UNIVERSITY OF CALIFORNIA, SAN DIEGO

Integrin activation is required for regulatory T cell homeostasis and function

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biomedical Sciences

by

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The dissertation of Jane Elizabeth Klann is approved, and it is acceptable in quality and form for publication on microfilm or electronically:

Chair

University of California, San Diego

2018

DEDICATION

To my Dad, Mom, Mary, Eric, Sally, Woody and Nellie

EPIGRAPH

Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.

Marie Curie

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LIST OF ABBREVIATIONS

CFSE	Carboxyfluorescein succinimidyl ester
DC	Dendritic cell
GO	Gene Ontology
ICAM-1	Intracellular adhesion molecule 1
I.P.	Intraperitoneal
IS	Immunological synapse
I.V.	Intravenous
LFA-1	Lymphocyte function associated antigen 1
mAb	Monoclonal antibody
MFI	Mean Fluorescence Intensity
qPCR	Quantitative real-time polymerase chain reaction
RNA-seq	RNA sequencing
SEM	Standard error of the mean
TCR	T cell receptor
Treg cell	Regulatory T cell
VLA-4	Very late antigen 4

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ABSTRACT OF THE DISSERTATION

Integrin activation is required for regulatory T cell homeostasis and function

by

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Maintenance of the regulatory T (Treg) cell pool is essential for peripheral tolerance and prevention of autoimmunity. Integrins, heterodimeric transmembrane proteins consisting of α and β subunits that mediate cell-cell and cell-extracellular matrix interactions, play an important role in facilitating Treg cell contact-mediated suppression. Here we show that integrin activation plays an essential, previously unappreciated role in maintaining the identity and function of the Treg cell pool. Talin, a cytoskeletal protein essential in mediating β integrin activation, has been previously shown to be involved in the regulation of T cell proliferation and function. T cell-specific deletion of talin in *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice resulted in spontaneous lymphocyte activation,

primarily due to numerical and functional deficiencies of Treg cells in the periphery. Peripheral talin-deficient Treg cells were unable to maintain high expression of IL-2Rα, resulting in impaired IL-2 signaling and ultimately leading to increased apoptosis through downregulation of pro-survival proteins Bcl-2 and Mcl-1. Treg cell-specific loss of talin, or expression of talin(L325R), a mutant that selectively abrogates integrin activation, resulted in dysregulation of Treg cell identity and lethal systemic autoimmunity. This dysfunction could be attributed, in part, to a failure of Treg cells to maintain high expression of important Treg cell suppressive molecules and global dysregulation of the Treg cell transcriptome. Activation of α4β1 or αLβ2 integrins led to increased expression of IL-2Rα and boosted the size of the Treg cell population, respectively, suggesting that modulating integrin activation on Treg cells may be a useful therapeutic strategy for autoimmune and inflammatory disorders. Taken together, these results reveal a critical role for integrin-mediated signals in controlling peripheral tolerance by virtue of maintaining Treg cell identity and function.

CHAPTER I: INTRODUCTION

The role of talin in integrin activation

Integrins are heterodimeric transmembrane proteins made up of α and β subunits that mediate cell-to-cell and cell-to-extracellular matrix interactions. The regulation of the affinity of integrins for their extracellular ligands is involved in a multitude of signaling pathways that control cellular survival, proliferation and differentiation. Thus, mutations or genetic deficiencies in integrins or major components of the integrin signaling pathway can lead to defective organ development, immunodeficiency, cancer and autoimmune disease (1).

Integrins regulate the specificity and efficacy of T cell trafficking into secondary lymphoid organs as well as into inflamed peripheral tissues during infection or inflammatory events. Within these tissues, integrin binding to the extracellular matrix dictates where the cells move (2). At the initiation of the immune response, activation of integrins on T cells facilitates the formation of the immunological synapse, which forms between T cells and antigen presenting cells, leading to T cell activation. Both VLA-4, integrin α4β1, and LFA-1, integrin αLβ2, are highly expressed on T cells, and facilitate trafficking to lymph nodes and into inflamed tissues during infection (3). Because the ligands capable of activating integrins on T cells are expressed in diverse cell types, the activation of integrins must be tightly controlled to properly orchestrate the immune response.

The affinity of integrins for extracellular ligands is tightly regulated and critical for normal hematopoietic cell adhesion and function (4). Binding of the large cytoskeletal protein talin to the β integrin cytoplasmic domain is a key final step in inducing conformational changes in the integrin that confer high affinity (integrin activation) (5, 6). Talin activates β1, β2, and β3 integrins (5). The protein consists of a 190kD C-terminal flexible rod and a 47kD N-terminal

globular head domain containing four sub-domains (F0, F1, F2 and F3) (5-7). The F3 sub-domain of talin, which contains a phosphotyrosine-binding (PTB) domain, binds directly to a high-affinity binding site in the β-integrin cytoplasmic tail. Talin is essential for inside-out integrin activation, which regulates the affinity of integrins in accordance with changes to the extracellular environment sensed by the cell (8), and for outside-in integrin signaling, which is initiated by the binding of extracellular ligands (9). In addition to activating integrins, talin links integrins to the actin cytoskeleton and recruits essential signaling molecules such as phosphatidylinositol phosphate kinase (10) and TIAM1 to focal adhesions (11).

Germline deletion of talin results in embryonic lethality (11), but conditional knockout studies have revealed a role for talin in multiple immune cell types. Talin is required for fibrin clot retraction by platelets (12) and B cell homing to lymph nodes (13). In T cells, talin was one of the first proteins shown to be recruited to the immunological synapse (IS) (14), which forms between T lymphocytes and antigen-presenting cells (APCs) and facilitates T cell activation. The recruitment of talin to the IS has led to the hypothesis that talin may play a role in regulating this process. Studies with Jurkat T cells have demonstrated that talin is required for clustering and affinity regulation of the integrin LFA-1 (αLβ2) in order to facilitate contact between T cells and APCs (15, 16). Prior studies in mice with a T cell-specific deletion of talin revealed a role for the protein in the maintenance of T cell-APC contacts, contact-mediated T cell proliferation, and polarization of stable F-actin to the IS. Unexpectedly, these studies also showed that T cells isolated from the lymph nodes of these mice exhibited a phenotype consistent with prior activation, despite having reduced contact time with APCs and no defect in TCR signaling (17). Although these mice were also observed to have a reduced frequency of regulatory T (Treg) cells in the lymph nodes (17), it remains unknown whether talin is intrinsically required in naïve T

cells to prevent aberrant activation or if talin plays a specific role in Treg cells in order to maintain immune homeostasis.

Treg cells are essential to maintain immune homeostasis

Treg cells, a subset of $CD4^+$ T lymphocytes defined by expression of the transcription factor Foxp3, are indispensable for the maintenance of peripheral tolerance. Foxp3 deficiency in mice (18, 19) or humans (20, 21) leads to systemic inflammation and multi-organ autoimmunity. Deficiency in Treg cell numbers or suppressive function has been linked to various immune-mediated conditions such as inflammatory bowel disease, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosis, and multiple sclerosis (22, 23). Treg cells can originate in the thymus (nTreg cells), or turn on Foxp3 in response to antigen and TGF-β cytokine stimulation in the periphery (iTreg cells)(24). Treg cells are also characterized by high expression of IL-2Rα (CD25) (25). IL-2 signaling is known to reinforce the expression of Foxp3 (26, 27), and expression of Foxp3, in turn, directly upregulates the expression of IL-2Rα (28). Thus, IL-2 signaling plays a central role in the development, proliferation and homeostasis of Treg cells (24, 29-31). Multiple mechanisms of suppression and corresponding markers have been identified in Treg cells, including production of adenosine by CD39 and CD73; expression of the TNF family member GITR; capture of IL-2 through high expression of the high affinity IL-2 receptor chain; downregulation or blocking of co-stimulatory molecules, CD80 and CD86, on APCs through constitutive expression of CTLA-4; and production of anti-inflammatory cytokines IL-10 and TGF-β1 (24, 32).

The integrin LFA-1 is known to be required for contact-dependent suppressive mechanisms of Treg cells, such as the downregulation of co-stimulatory molecules on dendritic cells through

expression of CTLA-4 (33-35), cytolysis through the production of granzyme B in Treg cells (36), conversion of ATP to adenosine through the expression of both CD39 and CD73 on the surface of Treg cells (37) and clustering around activated dendritic cells to provide a physical barrier to prevent activation of naïve T cells (38). Intriguingly, recent studies revealed that LFA-1 expressed by Treg cells exhibits a stronger intrinsic adhesiveness compared to that expressed by conventional T cells, which can be attributed to reduced calpain levels in Treg cells that effectively slow the recycling of integrins from the cell surface (39, 40). Thus, these studies suggest that integrin expression and function may be regulated by different mechanisms in Treg cells compared to conventional T cells. Whether integrins play a role in Treg cell homeostasis beyond simply facilitating adhesion to mediate these contact-dependent suppressive mechanisms, however, remains largely unexplored.

