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

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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.

Keywords

Multiple sclerosis; Casitas-B lineage lymphoma-b; Experimental autoimmune encephalomyelitis; sphingosine-1-phosphate receptor 1; FTY720; T cell trafficking

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1. 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 [1–3]. The *CBLB* gene encodes for Cbl-b (Casitas B-lineage lymphoma-b), an E3 ubiquitin ligase that negatively regulates T cell activation [4–6]. Cbl-b has been shown to be relevant in regulating T cell responses in models of human disease such as allergic airway inflammation [7] and Cbl-b has been shown to be essential for TGF-β receptor signaling through direct inhibition of SMAD7 [8]. Importantly, Cbl-b deficiency in mice (Cbl-b$^{-/-}$ mice) leads to multi-organ cellular infiltration associated with T cell hyper-reactivity [4], co-stimulation independence in T cell activation [5], and T cell resistance to regulatory T cell (Treg)-mediated suppression [9, 10]. These abnormalities in Cbl-b$^{-/-}$ mice have also been documented in MS patients [11–15]. Consistent with this, Cbl-b$^{-/-}$ mice have been described to show increased susceptibility to experimental autoimmune encephalomyelitis (EAE), the murine model of MS [5, 16]. 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 [17]. 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 [17]. 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 $S1P_5$ [18]. Though still controversial, FTY720 theoretically mediates its therapeutic effect in MS by causing degradation of the lymphocyte homing receptor $S1P_1$ [19]. 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 [20]. As with all the

treatment options in MS, FTY720 is effective only in a proportion of patients with RRMS [21], 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 [22–25], 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.

2. Material and methods

2.1. 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).

2.2. 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. 0.9–1.4×10^6 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.

2.3. 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).

2.4. 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 peripheral blood leukocytes stained with anti-CD4-APC and anti-CD8α-FITC antibodies (Biolegend) and samples analyzed by flow cytometry.

2.5. 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 peripheral blood leukocytes stained with anti-CD4-APC and anti-CD8α-FITC antibodies (Biolegend), and samples analyzed by flow cytometry.

2.6. 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.

2.7. 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.

3. Results

3.1. 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 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 1B, 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 (Supplemental Figure 1). 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 1B and C). 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 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.

3.2. 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 [26, 27], 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 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 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 (Supplemental Figure 2A). 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 2B). MFI analysis also showed no difference in $S1P_1$ expression between WT and Cbl-b$^{-/-}$ CD4$^+$ and CD8$^+$ T cells in the mLNs (Supplemental Figure 2B). 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 (Supplemental Figure 3). 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.

3.3. 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 [28, 29]. 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 3A). MFI analysis revealed no statistical difference in CD69 expression levels between WT and Cbl-b$^{-/-}$ CD4$^+$ T cells (Supplemental Figure 4A), 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 3B). The frequency of CD69$^+$ Cbl-b$^{-/-}$ CD8$^+$ T cells in the mLNs was again comparable to that of WT CD8$^+$ T cells (Figure 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 (Supplemental Figure 4B). 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 (Supplemental Figure 5). 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 [30, 31]. 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.

3.4. 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) [32]. 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 [32]. 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 = 3.53×10^5/ml; Cbl-b$^{-/-}$ CD4$^+$ T cells = 5.23×10^5/ml; WT CD8$^+$ T cells = 1.98×10^5/ml; Cbl-b$^{-/-}$ CD8$^+$ T cells = 3.00×10^5/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 (Fig. 5*A* and *B*). 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 5A and B). 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 noninflammatory, naïve condition.

3.5. 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 [33]. SEW2871 directly binds to $S1P_1$ on T cells, resulting in $S1P_1$ internalization and subsequent entrapment of T cells in LNs [33]. 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 6A and B). 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.

3.6. 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 [23, 25, 34]. 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 [5, 16]. 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 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 7). These results suggest that FTY720 can inhibit EAE in the context of both Cbl-b deficiency and hyperactive T cell responses.

4. 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 [1–3]. 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 [35]. Interestingly, decreased Cbl-b mRNA and decreased Cbl-b protein expression in PBMCs of patients with RRMS have also previously been described [36]. 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 [4, 5], co-stimulation-independent [5], have a lower activation threshold [4, 5], and to be resistant to Tregs [9, 10], TGF-β [37], and the induction of anergy [38]. Interestingly, many of these abnormalities have also been documented in MS patients [11–15]. In addition, Cbl-b$^{-/-}$ mice have been shown to have increased susceptibility to EAE [5, 16].

