patients with active disease demonstrate enhanced TLR2 responses to Pam2CysK4

Upon ligation of a TLR2 ligand to a TLR2 receptor on the cell surface, TLR2 forms a heterodimer with either TLR1 or TLR6 in humans. Previously, increased levels of TLR1 expression in $CD4^+$ $CD25^+$ regulatory T cells of MS patients have been documented (122). While the status of TLR1 expression by $CD14^+$ monocytes of MS patients remains unclear, P3C (used in Figure 4-1) is a triacylated lipopeptide and it signals through TLR2/TLR1. Moreover, we previously found that L654 is a diacylated lipeptide that signals through TLR2/6 (unpublished observations). Because of the potentially increased TLR1 expression in MS $CD14^+$ monocytes, we next tested responses to Pam2CysK4 (P2C), a synthetic diacylated lipopeptide that acts as a TLR2/6 ligand.

It is possible that cells other than $CD14^+$ monocytes in the PB may play a role in the innate immune hyper-responsiveness in MS. Therefore, we next examined the whole PBMC TLR2 responses from MS patients. First we examined the TNFα production by the PBMCs of MS patients and healthy controls in response to varying doses of P2C by ELISA (**Figure 4-2A**). As we noted in the purified $CD14^+$ monocytes in response to P3C (**Figure 4-1A**), there was an

increased PBMC response to P2C by a subset of MS patients. (**Figure 4-2A**). Since most of the MS patients in our study were being treated with-treatment modalities which may alter composition of the PBMCs (**Table 4-2**), we examined the frequencies of "classical" $CD14^+$ monocytes, "non-classical" $CD14^+$ $CD16^+$ monocytes, and $CD19^+$ B cells in the PBMCs of each MS and control patient.

In contrast to the finding of Waschbisch *et al* documenting the reduced frequencies of $CD14^+$ $CD16^+$ or $CD16^{++}$ monocytes in the PBMCs of MS patients compared to healthy individuals (123), we found comparable frequencies of $CD14^+$ $CD16^+$ monocytes in the PBMCs of MS patients and healthy controls (**Figure 4-2B**). Additionally, the frequencies of $CD19^+$ B cells in the PBMCs between MS and healthy control cohorts were also comparable (**Figure 4-2B**). However, MS patients treated with Rituximab or Avonex showed an approximately two-fold increase in the frequency of $CD14^+$ monocytes in their PBMCs compared to other MS patients and healthy controls (**Figure 4-2B**). To account for the differential frequencies of $CD14^+$ cells among the patients and to negate the effect of increased $CD14^+$ monocyte numbers on the *ex vivo* TLR2 responses shown in **Figure 4-2A**, we normalized the TNFα levels from the each patient (**Figure 4-2A**) to the frequency of $CD14^+$ monocytes in the PBMCs of the same patient. After the normalization, a subset of MS patients still demonstrated increased TNFα levels compared to those of healthy controls. Since a statistically significant increase in TNFα levels in response to P3C was only revealed after stratifying MS patients based on disease activity (**Figure 4-1C**), we again stratified patients into active MS versus inactive MS based on their clinical history (**Table 4-2**). Importantly, there was a statistically significant increase in the normalized TNFα levels of MS patients with active disease in response to 2 μg/ml of P2C

compared to the levels seen in both healthy controls and MS patients with inactive disease (**Figure 4-2D**). At 10 μg/ml of P2C, there was also a statistically significant increase in the normalized TNFα levels from MS patients with active disease compared to those of MS patients with inactive disease (**Figure 4-2D**). It is interesting that at this early stage of our studies, the normalized TNFα levels of MS patients with inactive disease are lower than those of healthy controls, although these differences between the two cohorts did not reach statistical significance at all the doses of P2C tested (**Figure 4-2D**). Overall, these data suggest that based on the normalized TNFα production in response to P2C, MS patients with active disease demonstrates enhanced *ex vivo* TLR2 responses compared to healthy controls and MS patients with inactive disease.

Increased frequencies of TLR2-responsive CD14$^+$ monocytes in MS patients with active disease

After documenting the enhanced *ex vivo* TLR2 responses to P2C and P3C from MS patients with active disease through the ELISA approach, we examined the differences of TNFα production specifically from the PB CD14$^+$ monocytes using intracellular cytokine staining**.** Without stratifying MS patients based on their disease activity, we did not detect any statistically significant differences in the frequencies of TNFα-producing CD14$^+$ monocytes between MS and healthy controls (**Figure 4-3A**) or the mean fluorescence intensity (MFI) of the TNFα expression by CD14$^+$ monocytes between the two cohorts (**Figure 4-3B**). However, when we stratified the MS patients based on disease activity, the MS patients with active disease demonstrated significantly increased frequencies of TNFα-producing CD14$^+$ monocytes compared to MS patients with inactive disease (did not reach statistical significance as of yet when comparing

with healthy controls, however) (**Figure 4-3C**). Thus, based on these results, the MS patients with active disease demonstrate increased frequencies of PB $CD14^+$ monocytes that are responsive to TLR2 stimulatory signals.

Finally, we attempted to examine the *ex vivo* TLR responses of "non-classical" $CD14^+$ $CD16^+$ monocytes in MS patients. However, we found that the expression of CD16 in the PB $CD14^+$ monocytes becomes rapidly and drastically downregulated in response to both P2C and LPS stimulation in most individuals studied, which prevented us from being able to analyze the TNFα production by these cells.

B cells of MS patients and healthy controls are minimally responsive to TLR2 ligand-induced stimulation in vitro

In addition to PB $CD14^+$ monocytes, B cells express pattern recognition receptors including TLR2 and are capable of responding to PAMPs and DAMPs. Furthermore, B cells are a major cytokine producer and act as antigen presenting cells (APCs). Importantly, the therapeutic efficacy of anti-CD20 antibody (Rituximab) in the treatment of MS underscores the pathogenic role of B cells in MS. Therefore, we examined the *ex vivo* TLR2 responses of $CD19^+$ B cells from MS patients versus healthy controls by intracellular cytokine staining and flow cytometry. The frequencies of PB $CD19^+$ B cells in the PBMCs were comparable between MS patients and healthy controls, ranging between 5-6% of the PBMC populations, with exception of MS patients being treated with anti-CD20 (Rituximab). In these patients, the $CD19^+$ B cell populations were almost completely depleted from the peripheral blood and thus they were not included in this part of the study (**Figure 4-2B**). In contrast to $CD14^+$ monocytes in which

approximately 20-30% of the population produced TNFα in response to P2C, the frequencies of $CD19^+$ B cells producing TNFα in response to P2C remained lower than or equal to 10% of the total B cell populations in both MS patients and healthy controls (**Figure 4-4A**). Furthermore, based on the TNFα MFI, there was minimal upregulation of TNFα in $CD19^+$ B cells in response to P2C compared to the baseline levels (**Figure 4-4B**). More importantly, we did not detect any differences in the frequencies of TNFα-producing $CD19^+$ B cells or TNFα MFI of these cells between MS patients and healthy controls. The relatively low frequencies of $CD19^+$ B cells producing TNFα and the minimal upregulation of TNFα based on the MFI were also seen in response to LPS for both healthy controls and MS patients (**Figure 4-4C and D**). These results suggest that $CD19^+$ B cells most likely do not significantly contribute to the enhanced *ex vivo* TLR2 responses seen in the PBMCs of MS patients with active disease.

Lipid 342 as a potential blood biomarker for MS

Our laboratory has previously identified and characterized a unique bacterially derived lipodipeptide, Lipid 654 (L654), which is produced by the commensal bacteria *Bacteroidetes* that reside in the human GI tract (**Table-4-3**). We previously reported that low levels of L654 may be useful as a serum biomarker for MS diagnosis with a specificity ranging from 83 to 99% and an associated sensitivity ranging from 82 to 94%. However, in our ongoing effort to study the levels of 654 in our newly recruited patient blood samples, we have encountered a series of technical issues with a reduced sensitivity of L654 detection through the high performance liquid chromatography (HPLC) and the MRM-MS approach. This inevitably blunted the progress of our study involving L654.