Recent evidence has revealed that Treg cells exhibit phenotypic and functional heterogeneity. Treg cells can be categorized as central (cTreg) and effector Treg (eTreg) cells based on phenotypic markers ($CD62L^{hi}CD44^{lo}$ and $CD62L^{lo}CD44^{hi}$), respectively, as well as localization and homeostatic requirements (41). cTreg cells express high levels of the chemokine receptor CCR7, enabling them to recirculate through lymphoid tissues. These cells are quiescent and relatively long-lived due to high expression of the anti-apoptotic proteins Bcl-2 and Mcl-1. In addition, cTreg cells are believed to be more highly dependent on IL-2 signaling and correspondingly express high levels of IL-2Rα (41). By contrast, eTreg cells are highly proliferative, localize mainly to non-lymphoid tissues, such as the liver and lung, and are short-lived and highly apoptotic (41, 42). Compared to cTreg cells, eTreg cells are also responsive to IL-2, but depend more highly on ICOS signaling for the maintenance of homeostasis and correspondingly express higher levels of ICOS (41). eTreg cells are also characterized by high

expression of GITR and CD103, which facilitate trafficking to tissues (43). Most Treg cells exiting the thymus are characterized as cTreg cells, as they express high levels of CCR7 and CD62L. The subsequent differentiation of cTreg cells into eTreg cells has been shown to occur in the periphery and requires TCR signals (44, 45) and the expression of the transcription factors IRF4 and Blimp-1 (46). These subsets are functionally distinct, as cTreg cells have been proposed to prevent aberrant T cell priming in the lymphoid organs, while eTreg cells are thought to control effector T cell responses at the non-lymphoid tissue sites (41). Thus, optimal differentiation and maintenance of both subsets is required for the preservation of immune homeostasis.

Because Treg cells are essential to maintain peripheral tolerance, the stability of this population is also required for immune homeostasis. The stability of Treg cells can be defined as the maintenance of suppressive capacity and expression of Foxp3, and the absence of pro-inflammatory effector function, which could include production of IL-2, production of pro-inflammatory cytokines, cytolysis etc (47). Sustained expression of Foxp3 is thought to be controlled, in part, through epigenetic regulation. Specifically, regions within the Foxp3 gene locus, including the Treg cell specific demethylated region (TSDR), also known as the CNS2, are consistently hypomethylated in comparison to conventional T cells (48, 49). Dysregulated methylation in this region has been linked to loss of Foxp3 expression and conversion of Treg cells to other effector T cell phenotypes. Treg cells are prone to downregulate Foxp3 in response to deprivation of IL-2, exposure to pro-inflammatory cytokines and TCR stimulation (47). It is important to understand that Treg cells can lose their suppressive capacity while still maintaining their expression of Foxp3, as expression of FOXO, Eos and Nrp1 have all been shown to be required for Treg cell stability (48, 50, 51).

Integrin activation is required for Treg cell-mediated maintenance of immune homeostasis

In this dissertation, we explored the role of talin in maintaining immune homeostasis. We first analyzed *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice with a T cell-specific deletion of talin, which harbor spontaneously activated CD4$^+$ and CD8$^+$ T cells. This immune dysregulation could be attributed, in part, to a substantial deficiency in both the number and function of Treg cells. Strikingly, talin-deficient Treg cells were unable to maintain high expression of IL-2Rα, which led to increased apoptosis of these cells. Together, these findings suggest that talin plays a critical role in regulating Treg cell function, homeostasis and survival. From there, to understand the exact role of talin in Treg cells, we created a mouse model in which talin was specifically deleted from Treg cells. *Tln1*$^{fl/fl}$*Foxp3*^{Cre} male mice exhibited spontaneous lethal autoimmunity, demonstrating an essential role for talin in maintaining Treg cell homeostasis and function. Mice harboring Treg cells expressing a mutant form of talin, talin(L325R), a mutant that selectively abrogates integrin activation (12, 52, 53), developed immune-mediated pathology resembling that observed in mice with talin-deficient Treg cells, indicating that talin is required in Treg cells owing to its role in integrin activation. Conversely, activation of VLA-4 or LFA-1 integrins led to increased expression of IL-2Rα on Treg cells and boosted the size of the Treg cell population, respectively. Together these findings suggest that talin and integrin activation play a critical role in controlling Treg cell-mediated peripheral tolerance and raise the possibility that activating integrins in Treg cells may be a useful therapeutic strategy in the treatment of immune-mediated disorders.

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CHAPTER II: T CELL-SPECIFIC DELETION OF TALIN RESULTS IN SPONTANEOUS LYMPHOCYTE ACTIVATION DUE TO FUNCTIONAL AND NUMERICAL DEFECTS IN TREG CELLS

2.1 Introduction

Using a $CD4^{Cre}$ conditional mouse model in which talin was deleted in all T cells during the double positive stage of development in the thymus, Huttenlocher and colleagues previously showed that talin is required for T cell-APC contacts, contact-mediated T cell proliferation, and polarization of stable F-actin to the IS (17). These studies also demonstrated that conventional T cells isolated from the lymph nodes of $Tln1^{fl/fl}Cd4^{Cre}$ mice exhibited an activated phenotype, despite significantly reduced contact time with APCs (17). While these mice were also shown to harbor a significantly reduced Treg cell population in the lymph nodes (17), it remains unknown whether talin is intrinsically required in naïve T cells to maintain a naïve phenotype or if talin is specifically required in Treg cells to maintain immune homeostasis. To explore the role of talin in maintaining immune homeostasis and T cell activation, we further analyzed the same $Tln1^{fl/fl}Cd4^{Cre}$ mice with a T cell-specific deletion of talin. We found that talin-deficient $CD4^+$ and $CD8^+$ T cells exhibited spontaneous activation, which was not due to defects in T cell development or aberrant expression of co-stimulatory or inhibitory molecules by naïve peripheral T cells. Instead, $Tln1^{fl/fl}Cd4^{Cre}$ mice exhibited a substantial deficiency in both the number and function of Treg cells.

2.2 Results

2.2.1 Spontaneous lymphocyte activation in mice with a T cell-specific deletion of talin

To investigate the role of talin in maintaining peripheral tolerance, we generated mice with a T cell-specific deletion of talin1 by crossing floxed talin1 mice with $Cd4^{Cre}$ mice ($Tln1^{fl/fl}Cd4^{Cre}$) in

which talin was deleted at the $CD4^+CD8^+$ double-positive stage during thymic development. Compared to control *Tln1*$^{fl/fl}$ mice, *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice were born at expected frequencies, developed normally with no overt signs of pathology and appeared healthy (**Fig. 2.1A**). Examination of thymi from control and *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice revealed similar frequencies and numbers of CD4 and CD8 double-positive and single-positive thymocytes (**Fig. 2.1B, 2.1C**), suggesting that thymic T cell development after the double-positive stage was not affected in the absence of talin. Analysis of splenic T cells isolated from *Tln1*$^{fl/fl}$*Cd4*^{Cre} and control mice showed that although there were changes in the proportion of talin-deficient $CD4^+$ and $CD8^+$ T cells (Fig. 1D), no significant differences in total cell numbers were observed (**Fig. 2.1E**).

Further examination of the $CD4^+$ and $CD8^+$ T cell compartments revealed that talin-deficient lymphocytes in the spleen displayed an activated, antigen-experienced ($CD44^{hi}CD62L^{lo}$) phenotype (**Fig. 2.2A, 2.2B**). Consistent with this activated phenotype, $CD4^+$ T cells isolated from *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice displayed an increased proliferative capacity, as evidenced by high Ki67 expression (**Fig. 2.2C**). Talin-deficient $CD4^+$ T cells produced high levels of pro-inflammatory cytokines TNFα and IL-17A compared to control cells (**Fig. 2.2D**). Taken together, these data show that talin is required to maintain quiescence and prevent activation of naïve T lymphocytes.

2.2.2 Spontaneous lymphocyte activation is controlled by factors extrinsic to naïve T cells

We next sought to determine the mechanisms by which deletion of talin causes aberrant T cell activation. To investigate whether T cell activation occurred during development, we examined the activation status of developing thymic T cells from *Tln1*$^{fl/fl}$*Cd4*^{Cre} and control mice. Thymic $CD4^+$ or $CD8^+$ single-positive T cells did not display an activated phenotype based on CD44

expression (**Fig. 2.3**), suggesting that the aberrant T lymphocyte activation we observed in the periphery did not occur during T cell development. We next hypothesized that the absence of talin might lead to increased expression of co-stimulatory receptors, or conversely, decreased expression of inhibitory receptors on naïve T cells, thereby influencing their activation threshold in the periphery. Examination of co-stimulatory (CD28, OX40, ICOS) and inhibitory receptors (PD1 and CTLA-4), however, revealed comparable expression by naïve ($CD44^{lo}$) talin-deficient and control $CD4^+$ and $CD8^+$ T cells (**Fig. 2.4**). Thus, factors intrinsic to naïve T cells did not appear to be responsible for the systemic T cell activation observed in *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice.