Sturner *et al*. recently studied T cell functional abnormalities associated with the *CBLB* SNP – MS-risk allele, rs12487066 [17]. 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 [17]. 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$_5$ [18]. Though controversial, FTY720 theoretically mediates its therapeutic effect in MS by causing the degradation of the lymphocyte homing receptor S1P$_1$ [19], 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 [20]. As with all present treatment choices for MS, FTY720 is not effective in all of RRMS patients [21]. Importantly, pre-clinical studies demonstrating the efficacy of this drug were carried out in C57BL/6 mice [22–25], 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 [39] and thus affects many targets downstream of this pathway including the S1P$_1$-regulating transcription factor KLF2 [40, 41]. 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 [42, 43]. Cbl-b normally negatively regulates the PI3K-Akt pathway [39, 44, 45] and the deletion of Cbl-b is known to enhance the activation of this pathway [44, 45]. This enhanced activation of the PI3K-Akt pathway in Cbl-b$^{-/-}$ mice leads to increased inactivation of Foxo1 [44], 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 $S1P_1$ [28], 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 [46]. 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 [47]. 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.* [17], 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

CBLB/**Cbl-b**	Casitas-B lineage lymphoma-b
Cbl-b$^{-/-}$	Cbl-b-deficient
RRMS	relapsing remitting MS
S1P	sphingosine-1-phosphate
S1P$_1$	sphingosine-1-phosphate receptor 1
THI	2-acetyl-4-tetrahydroxybutyl imidazole
SEW2871	5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole

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Highlights

- Polymorphisms in the *CBLB* gene have been associated with MS

- Cbl-b$^{-/-}$ T cell lymph node accumulation is abnormal after transfer into RAG-1$^{-/-}$ mice

- Activated Cbl-b$^{-/-}$ T cells express enhanced S1P$_1$ and decreased CD69

- Despite trafficking abnormalities the MS-approved drug FTY720 significantly inhibits EAE in Cbl-b$^{-/-}$ mice

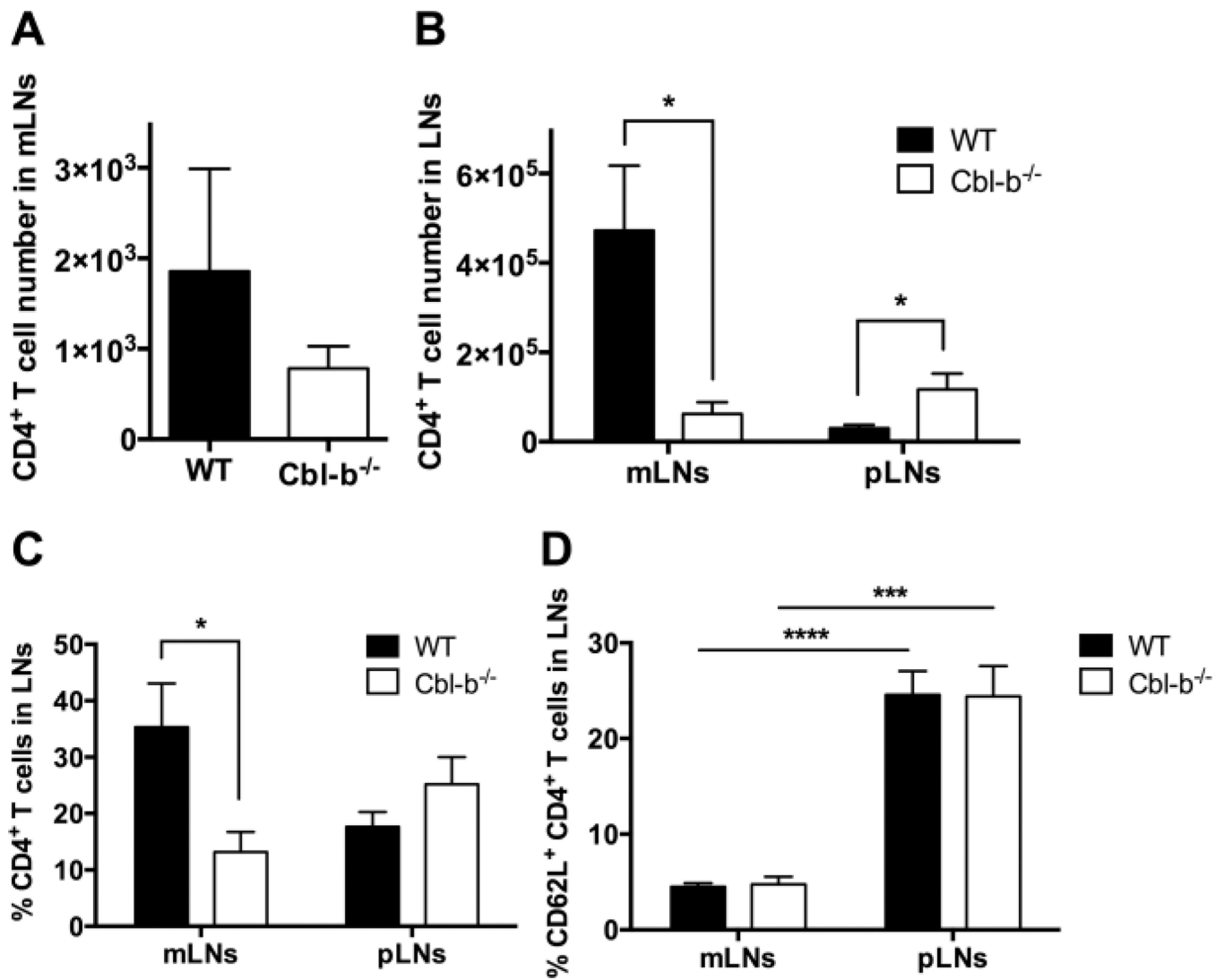- Cbl-b$^{-/-}$ mice may represent a new model for testing therapeutic approaches in MS