Recently, we have made a significant breakthrough in identifying and characterizing another lipid named Lipid 342 (L342). L342 is a breakdown product of L654 with a molecular weight of 342 and contains a single acyl chain and a single glycine residue originating from L654 (**Figure 4-5)**. Similarly to L654, L342 was detectable in the plasma of all individuals tested. Importantly, there is a robust difference between the approaches in analyzing L342 and L654 in that L342 can be easily and accurately quantified through the use of Gas-Chromatography (GC) Mass Spectrometry (MS) (GC-MS) without the need for HPLC and MRM-MS approach. To measure the levels of L342 in the plasma in MS patients and healthy controls, plasma lipid fractions were first extracted from 500 µl of each plasma sample, and a known amount of an internal standard was added to each sample and subjected to the GC-MS. After the GC-MS steps, the plasma levels of L342 were quantified by calculating the area under the curve (AOC) for each patient. Most importantly and strikingly, we found that the levels of L342 detected in the plasma of MS patients were significantly lower than the levels in the plasma of healthy controls (Mann-Whitney test: *p = 0.0164) (**Figure 4-6**).

Despite the technical difficulties that we faced with reproducing our previous results with L654, we identified and characterized a bacterial breakdown product of Lipid 654, L342, which is also present in the plasma of all subjects examined, and most importantly, present at significantly lower levels in the plasma of MS patients compared to the levels of healthy controls.

Lipid 342 stimulates hIL-8 production from HEK-Blue™ hTLR2 cells

L654, a related lipid to L342, is both a human and a mouse TLR2 ligand. To determine whether L342 also acts as a human TLR2 ligand, we utilized the HEK293 cells transfected to overexpress

hTLR2 and hCD14 (HEK-Blue™ hTLR2 cells). Samples of bacterially derived L342 extracted from *Porphymonas gingivalis* were then used to stimulate **HEK-Blue™ hTLR2** cells *in vitro* for 24 hours, and the levels of hIL-8 in the supernatant were measured by ELISA. L342 stimulated substantial amounts of hIL-8 secretion by HEK-Blue™ hTLR2 cells at a concentration of 2.5 μg/ml (**Figure 4-7A**).

For all bacterially derived lipid samples, there is a chance for the potential contamination of these samples with other microbial TLR2 ligands that may also stimulate HEK-Blue™ hTLR2 cells. Therefore, in order to more accurately determine the property of L342 as a TLR2 ligand, we also tested the activity of a synthetic form of L342. Importantly, stimulation of HEK-Blue™ hTLR2 cells with the synthetic L342 led to a secretion of hIL-8 by these cells albeit at a lower level compared to the levels secreted in response to the *P.gingivalis*-derived L342 (**Figure 4-7B**). Overall, these results suggest that L342, a bacterially derived lipid that is a breakdown product of L654 is present at lower levels in the plasma of MS patients (**Figure 4-6**), is able to signal through TLR2.

DISCUSSION

During the last decade, the relationship between the human gut commensal microbiota and autoimmune disease has become a significant focus of research worldwide. However, very little is known about how the gut microbiome specifically interacts with the systemic immune system at the present time.

In studying two communities with genetic similarities, Stein *et al* documented the negative correlation between the prevalence of childhood asthma and the levels of endotoxins in house dust, from which they postulated that the exposures to endotoxins may predetermine the susceptibility to asthma (119). In MS, Jangi *et al* identified positive correlations between abundance levels of certain bacterial species in fecal samples and the transcriptional changes in the MS-associated genes, suggesting the potential interactions between the alterations in the gut commensal bacteria and epigenetic regulation of the immune responses (17). While these studies strongly implicate the role of human gut microbiota in regulating systemic immunity, neither of them identified bacterially derived factors that are present in the systemic circulation to directly interact with the immune system.

Previously, we have identified the unique bacterially derived lipodipeptide L654 produced by the commensal *Bacteroidetes* species in the human GI tract. L654 acts as a TLR2 ligand, and is expressed at significantly lower levels in the serum of patients with MS. Based on 1) the reports on the role of the human gut microbiome in regulating systemic autoimmunity, 2) the lower levels of L654 in MS patients, and 3) the therapeutic efficacy of TLR2 tolerance during EAE, we have proposed that TLR tolerance may be defective in MS patients, and that the defective TLR tolerance may result in TLR hyper-responsiveness in MS patients (**Figure 4-8**). Here, we document for the first time that the purified peripheral blood (PB) $CD14^+$ monocytes of a subset of MS patients demonstrate an increased TLR2 response compared to those of healthy controls. This enhancement in TLR2 response appears to correlate with disease activity as MS patients with inactive disease demonstrated a comparable or lower TLR2 response of PB $CD14^+$

monocytes as those of healthy controls. Additionally, we found comparable levels of TLR2 expression between the PB $CD14^+$ monocytes of MS patients and those of healthy controls.

Since the whole PBMC population is a better physiological representation of the systemic immunity of MS versus healthy control rather than the purified PB $CD14^+$ monocytes, we also examined the TLR2 response of the PBMCs. The enhanced TLR2 responses in MS patients with active disease was seen regardless of whether only the purified $CD14^+$ monocytes were used or the whole PBMCs were used. Importantly, the enhanced TLR2 response of MS patients with active disease was seen regardless of whether the ligand signals through TLR2/1 (P3C) or TLR2/6 (P2C). Furthermore, we found that the increased TLR2 response in a subset MS patients with active disease was due to an increased frequency of TNFα-producing $CD14^+$ monocytes and not due to the increased expression of TNFα on a per cell basis (TNFα MFI) as demonstrated by flow cytometry.

Chuluundorj *et al* previously documented that PB $CD14^+$ monocytes of MS patients have increased levels of expression for activation markers CD86, HLA-DR and CD64 (124), suggesting that these cells have an activated phenotype and may demonstrate enhanced capacity to prime T cells and provide co-stimulatory signals. It will be interesting to examine whether TLR2 stimulation results in enhanced capacity of $CD14^+$ monocytes of MS patients to prime T cells compared to those of healthy controls. Furthermore, the findings by Chuluundorj also suggest that $CD14^+$ monocytes of MS patients may produce increased baseline levels of TNFα compared to those of healthy controls due to their activated phenotype (124). However, we found

that this was not the case – the baseline TNFα production by $CD14^+$ monocytes of MS patients and healthy controls were both equally minimal and close to zero.

In addition to the "classical" $CD14^+$ $CD16^-$ monocytes that we have studied, "non-classical" $CD14^+$ $CD16^+$ monocytes have also been implicated in the pathogenesis of MS (123). Thus we also attempted to study the TLR2 response of PB $CD14^+$ $CD16^+$ monocytes. Chuluundorj *et al* documented decreased frequency of $CD14^+$ monocytes and the increased frequency of $CD16^+$ subset of $CD14^+$ monocytes in MS patients (124). However, we did not observe these differences between MS patients and healthy controls. Moreover, we were unable to examine the *ex vivo* TLR2 response of $CD14^+$ $CD16^+$ monocytes since we found that the expression of CD16 molecule became rapidly downregulated in $CD14^+$ monocytes upon TLR2 stimulation in the PBMCs. In the future, $CD14^+$ $CD16^+$ monocytes will be purified prior to the TLR2 stimulation for examining their responses *ex vivo*.

At present, we have observed an increased TLR2 response of $CD14^+$ monocytes from only in a subset of MS patients with active disease. However, Jangi *et al* documented robust transcriptional changes in the MS-associated immunological genes between MS patients and healthy controls through the nanostring technology in which the average value of EDSS of the MS cohort was relatively low (1.2). Although this type of analysis may be much more sensitive than the stimulation assays that we utilize in our study, it is also possible that if we further lower the doses of the TLR2 ligand P2C, we may find that not only the subset of MS patients with active disease but also those with inactive disease also demonstrate enhanced TLR2 responses. Nevertheless, it will be crucial to continue examining the potential correlation between disease

activity and the level of TLR2 response in MS patients. Since TLR2 has been shown to cross-tolerize to TLR4 (121), we also examined whether TLR4 responses differ between the PB $CD14^+$ monocytes or PBMCs of MS patients and healthy controls. We found that for the most part the two cohorts demonstrate comparable levels of TNFα production in response to LPS.