To determine if extrinsic factors were contributing to spontaneous activation of naïve T lymphocytes in *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice, FACS-sorted naïve ($CD44^{lo}CD62L^{hi}$) wild-type or talin-deficient T cells were labeled with the fluorescent division dye CFSE and adoptively transferred into congenic wild-type or *Tln1*$^{fl/fl}$*Cd4*^{Cre} recipients. Neither donor wild-type nor talin-deficient T cells proliferated when adoptively transferred into wild-type recipients. By contrast, both wild-type and talin-deficient T cells underwent proliferation when transferred into *Tln1*$^{fl/fl}$*Cd4*^{Cre} recipients (**Fig. 2.5**). In summary, these results suggest that the systemic lymphocyte activation observed in *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice resulted from factors extrinsic to naïve T cells.

2.2.3 Talin is required to maintain the number and function of Treg cells in the periphery

Because Treg cells are essential for controlling aberrant T cell activation, we hypothesized that a quantitative or qualitative deficiency in Treg cells might underlie the spontaneous lymphocyte activation exhibited by *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice. To test this hypothesis, we generated *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice expressing a GFP reporter for Foxp3 by crossing *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice with Foxp3 GFP reporter mice (54). We observed significant reductions in the frequencies and absolute numbers

of Treg cells in the spleens of *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice (**Fig 2.6A, 2.6B**), consistent with the previously published observation that lymph nodes from these mice harbor reduced frequencies of Foxp3$^+$ Treg cells (17). In addition, talin-deficient Treg cells expressed significantly less Foxp3 on a per cell basis (**Fig. 2.6C**).

We next assessed whether expression of talin was required for Treg cell function. Using an *in vitro* suppression assay, we observed that Treg cells lacking talin were functionally deficient on a per cell basis (**Fig. 2.6D**). Examination of suppressive molecules revealed that talin-deficient Treg cells exhibited reduced expression of IL-2Rα, CD39, GITR and CTLA-4, but not CD73 (**Fig. 2.6E**). Analysis of anti-inflammatory cytokines at the mRNA level in talin-deficient Treg cells revealed no significant defect in the production of TGFβ-1, but a significant reduction in IL-10 production (**Fig. 2.6F**). Taken together, these data suggest that the activated phenotype of CD4$^+$ and CD8$^+$ T cells in *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice may be attributable to both the reduced suppressive capacity as well as reduced size of the Treg cell pool.

We next investigated whether the numerical deficiency of peripheral Treg cells in *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice may be due to a defect thymic development. However, we observed similar frequencies and numbers of Treg cells in the thymi of control and *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice (**Fig. 2.7A, 2.7B**) as well as comparable Foxp3 expression on a per cell basis (**Fig. 2.7C**). In addition, talin-deficient Treg cells in the thymus did not display defects in the expression of Treg cell suppressive molecules, indicating that talin may be dispensable during Treg cell development (**Fig. 2.7D**). Overall, these data suggest that talin may play a specific role in maintaining peripheral Treg cell homeostasis and survival.

2.2.4 Spontaneous lymphocyte activation in Tln1$^{fl/fl}$*Cd4*^{Cre} *mice is Treg cell-dependent*

The numerical and functional deficiencies we observed in talin-deficient Treg cells led us to hypothesize that talin may be specifically required for Treg cells to prevent spontaneous lymphocyte activation. To test this possibility, we generated a series of bone marrow chimeras in which lethally irradiated RAG1-deficient mice were reconstituted with bone marrow cells from *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice alone or in combination with bone marrow cells from wild-type or Foxp3-deficient mice (**Fig. 2.8**). Reconstitution with *Tln1*$^{fl/fl}$*Cd4*^{Cre} bone marrow cells alone (*Cd4*^{Cre}) or in combination with Foxp3-deficient bone marrow cells (*Cd4*^{Cre}:Foxp3null) yielded mice exhibiting systemic CD4^{+} T cell activation that recapitulated the phenotype we observed in *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice (Fig. 4A). In contrast, reconstitution with *Tln1*$^{fl/fl}$*Cd4*^{Cre} bone marrow cells along with wild-type bone marrow cells (*Cd4*^{Cre} :WT) was sufficient to prevent systemic T cell activation, as the percentage of activated CD4^{+}CD44^{hi}CD62L^{lo} cells in mixed bone marrow chimeras was similar to that in bone marrow chimeras reconstituted with wild-type bone marrow (WT:WT) or wild-type bone marrow in combination with Foxp3-deficient bone marrow (WT:Foxp3null) (**Fig. 2.9A**).

Analysis of Treg cell phenotype revealed that talin-deficient Treg cells isolated from *Cd4*^{cre} chimeras were present at significantly lower frequencies and absolute numbers and expressed significantly less Foxp3 on a per cell basis compared to wild-type Treg cells isolated from WT:WT chimeras. Moreover, talin-deficient Treg cells isolated from mixed *Cd4*^{cre}:WT chimeras were also numerically deficient and exhibited a significant reduction in Foxp3 expression compared to wild-type Treg cells isolated from the same mixed chimera (**Fig. 2.9B-D**), indicating that talin deficiency has a cell intrinsic effect on Treg cell population size. Additional analysis revealed that talin-deficient Treg cells isolated from *Cd4*^{Cre}:WT and *Cd4*^{Cre} bone marrow chimeras exhibited decreased expression of CD39, GITR and CTLA4 compared to

wild-type Treg cells, consistent with the phenotype of Treg cells isolated from *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice (Fig. 4E). By contrast, there was no significant difference in the expression of IL-2Rα between wild-type Treg cells and talin-deficient Treg cells isolated from WT:WT, *Cd4*^{cre}:WT, *Cd4*^{Cre}, and *Cd4*^{Cre}:Foxp3null bone marrow chimeras (Fig. 1.10, Supplemental Fig. 2). However, this finding did not appear to result from a rescue of talin-deficient Treg cells through exposure to a wild-type environment; instead, it may be an artifact of the bone marrow chimera system, as IL-2Rα expression by wild-type Treg cells from WT:WT bone marrow chimeras was significantly reduced (**Fig. 2.10, Fig. 2.11**) relative to that by wild-type Treg cells from control *Tln1*$^{fl/fl}$ mice (**Fig. 2.6E, Fig. 2.11**). To test this possibility, we adoptively transferred talin-deficient Treg cells into congenically marked wild-type recipients and analyzed them four days after transfer. Analysis of talin-deficient Treg cells failed to demonstrate a significant increase in the expression of IL-2Rα, Foxp3 or any of the Treg cell suppressive molecules we examined (**Fig. 2.12F-I**), suggesting that neither the presence of wild-type Treg nor non-Treg T cells can rescue the number, phenotype or function of talin-deficient Treg cells. Taken together, these data indicate that the spontaneous lymphocyte activation observed in *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice is primarily due to intrinsic defects in the Treg cell compartment.

2.2.5 Talin is required for the homeostasis of cTreg and eTreg cells

We next investigated the mechanisms accounting for the observed numerical deficiency of talin-deficient Treg cells. We initially hypothesized that talin-deficient Treg cells might have an impaired proliferative capacity. However, splenic Treg cells from *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice exhibited increased proliferation based on Ki67 expression (**Fig. 2.13A**). We next investigated whether talin-deficient Treg cells were more apoptotic than their wild-type counterparts. We

assessed the expression of the pro-survival proteins Bcl-2 and Mcl-1 (55-57) and found that talin-deficient Treg cells expressed significantly lower mRNA levels of both molecules (**Fig. 2.13B**). Correspondingly, talin-deficient Treg cells expressed higher mRNA levels of the apoptosis executioner protein Caspase 7 (Casp7) (**Fig. 2.13B**), indicating that signals that promote survival may be impaired in the absence of talin.

We next investigated whether the dysregulation in the balance of pro- and anti-apoptotic signals observed in the total Treg cell pool affected both eTreg and cTreg subsets. Although we observed an increase in the frequency of $CD62L^{lo}CD44^{hi}$ eTreg cells and a corresponding decrease in the frequency $CD62L^{hi}CD44^{lo}$ cTreg cells (**Fig. 2.14A**), only the absolute number of cTreg cells was significantly altered (**Fig. 2.14B**), suggesting that talin may be more critical in the maintenance of the cTreg cell population. Nonetheless, both talin-deficient cTreg and eTreg cells were more highly apoptotic then their control counterparts, based on analysis using Mito Flow, a dye that measures mitochondrial membrane potential (**Fig. 2.14C**). Additionally, both talin-deficient eTreg and cTreg cells exhibited reduced expression of Foxp3 and IL-2Rα on a per cell basis (**Fig. 2.14D-F**), indicating that talin is required for the homeostasis of both Treg cell subsets. Lastly, characterization of molecules highly expressed by eTreg revealed reduced expression of GITR and ICOS, but not CD103 or IRF4, by talin-deficient eTreg cells (**Fig. 2.14G, 2.14H**). Further characterization of molecules highly expressed on cTreg cells revealed reduced expression of Bcl-2 by talin-deficient cTreg cells (**Fig. 2.14I, 2.14J**). Taken together, these findings indicate that although cTreg cells appear to be more dependent on talin for survival than eTreg cells, talin is nonetheless required for the function and homeostasis of both Treg cell subsets.