Figure 1.

Decreased accumulation of Cbl-b$^{-/-}$ CD4$^+$ T cells in mLNs of RAG-1$^{-/-}$ mice after adoptive transfer. 0.9–1.4 × 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.
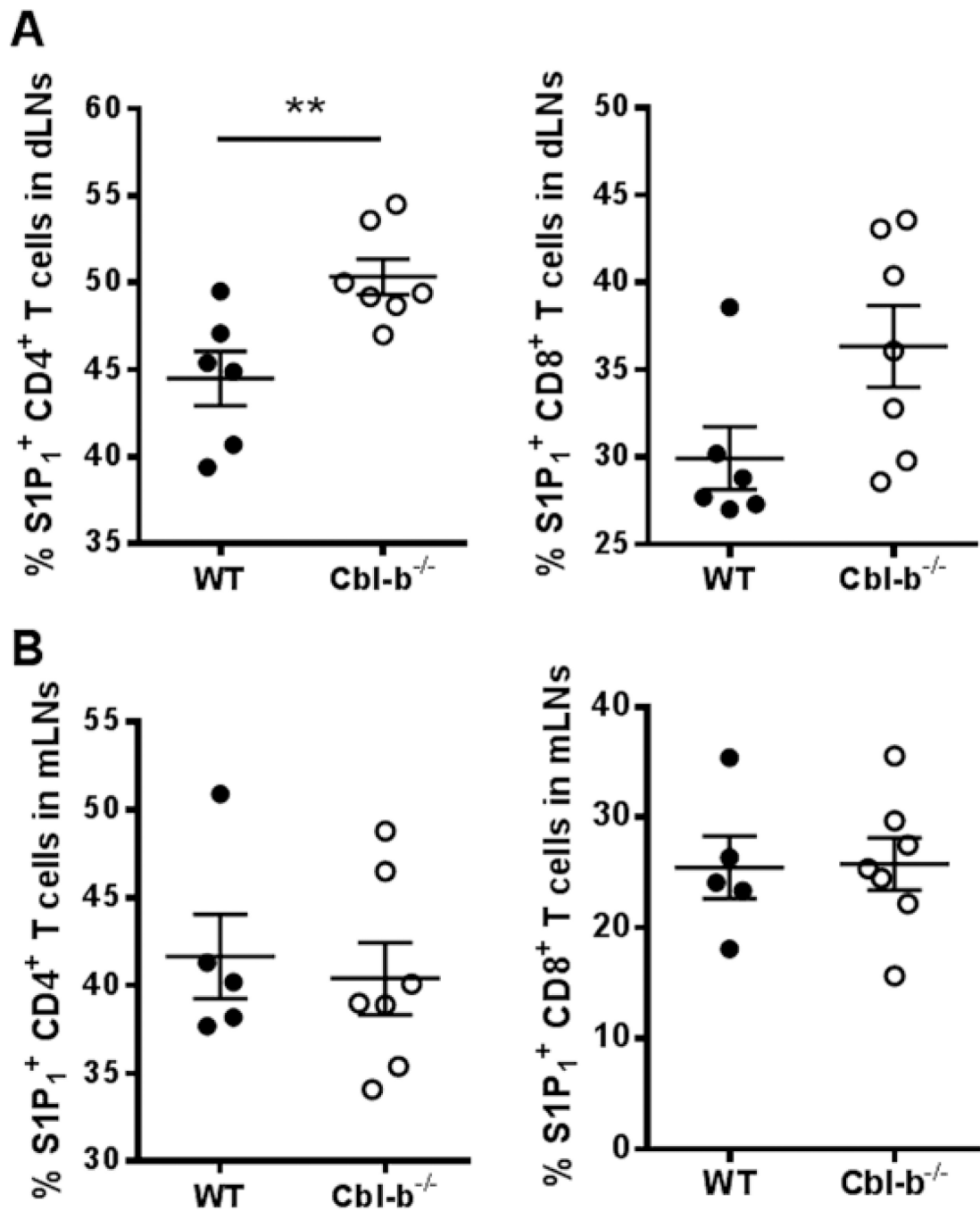
Figure 2.
Increased frequency of $S1P_1^+$ $CD4^+$ T cells in draining LNs of Cbl-b$^{-/-}$ mice after CFA/MOG$_{35-55}$ immunization. WT and Cbl-b$^{-/-}$ mice were immunized s.c. in the footpads with 150 