Lastly, since B cells have been implicated in the pathogenesis of MS as per the use of anti-CD20 (Rituximab) in MS, we examined the contribution of PB $CD19^+$ B cells in the *ex vivo* TLR2 response. Importantly, the levels of TNFα produced by $CD19^+$ B cells and the frequency of TNFα-producing $CD19^+$ B cells in response to TLR2 stimulation were much lower compared to those of PB $CD14^+$ monocytes in both MS patients and healthy controls and there was no significant differences noted between MS and controls patient $CD19^+$ responses. These results suggest that the contribution of B cells to the TLR2 response is minimal compared to that of $CD14^+$ monocytes in the PB.

Recently, we have identified and characterized a new breakdown product of L654 termed Lipid 342 (L342). Similarly to L654, L342 was detectable in the plasma of all individuals tested. Here, we report for the first time that, with striking similarities to our findings with L654, the levels of L342 in the plasma of MS patients are significantly lower than those in the plasma of healthy controls. Moreover, L342 is a TLR2 agonist as it stimulated hIL-8 production in HEK293 cells transfected with human TLR2 and CD14. In the future, we plan to stimulate human PBMCs with L342 and examine whether it triggers a cytokine response in a TLR2 dependent manner.

Currently, mechanisms responsible for the lower levels of L342 (and L654) in their systemic circulation of MS patients still remain unclear. Miyake *et al* previously documented the decreased abundance levels of commensal bacterial species belonging to *Clostridia* clusters XIVa and IV, as well as several *Bacteroidetes* species in fecal samples of MS patients compared to those of healthy individuals (18). In particular, there was a striking degree of reduction in *Prevotella copri* in MS patients compared to healthy controls. Since L654 is produced by many of the *Bacteroidetes* species including *Prevotella copri*, alterations in commensal gut bacteria composition may potentially be responsible for the differential levels of these products (18).

At this point in our studies, all MS patients appear to have lower levels of L342 regardless of disease activity. In future studies, we will increase the sample size of our MS and healthy control cohorts for our study of L342 to better determine if there is a critical correlation between disease activity and the levels of 342. Furthermore, it would be essential to perform a longitudinal study on the plasma levels of L342 over time in MS patients with the goal of finding potential correlations between the L342 levels and disease progression.

Housley *et al* documented that the numerous genetic variants associated with MS susceptibility result in enhanced activation of NF-κB pathway. This is possible that expression of such variants would result in greater responsiveness to inflammatory stimuli such as TLR ligands (125). However, as we have previously predicted for L654, we speculate that the levels of L342 in the systemic circulation may potentially correlate with the degree of TLR2 responsiveness of MS patients. Specifically, based on our postulate on the defective TLR tolerance in MS, we predict that there will be a negative correlation between the levels of L342 and the degree of

TLR2 responses. As illustrated by Jangi *et al* and their findings of the correlation between the altered gut bacterial compositions and immunological changes, whether the microbial changes drive immunological changes or vice versa still remains to be determined. In our future studies, we will also determine whether there is a critical correlation between the plasma levels of L342 and the *ex-vivo* TLR2 responses.

In sum, we now report that there is an increased TLR2 responsiveness of peripheral blood $CD14^+$ monocytes in a subset of MS patients, and this may correlate with disease activity. Furthermore, we identified and characterized Lipid 342, a unique bacterially derived product derived from the human GI that acts as a human TLR2 ligand. Importantly, we document for the first time that the patients with MS express significantly lower levels of Lipid 342 in the plasma compared to healthy individuals. These results suggest a novel mechanism of disease pathogenesis in MS that connects the human gut microbiota to human systemic autoimmunity, and indicate a potential use of a bacterially derived factor as a blood biomarker for MS.

Age	Gender	Ethnicity
53	M	Caucasian
44	F	Caucasian
57	F	Caucasian
37	M	Caucasian
28	F	Hispanic
62	F	Caucasian
66	F	Caucasian

Table 4-1: Study subjects: Healthy individuals

Age	Gender	Ethnicity	Duration	MS	Last relapse	Treatment
53	M	Caucasian	8 yrs	RR	3 yrs ago	Copaxone
39	F	Caucasian	12 yrs	RR	5 yrs ago	n/a
59	F	Caucasian	9 yrs	RR	5 yrs ago	Tysabri
60	F	Caucasian	7 yrs	PP	--	Aubagio
41	F	Caucasian	11 yrs	RR	--	Tysabri
68	F	Caucasian	8 yrs	PP	--	n/a
37	M	Caucasian	13 yrs	RR	2 yrs ago	Rituximab
65	F	Caucasian	20 yrs	PP	--	Avonex

Table 4-2: Study subjects: Multiple sclerosis Patients

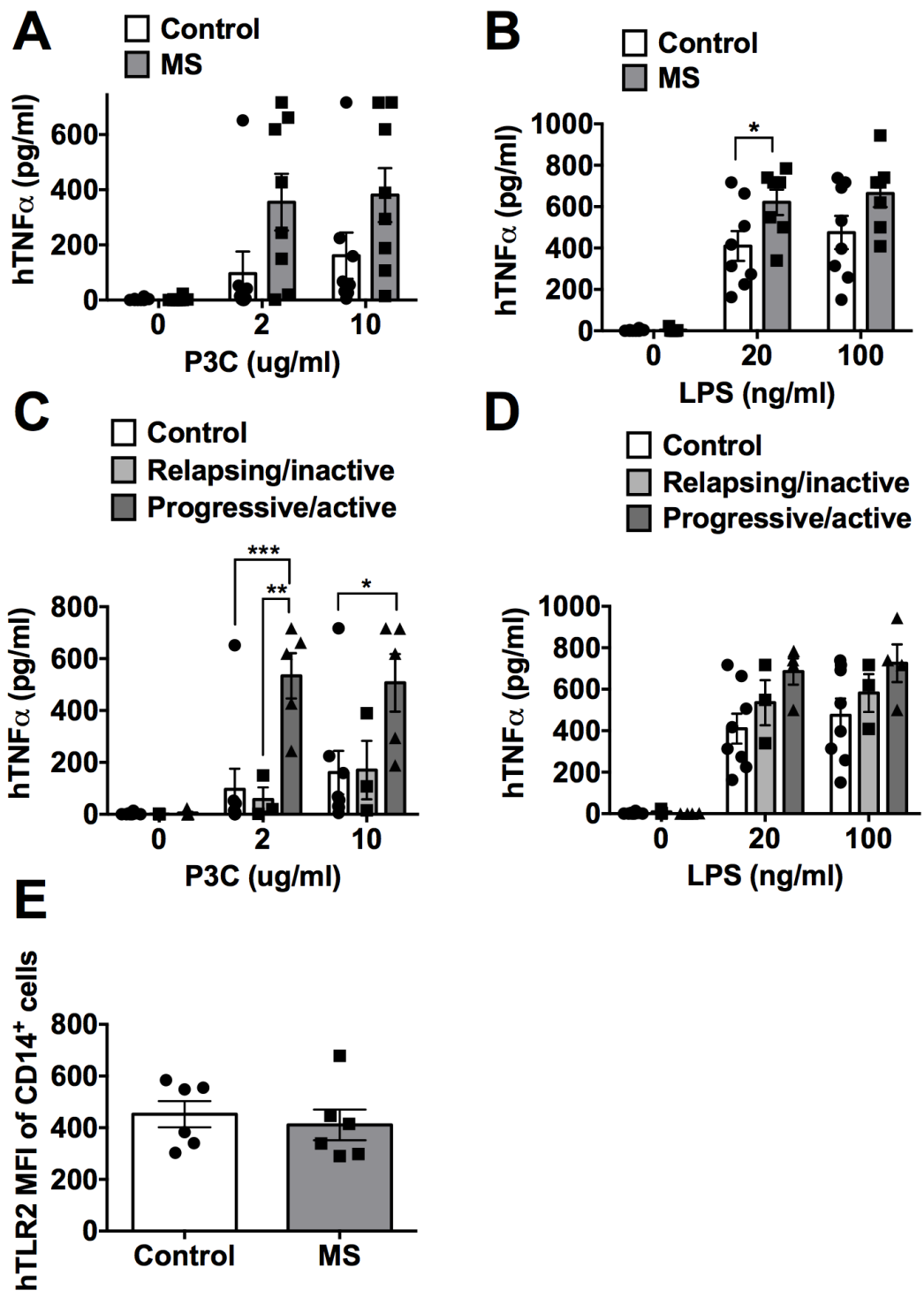


Figure 4-1. The *ex vivo* TLR2 responses of the PB CD14$^+$ cells isolated from healthy controls and MS patients. Peripheral blood CD14$^+$ monocytes were isolated from blood obtained from patients with MS or healthy controls. 2 x 10^4 cells/well of CD14$^+$ cells were left