2.2.6 Talin mediates IL-2 responsiveness in Treg cells

IL-2 signaling is essential for the homeostasis and survival of Treg cells (58) and has been shown to be a positive regulator of Foxp3, Mcl-1 and Bcl-2 expression in Treg cells (26, 27, 55). Moreover, IL-2 signaling directly upregulates expression of IL-2Rα (58). Having observed decreased cell surface expression of IL-2Rα (**Fig. 2.6E, Fig. 2.14E, 2.14F**) and mRNA expression of Mcl-1 and Bcl2 (**Fig. 2.13B**) in talin-deficient Treg cells, we hypothesized that IL-2 signaling in Treg cells might be impaired in the absence of talin.

Transcript levels of *Il2ra* were significantly lower in talin-deficient Treg cells compared to control cells (**Fig. 2.15A**), suggesting that IL-2 signaling is impaired as a consequence of talin deficiency. We therefore examined STAT5 phosphorylation, which occurs directly after activation of the IL-2R (58). When assessed directly *ex vivo*, talin-deficient Treg cells exhibited significantly lower levels of phosphorylated (p)STAT5 (**Fig. 2.15B**). Moreover, lower levels of pSTAT5 were observed even in IL-2Rα^{hi} talin-deficient Treg cells compared to their control IL-2Rα^{hi} counterparts. Both IL-2Rα^{lo} talin-deficient and IL-2Rα^{lo} control Treg cells (**Fig. 2.15B**) exhibited low levels of pSTAT5. We next investigated whether the inability of talin-deficient Treg cells to phosphorylate STAT5 could be rescued with high levels of IL-2 *in vitro* and *in vivo* with IL-2/IL-2 monoclonal antibody (mAb) complexes, which have been shown to target IL-2 specifically to Treg cells to induce a rapid expansion of the Treg cell pool (59, 60). Addition of exogenous IL-2 *in vitro* was capable of increasing STAT5 phosphorylation in talin-deficient IL-2Rα^{hi} Treg cells to levels observed in wild-type cells (**Fig. 2.15C**). However, neither wild-type nor talin-deficient IL-2Rα^{lo} Treg cells were capable of increasing STAT5 phosphorylation in response to exogenous IL-2 *in vitro* (**Fig. 2.15C**). Similarly, *in vivo* targeting of IL-2 to talin-deficient Treg cells with IL-2/IL-2 mAb complexes resulted in an increased frequency and

absolute number of Foxp3$^+$ talin-deficient Treg cells, along with increased Foxp3 expression on a per cell basis (**Fig. 2.16A-C**). Lastly, treatment with IL-2/IL-2 mAb complexes restored or increased the expression of suppressive molecules IL-2Rα, CD39, GITR and CTLA-4 in talin-deficient Treg cells to wild-type levels (**Fig. 2.16D**). Because only IL-2Rα^{hi}, but not IL-2Rα^{lo}, talin-deficient Treg cells were capable of phosphorylating STAT5 in response to exogenous IL-2 *in vitro* (**Fig. 2.15C**), administration of IL-2/IL-2 mAb complexes may have acted selectively on IL-2Rα^{hi} talin-deficient Treg cells to expand the numbers of these cells. Taken together, these data show that talin plays a critical role in the maintenance of high levels of IL-2Rα by Treg cells.

2.3 Discussion

Analysis of mice with a T cell-specific deletion of talin, the cytoskeletal regulatory protein involved in integrin activation, allowed us to determine that talin is extrinsically required to maintain the activation threshold of effector CD4$^+$ and CD8$^+$ T cells, but intrinsically required to maintain the size and function of the Treg cell pool. Maintenance of the Treg cell pool is essential for preventing autoimmunity, thus our work points to an essential and previously unappreciated role for talin in mediating immune homeostasis.

Mechanistically, our data suggests that talin is required for Treg cell to receive proper IL-2 signal, thus disrupting their survival and homeostasis. Strikingly, a reduced proportion of talin-deficient Treg cells were able to maintain high expression of IL-2Rα in the periphery; moreover, talin-deficient IL-2Rα^{hi} cells exhibited reduced STAT5 phosphorylation when assessed directly *ex vivo* compared to their control counterparts. Impaired IL-2Rα expression by IL-2Rα^{lo} talin-deficient Treg cells could not be rescued, even with the addition of exogenous IL-2. Thus, our

data suggest that talin, by virtue of its role in mediating integrin activation, is required for the maintenance of high IL-2Rα expression in peripheral Treg cells, thereby playing a critical role in their function, homeostasis and survival.

Chapter II, in full, is an adapted version of the material published in the *Journal of Immunology*. **Klann JE**, Remedios KA, Kim SH, Metz PJ, Lopez J, Mack LA, Zheng Y, Ginsberg MH, Petrich BG, Chang JT. Talin Plays a Critical Role in the Maintenance of the Regulatory T Cell Pool. *J Immunol.* 2017;198(12):4639-51. The dissertation author was the primary author of all material.

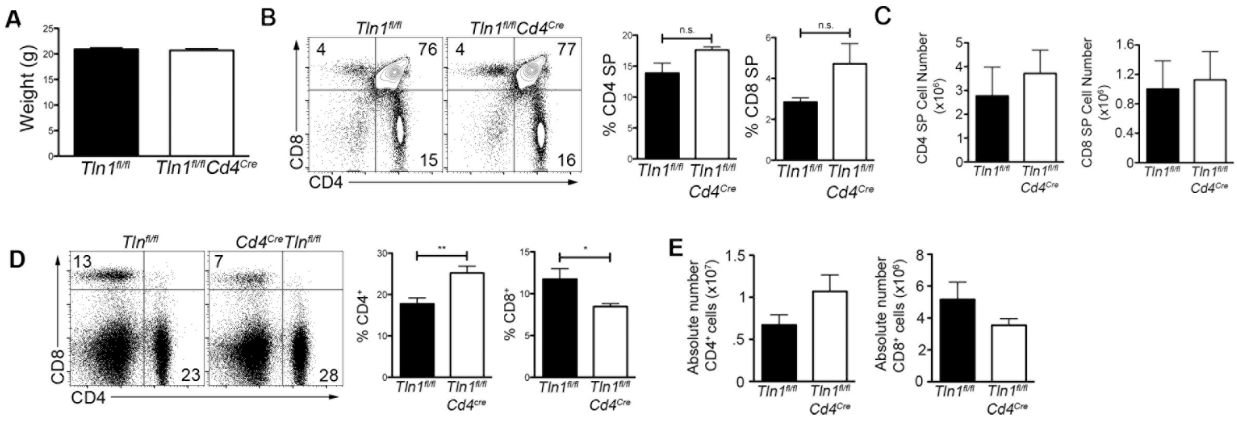

Figure 2.1 Deletion of talin does not affect the numbers of splenic or thymic T cells. (**A**) Weights of 8-week old *Tln1*$^{fl/fl}$ (WT) and *Tln1*$^{fl/fl}$*Cd4*^{Cre} (KO) mice (n=3). Percentages (**B**) and absolute number (**C**) of thymic CD4$^+$ and CD8$^+$ T cells isolated from WT and KO mice (n=3). Percentages (**D**) and absolute number (**E**) of CD4$^+$ and CD8$^+$ T cells isolated from WT or KO spleens (n=6). Data shown are mean ± SEM and are representative of at least 3 independent experiments. *, P < 0.05; **, P <0.01.