μg of CFA and 80 μg of MOG$_{35-55}$ and the popliteal LNs were harvested 4–5 days later. (A) Percentage 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.
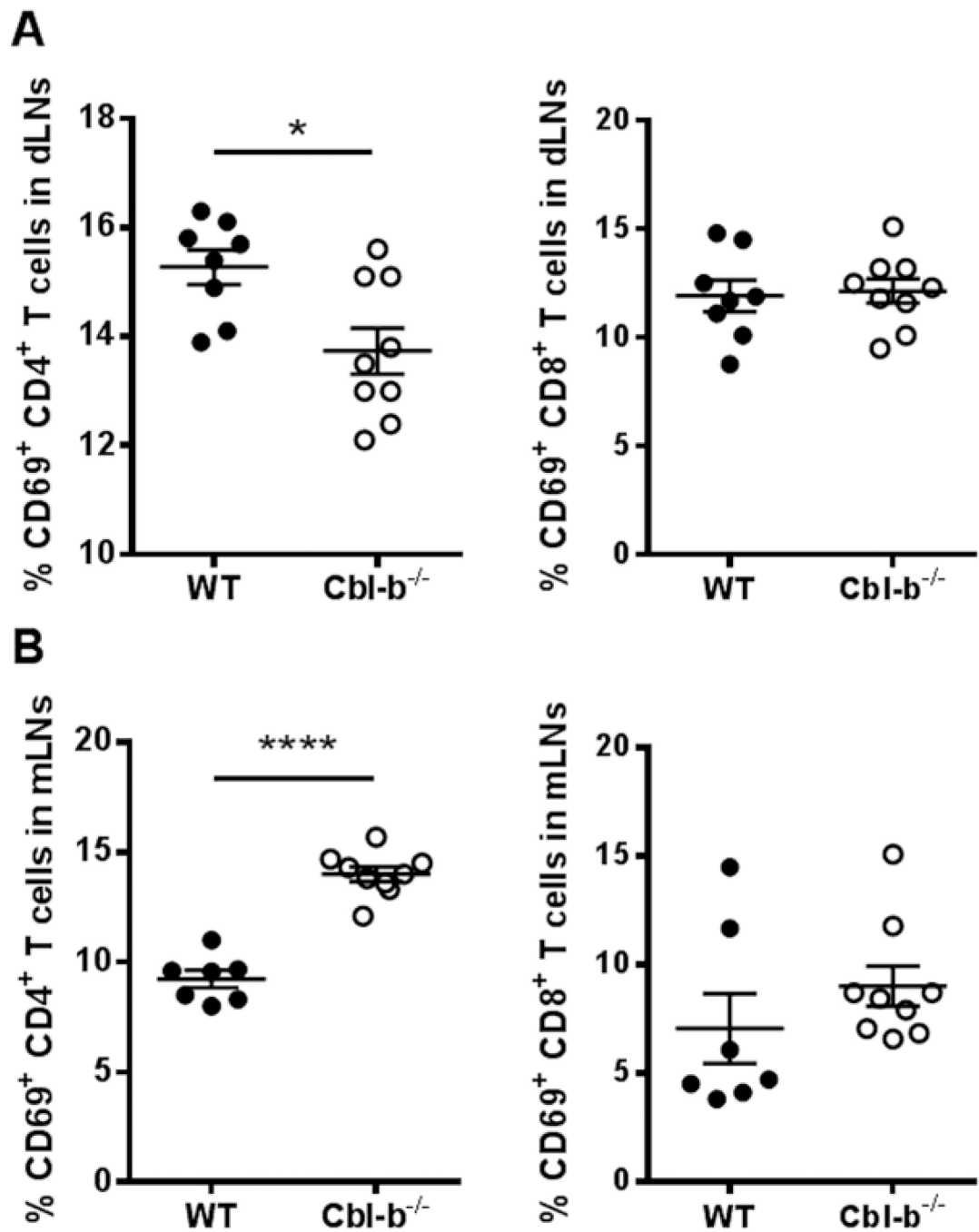
Figure 3.
Decreased frequency of CD69+ CD4+ T cells in draining LNs of Cbl-b−/− mice after CFA/MOG35–55 immunization. WT and Cbl-b−/− mice were immunized as in Figure 2 and the popliteal LNs were harvested 4–5 days later. (A) Percentage