unstimulated, or stimulated with P3C (A) or LPS (B) at varying doses and the levels of secreted TNFα in the supernatant were measured by ELISA. (C and D) The data shown in A for P3C response (C) and B for LPS responses (D) are represented for two categories of MS patients based on their clinical history: Relapsing/inactive (RR-MS and having currently inactive disease) or Progressive/active (progressive and having currently active disease). Four independent experiments combined, n = 3-6 per group. One-way ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$. (E) hTLR2 expression on $CD14^+$ cells isolated from healthy controls and MS patients was measured by flow cytometry. Mean fluorescence intensity (MFI) of hTLR2 staining is shown.

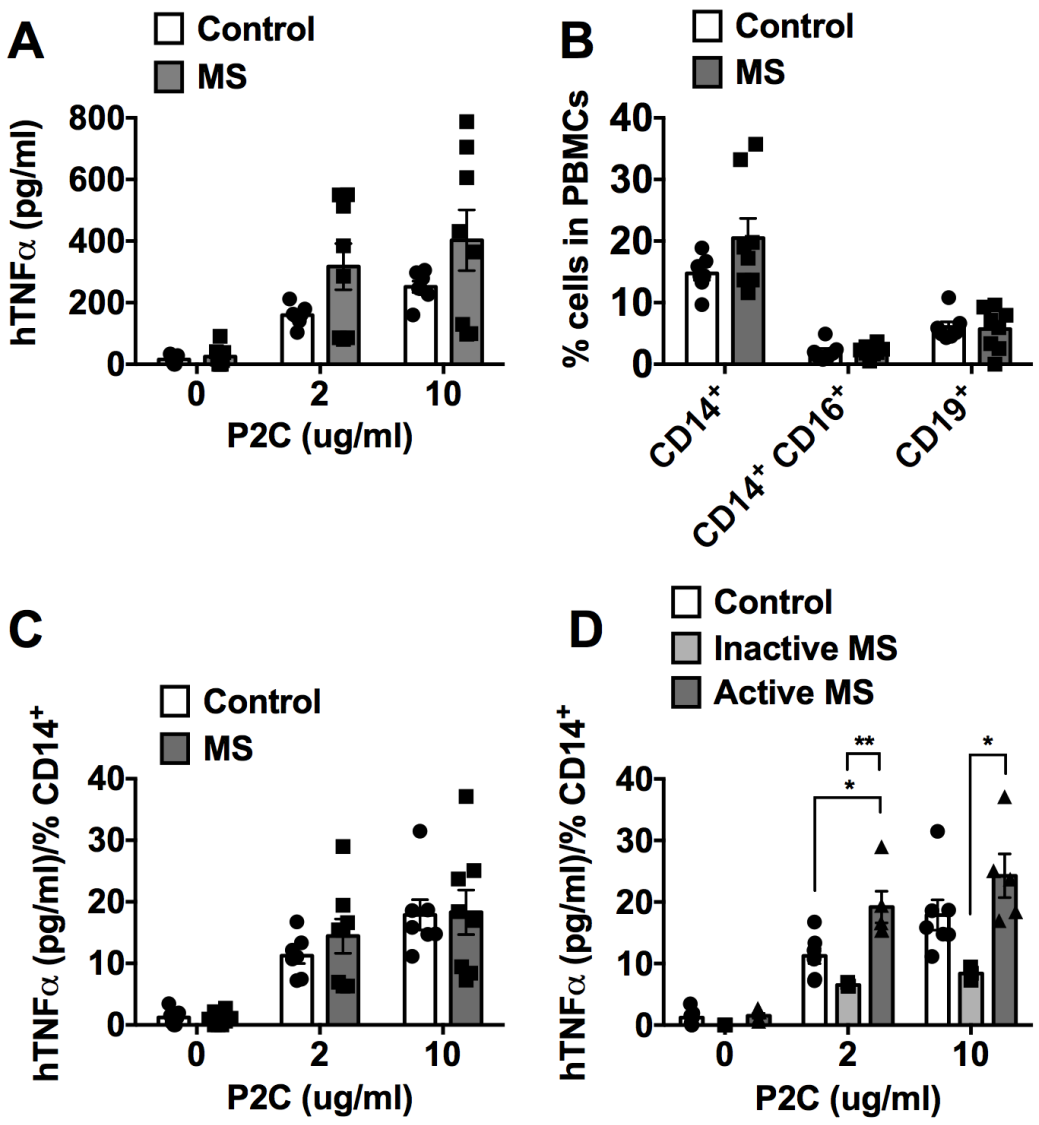


Figure 4-2. The *ex vivo* TLR2 responses of the PBMCs of MS patients vs. healthy controls. PBMCs were isolated from blood obtained from patients with MS or healthy controls. (A) 2 x 10^5 cells/well of PBMCs were left unstimulated, or stimulated with P2C at varying doses and the levels of secreted TNFα in the supernatant were measured by ELISA. (B) The frequencies of $CD14^+$ cells, $CD14^+$ $CD16^+$ cells and $CD19^+$ cells present in the PBMCs of each patient were determined by flow cytometry. (C) TNFα levels of the PBMCs in response to P2C stimulation shown in A are normalized to % $CD14^+$ cells of each patient of healthy control or MS cohorts.

For this, the TNFα level (pg/ml) of each patient was divided by the value of % $CD14^+$ cells in the PBMCs of the same patient. (D) The data shown in (C) are represented for two categories of MS patients: Inactive MS (RR-MS or PP-MS with long-standing inactive disease) or Active MS (RR-MS or PP-MS with currently active disease). Seven independent experiments combined; n = 7-8 per group. One-way ANOVA: *$p<0.05$, **$p<0.01$.