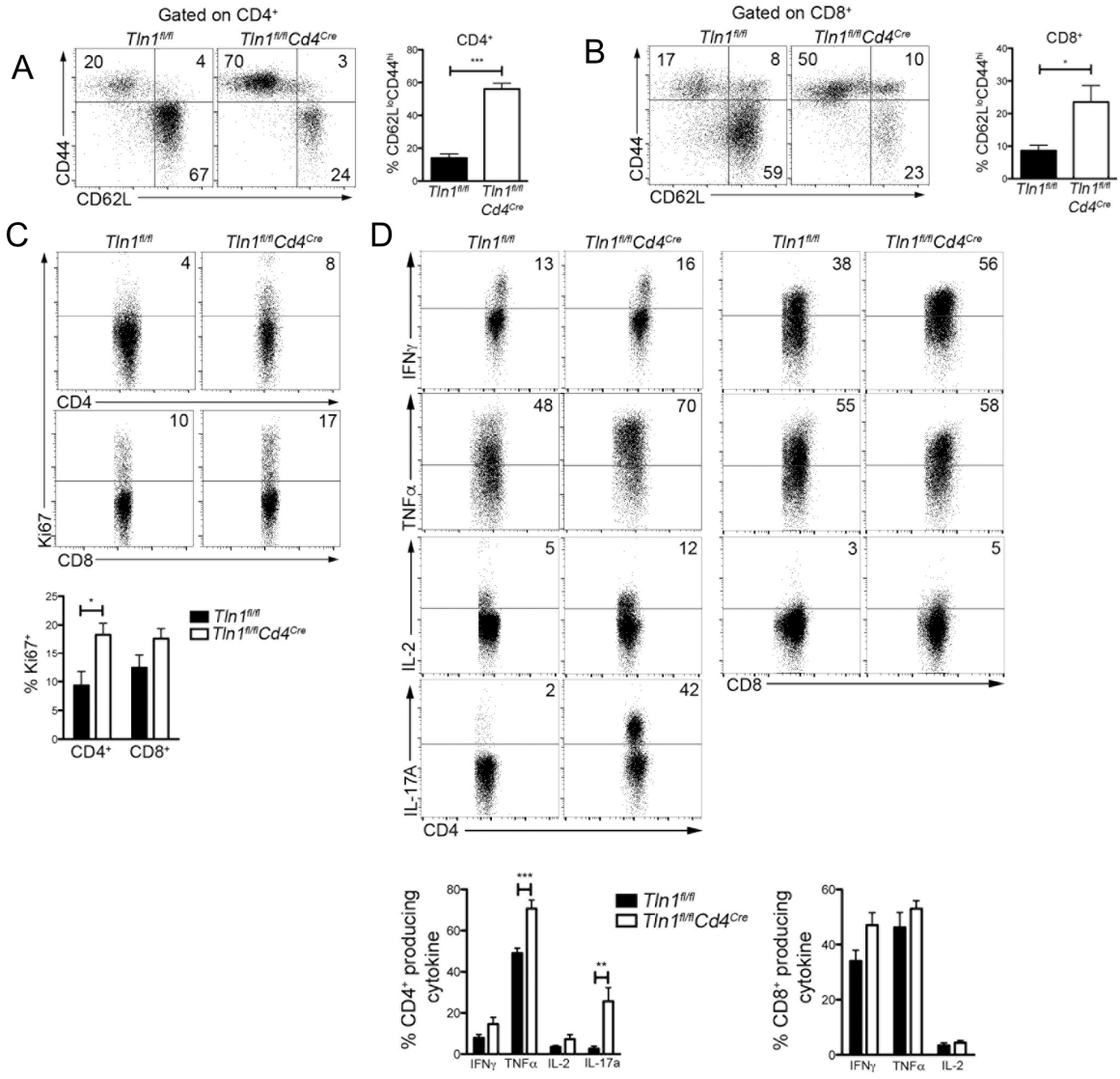

Figure 2.2 Spontaneous lymphocyte activation in mice with a T cell-specific deletion in talin. Expression of CD44 and CD62L in splenic $CD4^+$ (**A**) and $CD8^+$ (**B**) T cells from WT and KO mice (n=6). (**C**) Expression of Ki67 by $CD4^+$ and $CD8^+$ T cells (n=9). (**D**) IFNγ, TNFα, IL-2 and IL-17A expression by splenic $CD4^+$ (left) and $CD8^+$ (right) T cells from WT and KO mice after *in vitro* stimulation with PMA and ionomycin; displayed cells gated on $CD4^+CD44^{hi}$ or $CD8^+CD44^{hi}$ events (n=9). Data shown are mean ± SEM and are representative of at least 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

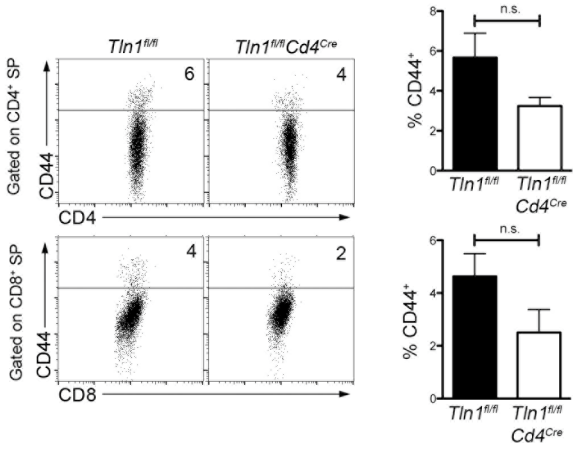

Figure 2.3 Talin does not affect the activation status of thymic T cells. Expression of CD44 by single-positive (SP) $CD4^+$ and $CD8^+$ thymocytes from *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice (n=3). Data shown are mean ± SEM and are representative of at least 2 independent experiments.

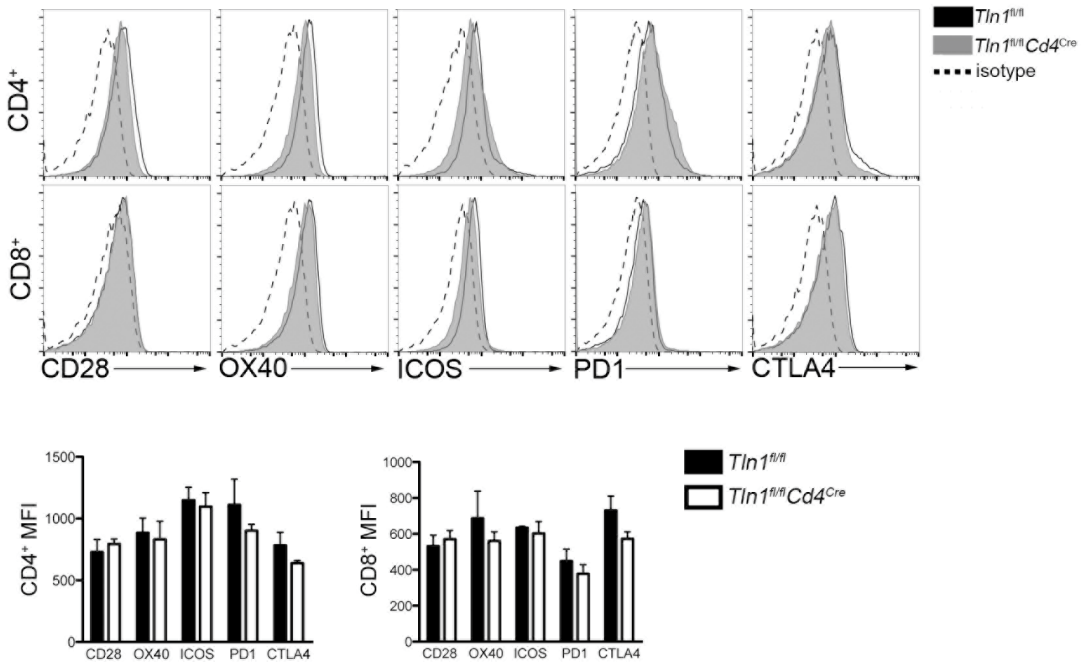

Figure 2.4 Talin is not required to maintain the expression of costimulatory or inhibitory molecules on naïve T cells. Expression of the indicated co-stimulatory (CD28, OX40, or ICOS) or inhibitory molecules (PD1 or CTLA4) by naïve cells from *Tln1*$^{fl/fl}$ (black line) and *Tln1*$^{fl/fl}$*Cd4*^{Cre} (gray histogram) mice compared to isotype control (dotted line); displayed cells were gated on CD4^{+}Foxp3^{-}CD44^{lo} or CD8^{+}CD44^{lo} cells (n=3). Data shown are mean ± SEM and are representative of at least 2 independent experiments.

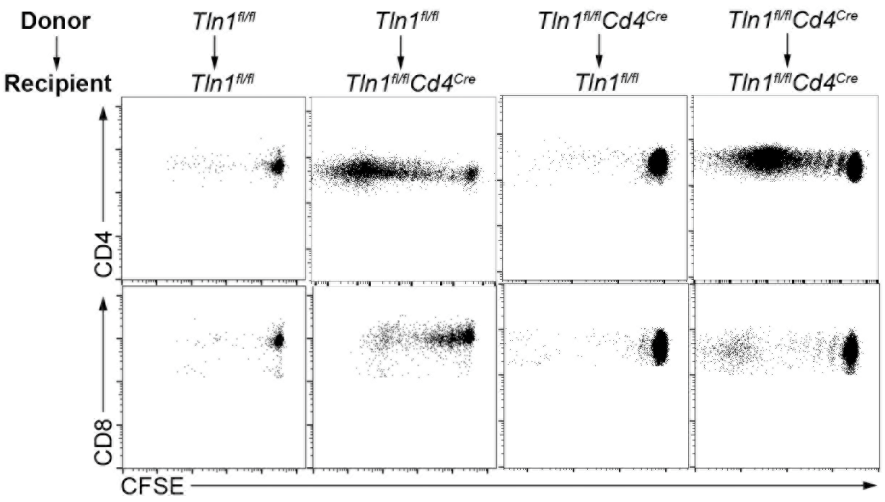

Figure 2.5 Spontaneous lymphocyte activation is controlled by factors extrinsic to naïve T cells. Adoptive transfer of CFSE-labeled CD45.2 *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Cd4*^{Cre} FACS-sorted naïve (CD44^{lo}CD62L^{hi}) T cells into CD45.1.2 *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Cd4*^{Cre} recipients, followed by flow cytometric analysis of splenocytes from recipient mice 5 days later; displayed cells were gated on CD4^{+} or CD8^{+} events. Data shown are representative of at least 2 independent experiments.