of CD4+ T cells (left) and CD8+ T cells (right) that are CD69+ in 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.
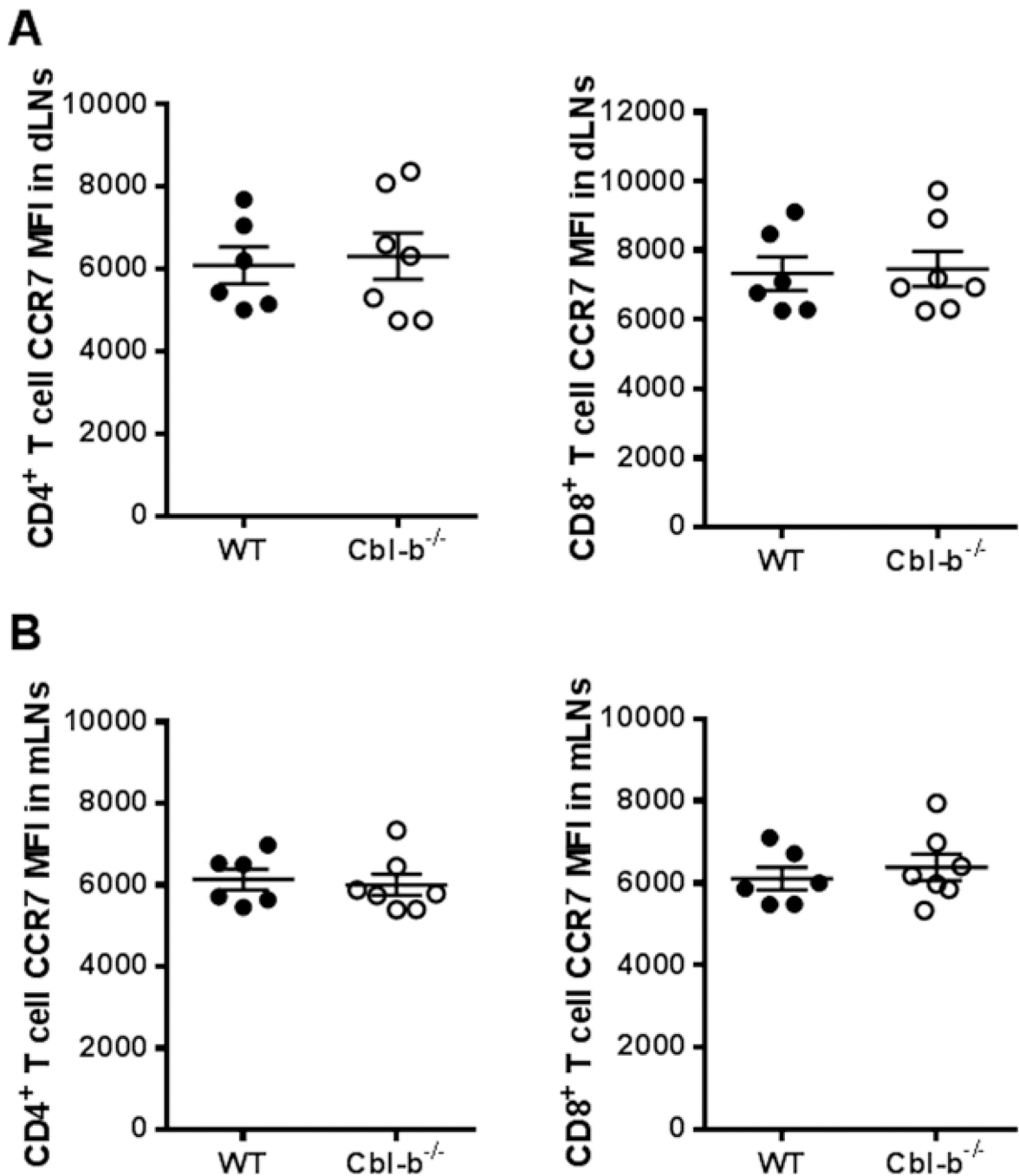
Figure 4.
CCR7 expression is unaltered in Cbl-b$^{-/-}$ T cells after CFA/MOG$_{35-55}$ immunization. WT and Cbl-b$^{-/-}$ mice were immunized, the popliteal LNs harvested as in Figure 2, and stained with human CCL19-Ig and anti-human IgG-Alexa Fluor 488. (A) MFI