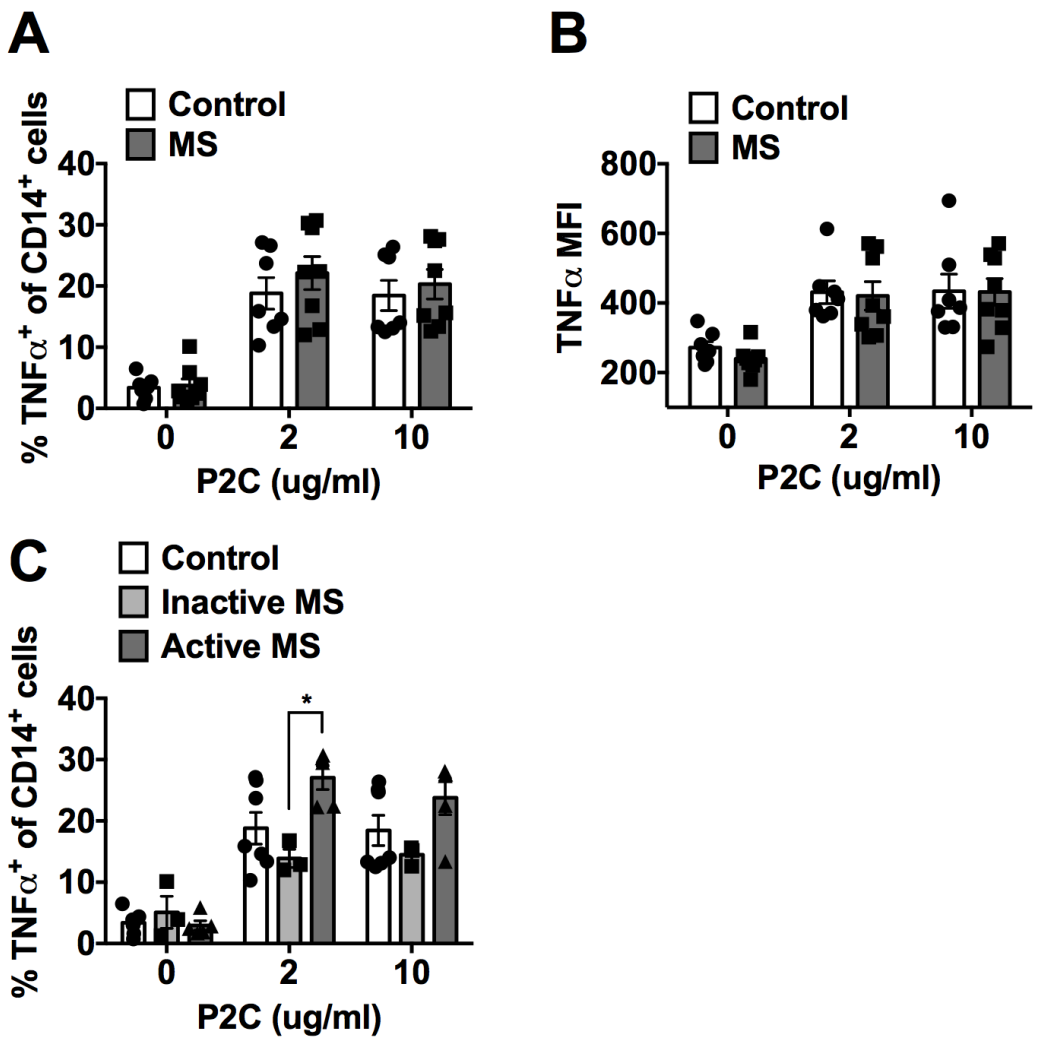


Figure 4-3. The *ex vivo* TLR2 responses of the peripheral blood CD14$^+$ cells in MS patients vs. healthy controls by flow cytometry. PBMCs were isolated from blood obtained from patients with MS or healthy controls. (A) 2 x 10^5 cells/well of the PBMCs were left unstimulated, or stimulated with P2C at varying doses and frequencies of TNFα$^+$ cells in CD14$^+$ cells (gated on live, single, non-autofluorescent cells prior to the CD14 gating) were determined by surface staining and intracellular cytokine staining for flow cytometry. (B) MFI of TNFα staining is shown for the data shown in A. (C) The data shown in (A) are represented for two

categories of MS patients: Inactive MS or Active MS as described above in A. Seven independent experiments combined; n = 7-8 per group (A and B); n = 3-8 per group (C). One-way ANOVA: *$p<0.05$, **$p<0.01$.

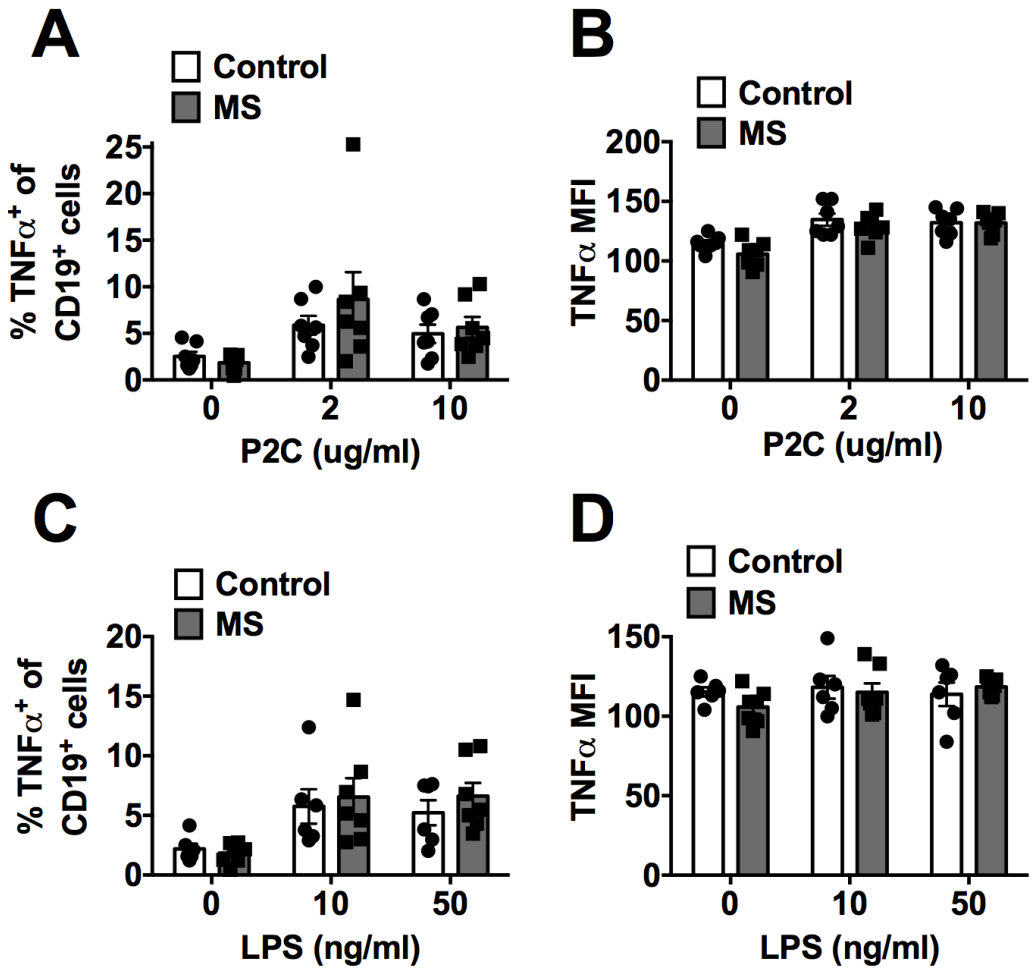
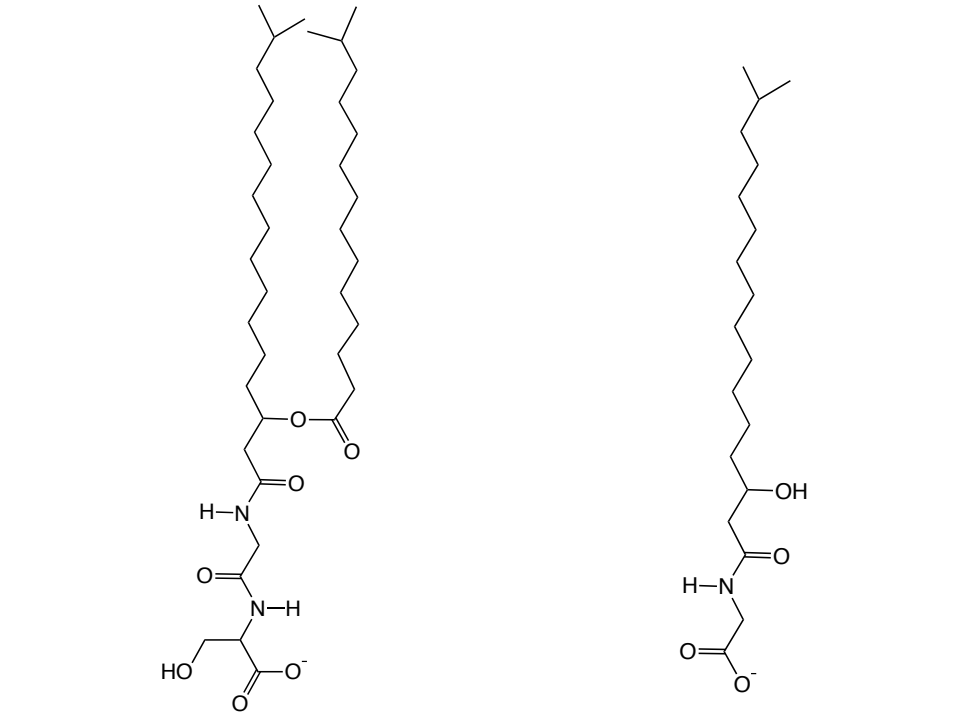