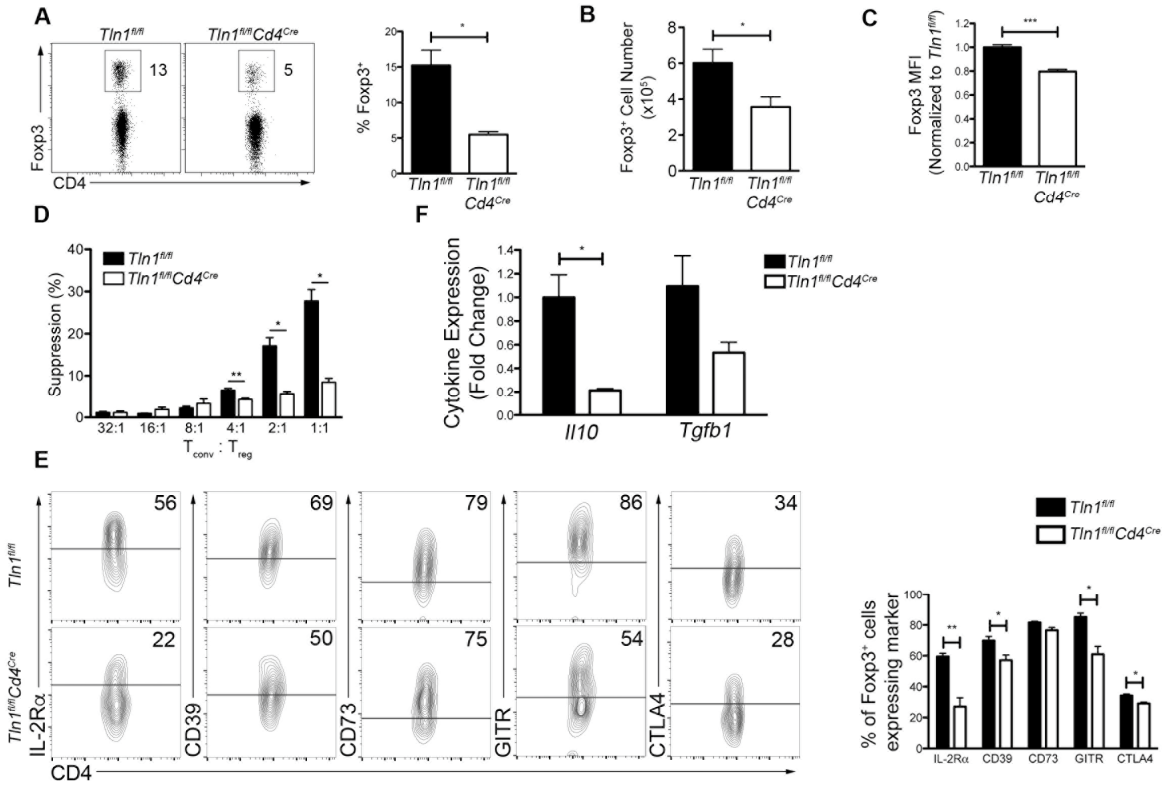

Figure 2.6 Talin deficiency leads to a reduction in Treg cell numbers and suppressive function. Frequency (**A**) and absolute number (**B**) of Treg cells isolated from the spleens of *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice; displayed cells were gated on CD4^{+} events (n=12). (**C**) Foxp3 expression on a per cell basis (mean fluorescence intensity, MFI) from Foxp3^{+}CD4^{+} splenic Treg cells (n=5). (**D**) Suppression by sorted Treg cells from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice at decreasing Tconv:Treg cell ratios, measured at 72 hours. (**E**) Expression of suppressive molecules IL-2Rα, CD39, CD73, GITR and CTLA4 on splenic Treg cells; displayed cells were gated on CD4^{+}Foxp3^{+} cells (n=5). (**F**) Quantitative real-time PCR of *Il10* and *Tgfb1* transcript expression by Treg cells isolated from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$ *Cd4*^{Cre}*Foxp3*^{GFP} mice. Cytokine mRNA expression was normalized to the abundance of *Rpl13* transcript and expressed relative to transcript abundance of control Treg cells, set to one (n=3). Data shown are mean ± SEM and are representative of at least 2 independent experiments. *, P < 0.05; **, P <0.01, ***, P <0.001.

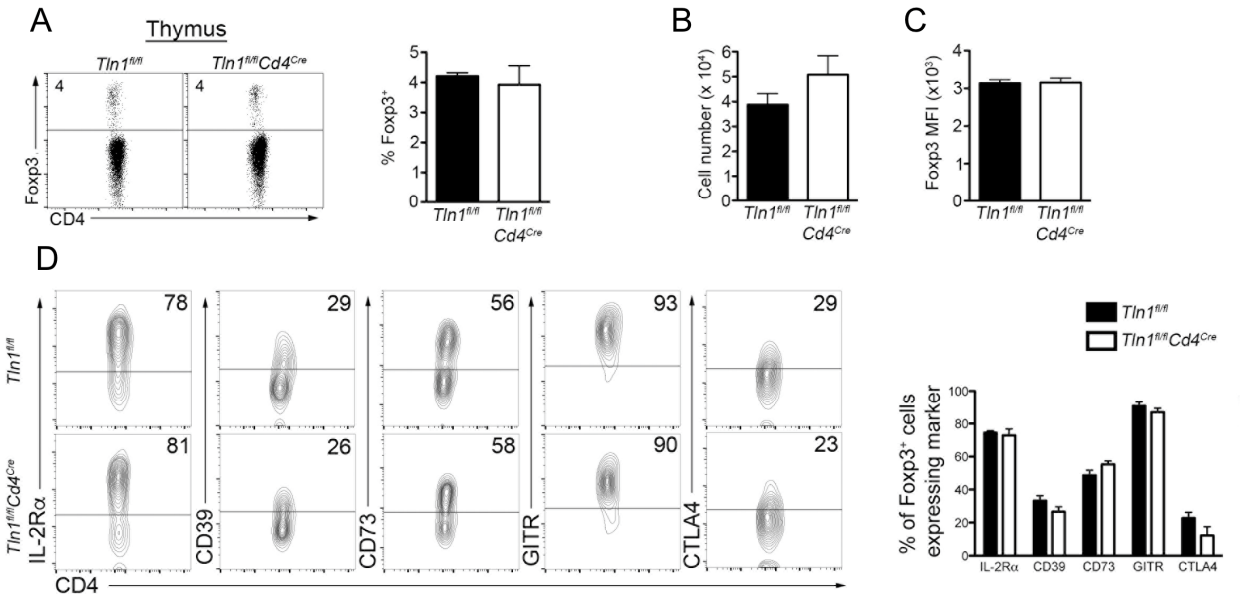

Figure 2.7 Talin is not required for development of Treg cells in the thymus. Percentage (**A**) and absolute number (**B**) of Foxp3-expressing thymic SP CD4$^+$ T cells from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice (n=3). (**C**) Foxp3 expression on a per cell basis (MFI) from Foxp3$^+$CD4$^+$ thymic Treg cells (n=3). (**D**) Expression of suppressive molecules IL-2Rα, CD39, CD73, GITR and CTLA4 on thymic Treg cells; displayed cells were gated on CD4$^+$Foxp3$^+$ cells (n=3). Data shown are mean ± SEM and are representative of at least 2 independent experiments.