of CCR7 expression on CD4$^+$ T cells (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.
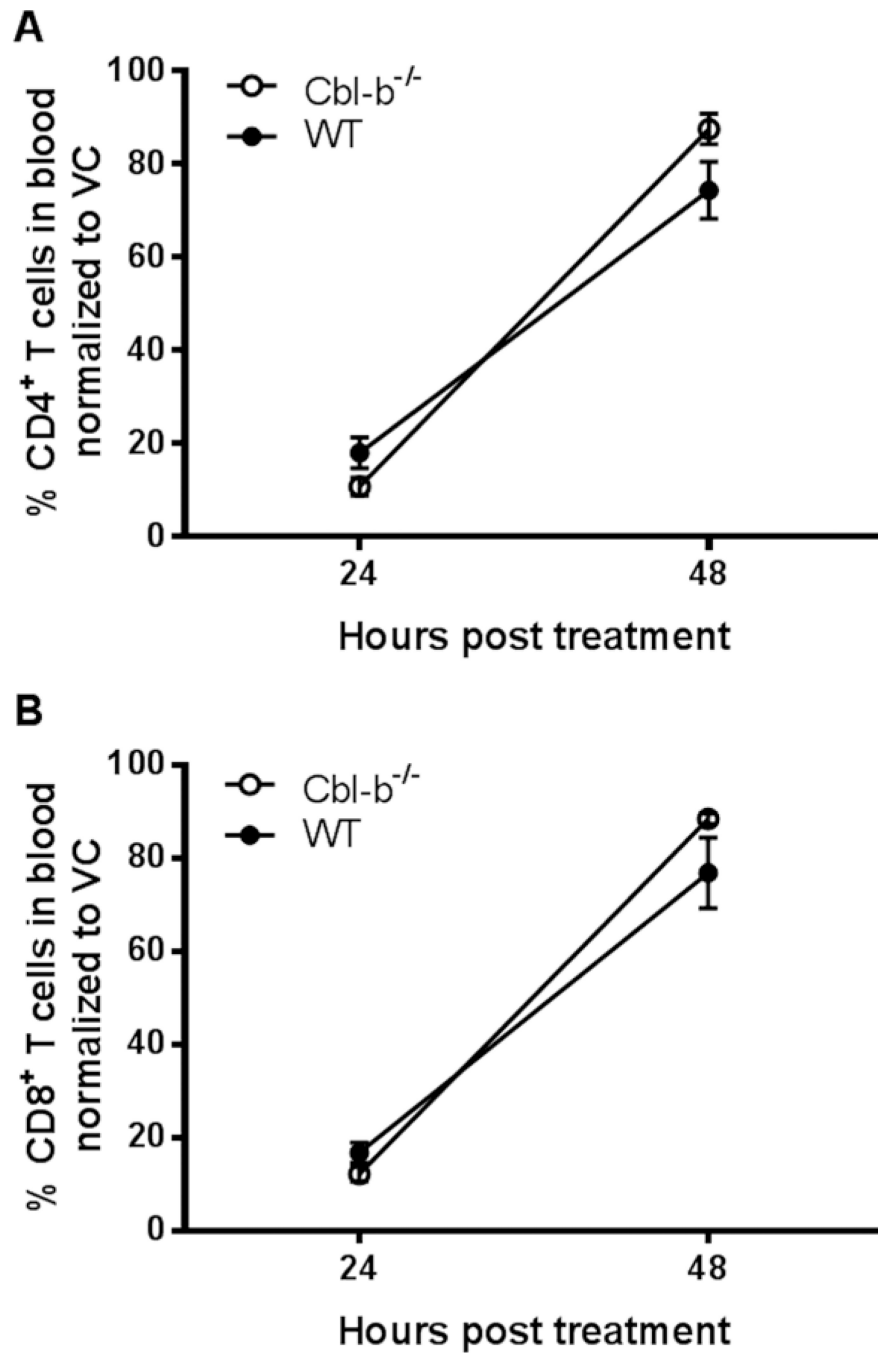
Figure 5.
Equal lymphopenic response of WT and Cbl-b$^{-/-}$ mice induced by the S1P lyase inhibitor, THI. WT and Cbl-b$^{-/-}$ mice were administered 10 mg/kg of THI or vehicle (VC) via gavage, and percent of CD4$^+$ and CD8$^+$ T cells in the total blood leukocytes was determined by flow cytometry. (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 6.