Figure 4-4: The *ex vivo* TLR2 responses of the peripheral blood CD19$^+$ B cells of healthy controls and MS patients. PBMCs were isolated from blood obtained from patients with MS or healthy controls. (A and C) 2 x 10^5 cells/well of the PBMCs were left unstimulated, or stimulated with P2C (A) or LPS (C) at varying doses and frequencies of TNFα$^+$ cells in CD19$^+$ cells (gated on live, single, non-autofluorescent cells prior to the CD19 gating) were determined by surface staining and intracellular cytokine staining for flow cytometry. (B and D) MFI of TNFα staining is shown for the data shown in A for P2C (B) and for the data shown in B for LPS (D). Seven independent experiments combined; n = 7-8 per group.



Chemical Formula: $C_{37}H_{69}N_2O_7^-$
Exact Mass: 653.51
Molecular Weight: 653.97
m/z: 653.51 (100.0%), 654.51 (40.0%), 655.52 (7.8%), 655.52 (1.4%)
Elemental Analysis: C, 67.96; H, 10.64; N, 4.28; O, 17.13

Chemical Formula: $C_{19}H_{36}NO_4^-$
Exact Mass: 342.26
Molecular Weight: 342.50
m/z: 342.26 (100.0%), 343.27 (20.5%), 344.27 (2.0%)
Elemental Analysis: C, 66.63; H, 10.60; N, 4.09; O, 18.68

Figure 4-5. Structures of Lipid 654 and Lipid 342. The left structure represents Lipid 654, with the chemical formula of $C_{37}H_{69}N_2O_7^-$ with the exact mass of 653.51. The right structure represents the Lipid 342, with the chemical formula of $C_{19}H_{36}NO_4^-$, with the exact mass of 342.26.

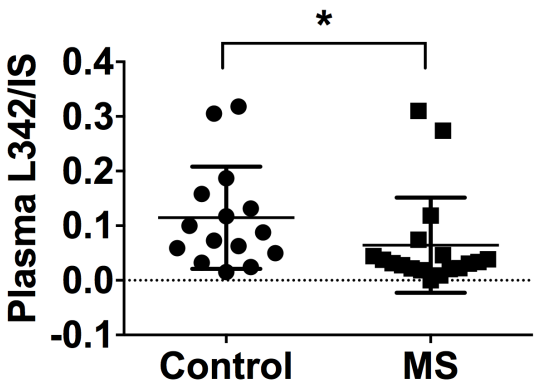


Figure 4-6. Plasma levels of L342 normalized to an internal standard.

Plasma was obtained from MS patients and healthy individuals (**Tables 4-1 and 4-2**) and lipids from the plasma were extracted and analyzed by the GC-MS for expression of L342. As an additional control for GC-MS efficiency, a defined quantity of an internal standard was added to each sample. MS patients: n = 18; healthy control: n = 15. Means: healthy controls = 0.1147; MS patients = 0.06440. Mann-Whitney test: *p = 0.0164.

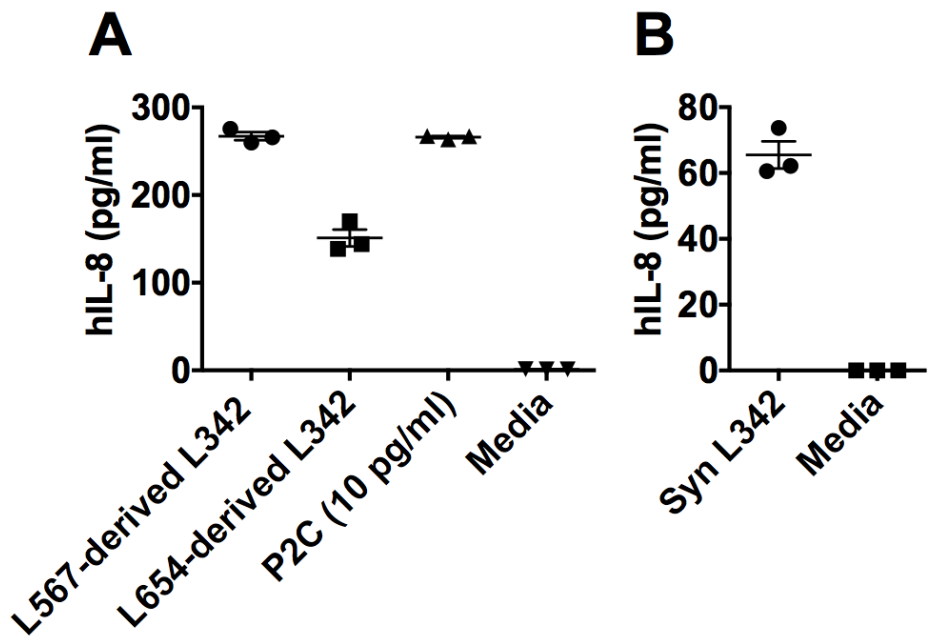


Figure 4-7. Both *P. gingvalis*-derived L342 and the synthetic form of L342 stimulate hIL-8 production from HEK-Blue™ hTLR2 cells *in vitro*.

2.5 x 10^4 cells/well of HEK-Blue™ hTLR2 cells were left unstimulated or stimulated with 2.5 µg/ml of two different *P.gingivalis*-derived L342 samples and the synthetic form of L342, or 10 pg/ml of P2C as a positive control for 24 hrs then the hIL-8 ELISA was performed to measure the levels of hIL-8 in the supernatant. All the lipid samples were sonicated in serum free complete HL-1 media prior to their in the assay. (A) The levels of hIL-8 produced by HEK-Blue™ hTLR2 cells that were stimulated by *P. gingivalis*-derived L342 samples (L567-derived or L654-derived) or by P2C as a positive control, or left unstimulated (media). (B) The levels of hIL-8 produced by HEK-Blue™ hTLR2 cells that were stimulated by the synthetic form of L342 or left unstimulated (media).

Oral Bacteroidetes	**Gastrointestinal Bacteroidetes**
Porphyromonas gingivalis	Prevotella copri
Porphyromonas endodontalis	Parabacteroides merdea
Prevotella intermedia	Parabacteroides distasonis
Tannerella forsythia	Bacteroides fragilis
Capnocytophaga ochracea	Bacteroides vulgatus
Capnocytophaga gingivalis	Bacteroides stercoris
Capnocytophaga sputigena	Bacteroides uniformis

Table 4-3: Commensal oral and gastrointestinal species of *Bacteroidetes* produce L654. Bacteria were grown, lyophilized, and extracted for total lipids as described in (53), and the expression of L654 was detected by the MRM-MS. The types of the commensal *Bacteroidetes* species from which L654 was detected from their lipid extracts are shown. Since L342 is a breakdown product of L654, L342 is derived from the same Bacteroidetes species listed above.