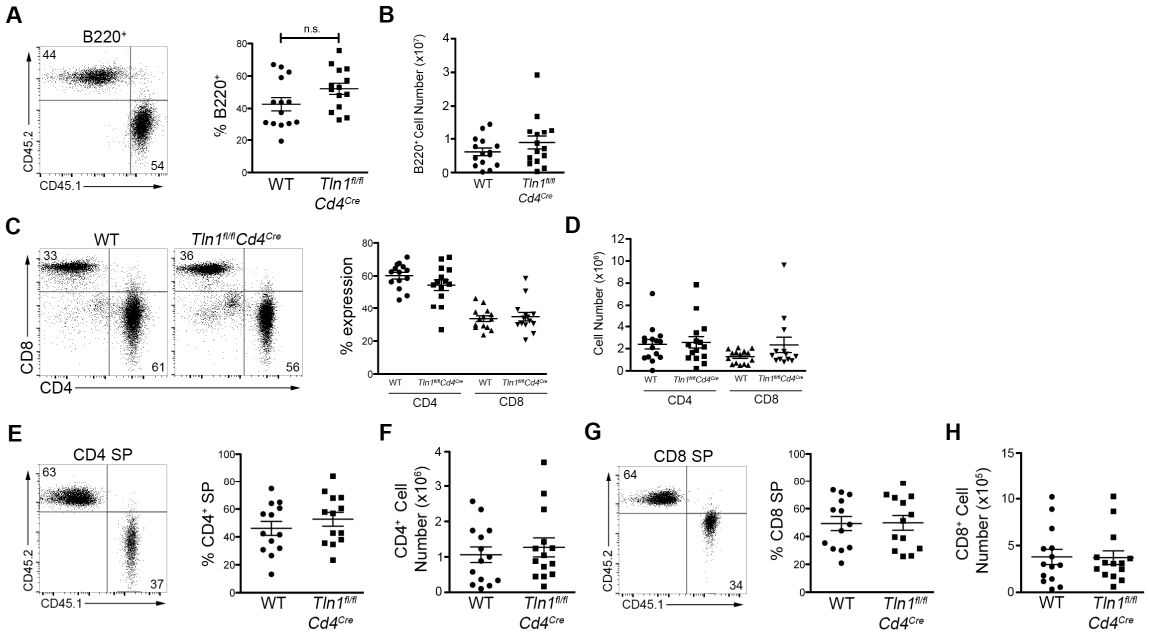

Figure 2.8 Reconstitution of mixed bone marrow chimeras. Analysis of mixed bone marrow chimeras reconstituted with bone marrow from *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice (CD45.2) and *Tln1*$^{fl/fl}$ wild-type mice (CD45.1) (n=14). Mice were analyzed by flow cytometry 8-10 weeks after reconstitution. (**A-H**) Frequency (**A**) and absolute number (**B**) of B220^{+} B cells. Frequency (**C**) and absolute numbers (**D**) of splenic CD4^{+} and CD8^{+} T cells. Frequency (**E**) and absolute numbers (**F**) of CD4 SP cells isolated from the thymus. Frequency (**G**) and absolute numbers (**H**) of CD8 SP cells isolated from the thymus. Data shown are mean ± SEM and are representative of at least 3 independent experiments.

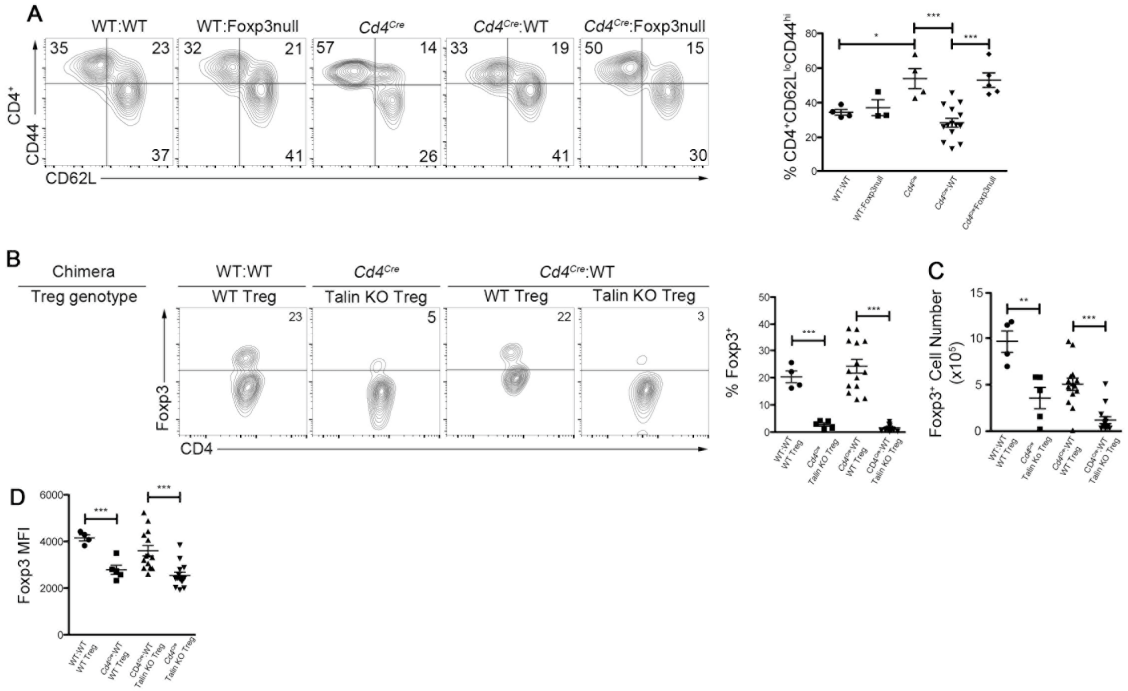

Figure 2.9 Spontaneous lymphocyte activation in Tln1fl/flCd4Cre mice is Treg cell-dependent. (**A-D**) Lethally irradiated RAG1-deficient mice were reconstituted with bone marrow cells from a CD45.2 wild-type donor in combination with cells from a CD45.1 wild-type donor (WT:WT, n=4), wild-type (CD45.2) bone marrow cells in combination with Foxp3-deficient (CD45.1) bone marrow cells (WT:Foxp3null, n=3), bone marrow cells from *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice (CD45.2) alone (*Cd4*^{Cre}, n=5), bone marrow cells from *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice (CD45.2) in combination with wild-type (CD45.1) bone marrow cells (*Cd4*^{Cre}:WT, n=14), and bone marrow cells from *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice (CD45.2) in combination with Foxp3-deficient deficient (CD45.1) bone marrow cells (*Cd4*^{Cre}:Foxp3-null, n=5). Mice were analyzed by flow cytometry 8-10 weeks after reconstitution. (**A**) Activation status of splenic CD4$^+$ T cells based on CD62L and CD44 expression; displayed cells were gated on CD4$^+$CD45.1$^+$ events in WT:WT, WT:Foxp3null, *Cd4* ^{Cre}:WT and *Cd4* ^{Cre}:Foxp3null bone marrow chimeric mice and on CD4$^+$CD45.2$^+$ events in *Cd4*^{cre} bone marrow chimeras. (**B**) Percentage of splenic CD4$^+$ cells expressing Foxp3$^+$; displayed cells were gated on CD4$^+$CD45.2$^+$ cells to identity talin-deficient Treg cells in *Cd4*^{cre} and *Cd4* ^{Cre}:WT bone marrow chimeras and on CD4$^+$CD45.1$^+$ cells in WT:WT and *Cd4* ^{Cre}:WT chimeras to identity wild-type Treg cells. (**C**) Absolute numbers of talin-deficient splenic CD4$^+$CD45.2$^+$ Foxp3$^+$ Treg cells in *Cd4*^{cre} and *Cd4*^{cre}:WT bone marrow chimeras and wild-type CD4$^+$CD45.1$^+$ Foxp3$^+$ cells in WT:WT and *Cd4* ^{cre}:WT chimeras. (**D**) Foxp3 expression on a per cell basis from talin-deficient (CD45.2$^+$) or wild-type (CD45.1$^+$) CD4$^+$Foxp3$^+$ splenic Treg cells from WT:WT, *Cd4*^{cre} and *Cd4* ^{Cre}:WT bone marrow chimeras. Data shown are mean ± SEM and are representative of at least 2 independent experiments. *, P < 0.05; **, P <0.01; ***, P<0.001.