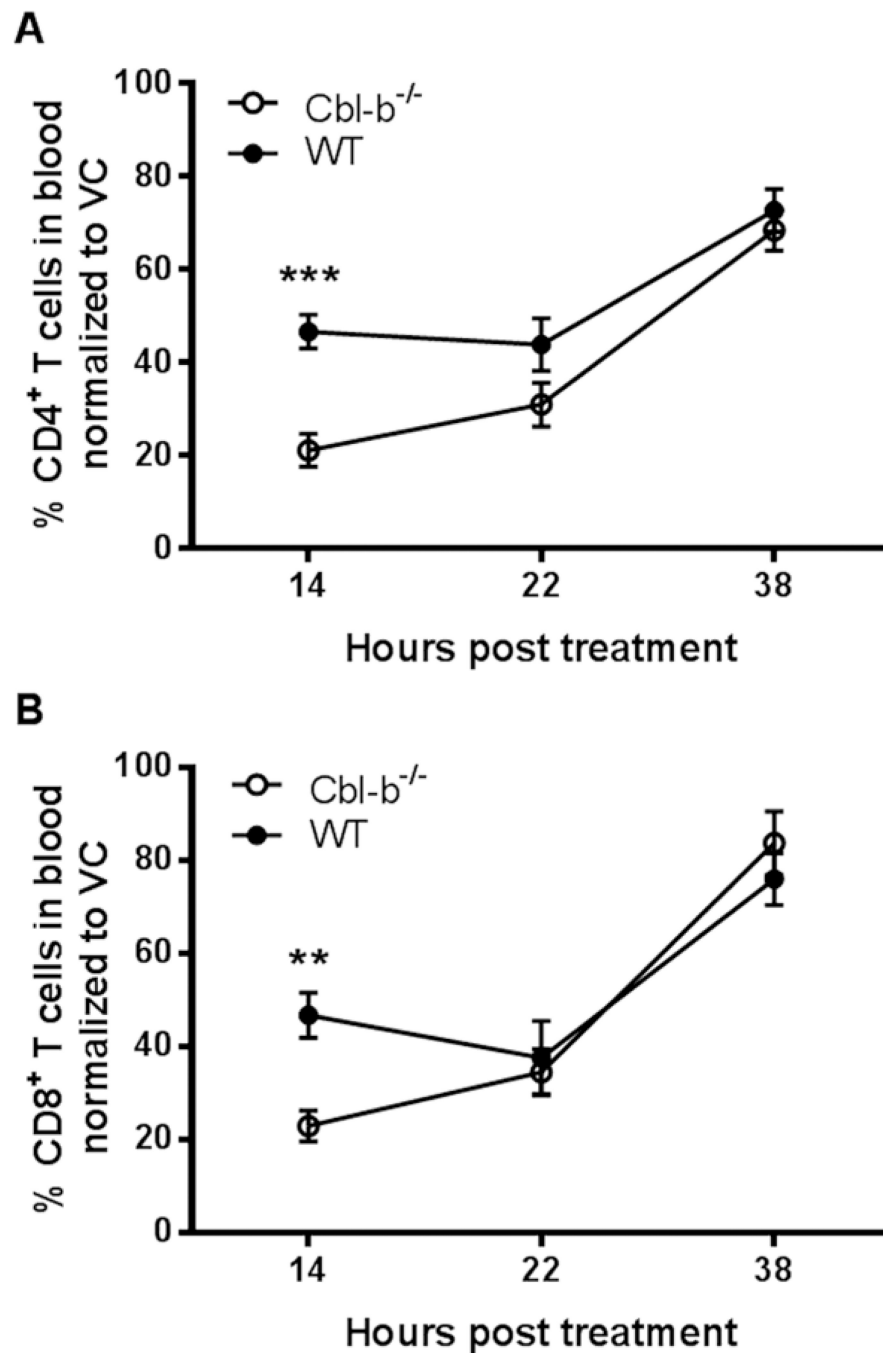Equal lymphopenic response of WT and Cbl-b$^{-/-}$ mice induced by the selective S1P$_1$ agonist, SEW2871. WT and Cbl-b$^{-/-}$ mice were administered 20 mg/kg of SEW2871 or vehicle (VC) via gavage, and percentage of CD4$^+$ and CD8$^+$ T cells in the total blood leukocytes was determined by flow cytometry. (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.