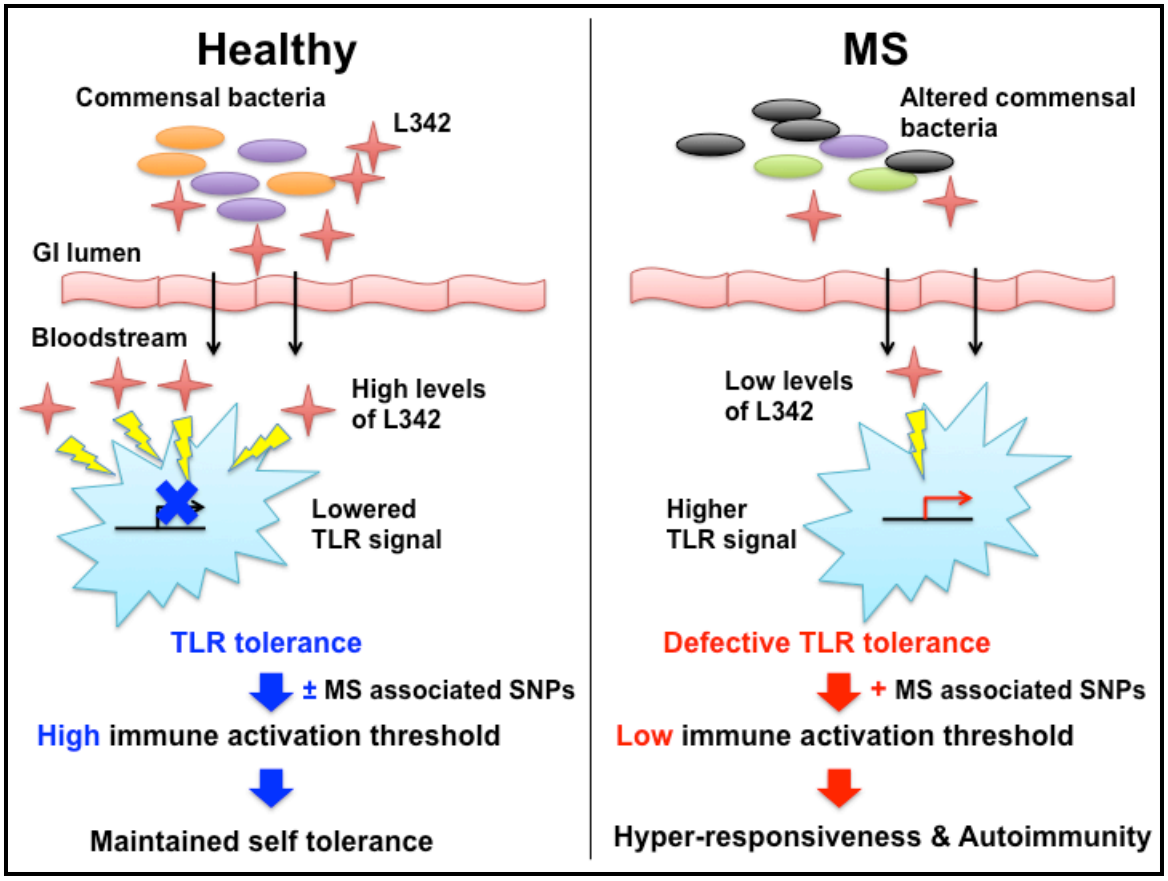


Figure 4-8: The hygiene hypothesis and the potential relevance of lower levels of L342 in MS. The diagram demonstrates our hygiene hypothesis in MS that we propose based on 1) the altered gut microbiota composition; 2) the lower levels of L342 (or L654) in the systemic circulation of MS patients; 3) potentially defective TLR tolerance in MS patients due to the "insufficient" levels of the tolerance-inducing bacterially derived products; 4) Higher threshold of immune activation in MS patients, which may be accompanied by MS-associated SNPs that may result in immune hyper-reactivity and contribute to MS susceptibility.

CHAPTER 5 Future Directions and Synthesis

Approaches toward personalized medicine in MS

In Chapter 2, we have put forth an idea to utilize Cbl-b$^{-/-}$ mice as a murine model to analyze therapeutic approaches in MS as a step toward personalized medicine. Although the concept of personalized medicine in MS has received significant interest in recent years, it is far from being implemented in clinical settings. The goal would be to identify parameters that would allow stratification of patients to potentially predict therapeutic outcomes of a patient to a certain drug. These parameters include genetic profiles as in GWAS, transcriptional or epigenetic profiles of MS-associated immunological genes as in RNASeq, the status of their immune reactivity as in TLR2 responsiveness, and the plasma levels of L342. The identification of any negative or positive correlation between these parameters and disease activity or therapeutic responses would aid in future implementation of a personalized medicine approach in MS.

Therapeutic targeting of Cbl-b in human cancer immunotherapy

The mechanism underlying resistance to PD-L1/PD-1 in Cbl-b$^{-/-}$ T and NK cells still remain unclear. Elucidation of this mechanism would provide further insights into molecular mechanisms of PD-L1/PD-1 mediated inhibition of T cells and NK cells. Additionally, resistance to PD-L1/PD-1 may contribute to disease pathogenesis in MS patients with potentially aberrant Cbl-b function. It would be important to examine whether any of the MS-associated *CBLB* variants is involved in mediating resistance to PD-L1/PD-1.

Now with our findings of resistance to PD-L1/PD-1 in murine Cbl-b$^{-/-}$ T cells, Cbl-b deficiency in mice results in resistance of T cells to: 1) CD4^{+} CD25^{+} Foxp3^{+} Tregs, 2) TGF-β, 3) CTLA-4 and 4) PD-L1/PD-1. If these findings in murine Cbl-b$^{-/-}$ T cells are confirmed in the human *CBLB*-silenced T cells, this would suggest that the therapeutic approach involving *CBLB* silencing or blockade of Cbl-b function would allow us to abrogate multiple immunosuppressive mechanisms or immune checkpoints altogether to maximally enhance anti-tumor responses in cancer patients. In sum, our findings in Chapter 3 add to the well-established role of Cbl-b in regulating the delicate balance between immunity and immunoregulation in the context of autoimmunity and anti-tumor immunity (**Figure 5-1**).

TLR responses in the peripheral blood CD14^{+} monocytes of MS patients

In our currently ongoing study discussed in Chapter 4, we only found significantly increased TLR2 responses in a subset of MS patients with active disease. However, the robust transcriptional changes in the MS-associated immunological genes were documented in MS patients of whom most of them demonstrated stable disease (mean EDSS of 1.2) (17). This may be due to a kinetic issue with the dosage of TLR2 stimuli used in our study, in which we may need to further decrease the doses of TLR2 ligands. With lower doses of the TLR2 stimuli, it is possible that MS patients with stable disease may also demonstrate enhanced TLR2 responsiveness compared to healthy individuals.

One of the essential objectives in our current study is to address the following question – Do MS-associated alterations in the human gut microbiome (or lower levels of L342 in MS) drive the changes in immunological gene expression documented in MS patients (or increased

TLR2 responsiveness), or does the disease (or the altered immunological status) state lead to changes in the gut microbiome (and lower levels of L342 in MS)? Based on our postulate that insufficient levels of tolerance-inducing PAMPs in MS patients result in defective TLR tolerance and TLR hyper-responsiveness, we speculate that the altered microbiota (lower levels of L342) induce the changes in immunological genes (TLR2 responsiveness) documented in MS patients. It is noteworthy that Jangi *et al* identified a positive or negative correlation between changes in the gut microbiome composition and transcriptional changes of immunological genes only within MS patients, and not in healthy controls (17). This MS-specific correlation between altered gut microbiome and transcriptional changes in immunological genes suggest that perhaps both microbiome alterations (levels of L342) and MS-associated genetic factors may both be actively involved in induction of MS pathogenesis (**Figure 5-2**).

There are currently over hundred variants in the immunological genes associated with MS (11-13). Additionally, the hyper-responsive status of T cells and monocytes of MS patients have been previously documented (125). Thus, it is possible that enhanced TLR2 responsiveness that we documented in a subset of MS patients may stem from MS-specific genetic alterations. Alternatively, the most widely accepted view in describing the etiology of MS is that MS is a manifestation of complex interactions between the environment and the genetic factors (**Figure 5-2**). Our goal is to increase the sample size of our MS and healthy control cohorts for both the plasma levels of L342 as well as the *ex-vivo* TLR2 responses so that we can begin to examine whether there is a critical correlation between these two parameters.