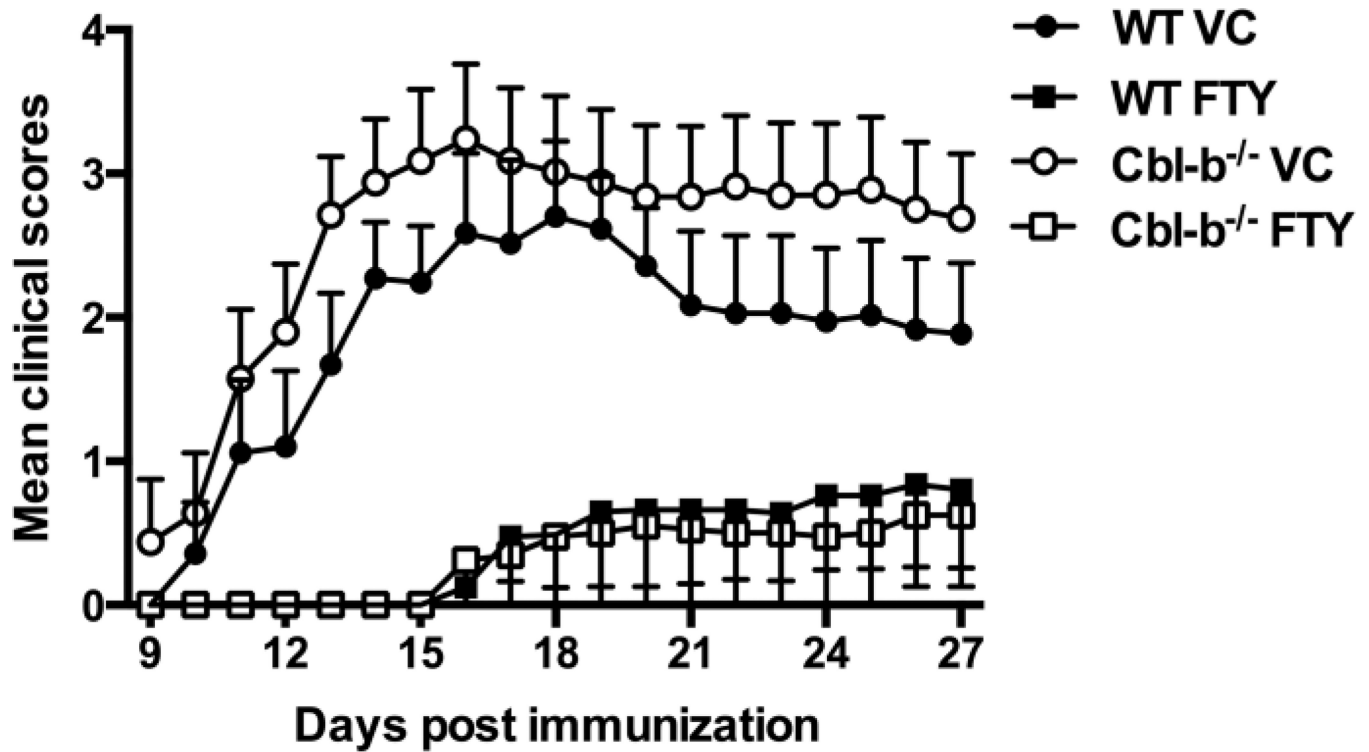
Figure 7.
Efficacy of FTY720 in inhibiting EAE in Cbl-b$^{-/-}$ mice. EAE was induced in WT and Cbl-b$^{-/-}$ mice by s.c. immunization with 165 μg of MOG$_{35-55}$ and 300 μg of CFA. 150 ng of Pertussis toxin was also given i.p. on day 0 and 2. On the day of EAE-induction, the mice were administered 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).