Testing the status of TLR tolerance in MS patients

It will be important to test the status of TLR tolerance in the peripheral blood (PB) $CD14^+$ monocytes of MS patients compared to healthy controls. It is possible that PB $CD14^+$ monocytes from MS patients and those from healthy controls may demonstrate comparable degrees of TLR2 tolerance, given the "sufficient" levels of tolerizing ligands being artificially introduced into the *in vitro* environment. This would suggest that it may be possible to therapeutically induce TLR tolerance in MS patients with the goal of increasing the homeostatic threshold for immune activation in these patients.

Numerous MS-associated variants have been documented to result in altered immune responses of MS patients (125). It is possible that some of these variants may alter the capacity of $CD14^+$ monocytes to undergo TLR2 tolerance *ex vivo*. In this case, we may find that PB $CD14^+$ monocytes from MS patients possibly carrying these SNPs demonstrate defective TLR2 tolerance *ex vivo*. In contrast, with the heightened degree of TLR2 responsiveness that we have observed in a subset of MS patients that we examined, it is possible that PB $CD14^+$ monocytes from these specific patients may undergo TLR2 tolerance *ex vivo* more efficiently than those from healthy controls or other MS patients with no changes in TLR2 responsiveness. In sum, examining the status of TLR2 tolerance in the peripheral blood $CD14^+$ monocytes of MS patients and in those of healthy individuals is a critical aspect of our future study.

The activity of Lipid 342 as a TLR2 ligand

In the future, we plan to confirm the TLR2 dependent nature of hIL-8 production by HEK-Blue™ hTLR2 cells by adding anti-hTLR2 blocking antibody along with L342 to the culture during the stimulation period. Furthermore, we also plan to stimulate either peripheral blood $CD14^+$ monocytes or PBMCs isolated from the whole blood of healthy controls with L342 *in vitro*, and examine whether L342 is also capable of stimulating hTNFα production from these cells.

The therapeutic efficacy of chronic administration of Lipid 342 in EAE

As it has been demonstrated for L654 (120), it would be crucial to test the therapeutic efficacy of L342 during EAE. Prior to this, the activity of L342 as a murine TLR2 ligand needs to be confirmed *in vitro* and *in vivo*. If L342 is identified to act as a murine TLR2 ligand, then the next step would be to identify the optimal doses for L342 in induction of *in vivo* TLR2 tolerance (120). Upon identifying the optimal doses of L342 for TLR2 tolerance *in vivo*, then the next step would be to test the therapeutic efficacy of this treatment regimen with L342 during EAE as it has been demonstrated for L654 (120).

TLR tolerance in other types of autoimmune disease

It will be important to examine the levels of L342 in patients with other autoimmune disease, such as RA, T1D, and SLE, to determine whether the lower levels of microbiome-derived products present in the systemic circulation such as L342 are universal across multiple types of autoimmune disease. If we find that L342 levels are also decreased in the plasma of patients with other autoimmune disease, then it would be important to test the status of TLR responses and

TLR tolerance in these patients to determine whether aberrant TLR responsiveness and/or defective TLR tolerance contribute to the disease pathogenesis of other systemic autoimmune disease.

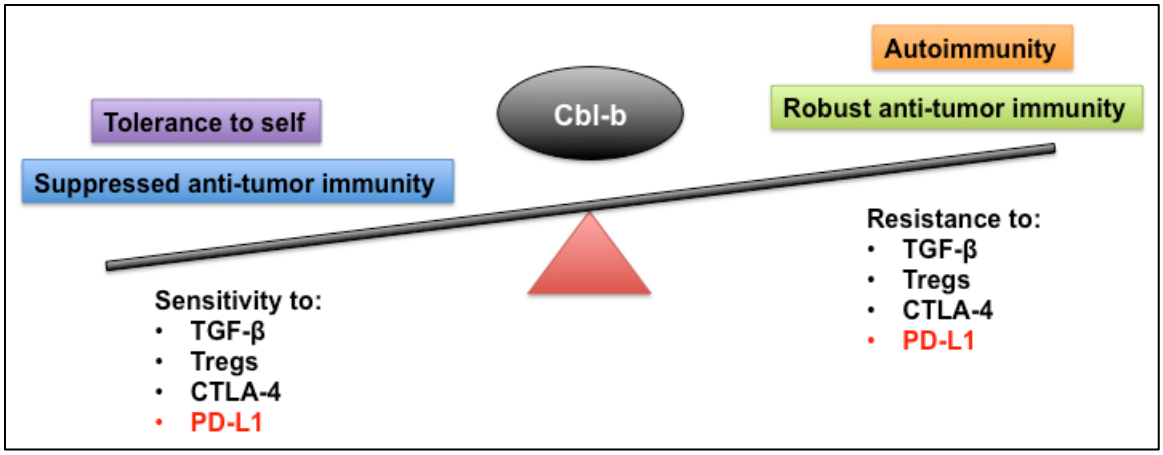


Figure 5-1. Mechanisms of immunoregulation mediated by Cbl-b.

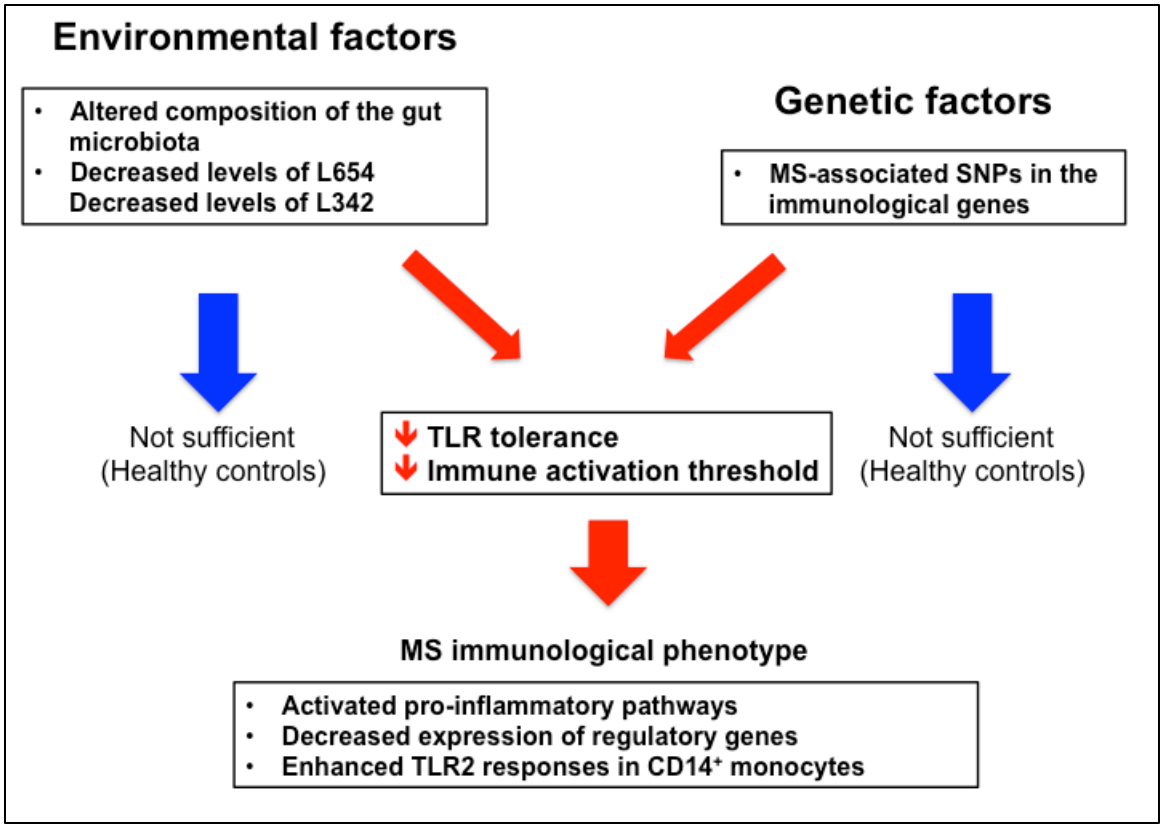


Figure 5-2. The proposed mechanism on the interaction between the human gut microbiome and MS-associated genetic alterations that leads to MS pathogenesis.

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