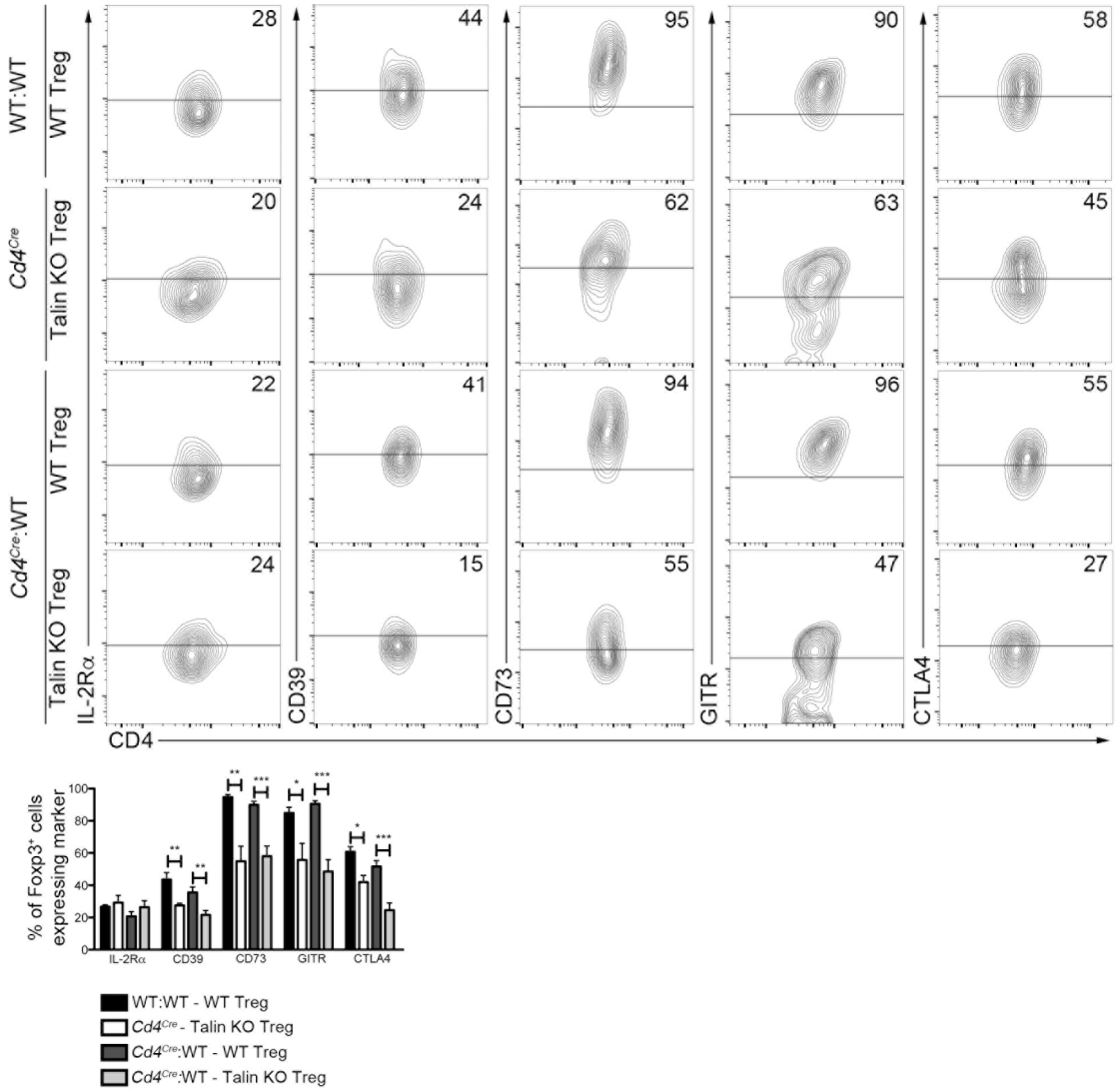

Figure 2.10 Talin deficiency leads to downregulation of multiple Treg cell suppressive molecules. Expression of suppressive molecules IL-2Rα, CD39, CD73, GITR and CTLA4 by splenic talin-deficient ($CD45.2^+$) or wild-type ($CD45.1^+$) $CD4^+Foxp3^+$ splenic Treg cells from WT:WT, *Cd4*^{cre} and *Cd4* ^{cre}:WT bone marrow chimeras (see Fig. 1.9). Data shown are mean ± SEM and are representative of at least 2 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

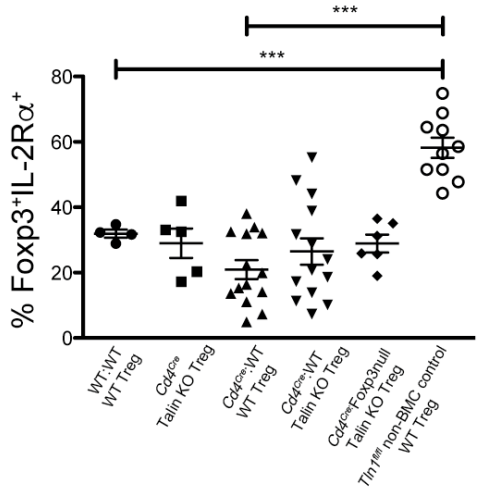

Figure 2.11 Analysis of IL-2Rα expression in bone marrow chimeric mice. Expression of IL-2Rα in Treg cells from each individual bone marrow chimera is shown (see Fig. 1.9); displayed cells were gated on $CD4^+CD45.2^+Foxp3^+$ cells to identify talin-deficient Treg cells in *Cd4*^{Cre}, *CD4*^{Cre}:WT and *Cd4*^{Cre}:Foxp3null mice, and gated on $CD4^+CD45.1^+Foxp3^+$ cells to identify wild-type Treg cells in WT:WT and *Cd4*^{Cre}:WT chimeras, compared to IL-2Rα expression in Treg cells from *Tln1*$^{fl/fl}$ control mice, gated on $CD4^+Foxp3^+$ cells (n=10). Data shown are mean ± SEM. *, $P < 0.05$; **, $P < 0.01$.

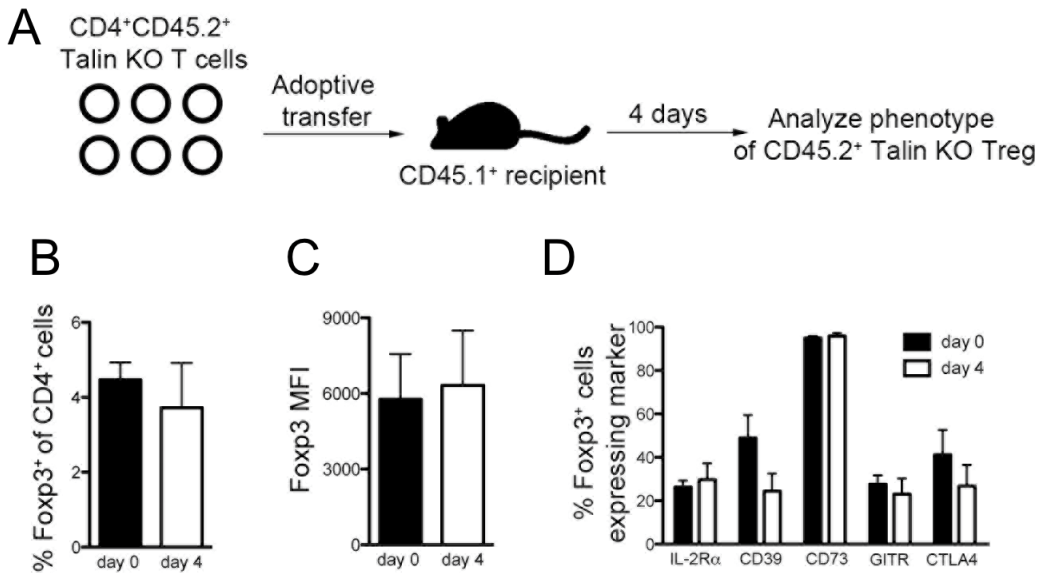

Figure 2.12 Phenotypic defects in talin-deficient Treg cells are cell intrinsic. (**A**) Experimental approach for the adoptive transfer of $CD4^+CD45.2^+$ T cells isolated from *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice into $CD45.1^+$ wild-type recipients. Expression (**B**) and MFI (**C**) of Foxp3 in talin-deficient $CD4^+CD45.2^+$ Treg cells isolated from the spleens of recipient mice. (**D**) Expression of IL-2Rα, CD39, CD73, GITR and CTLA4 by $CD4^+CD45.2^+GFP^+$ Treg cells isolated from the spleen of recipient mice. Data shown are mean ± SEM and are representative of at least 2 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

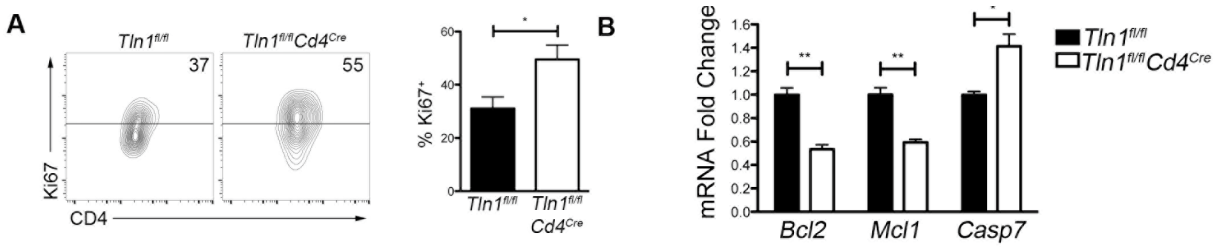

Figure 2.13 Talin-deficient Treg cells have an apoptotic gene signature. (**A**) Ki67 expression in splenic Treg cells; displayed cells were gated on $CD4^{+}Foxp3^{+}$ cells (n=5). (**B**) Quantitative real-time PCR of *Bcl2*, *Mcl1* and *Casp7* mRNA expression by sorted GFP^{+} Treg cells isolated from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice. mRNA expression was normalized to the abundance of *Rpl13* transcript and expressed relative to transcript abundance of control Treg cells, set to one (n=3). Data shown are mean ± SEM and are representative of at least 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

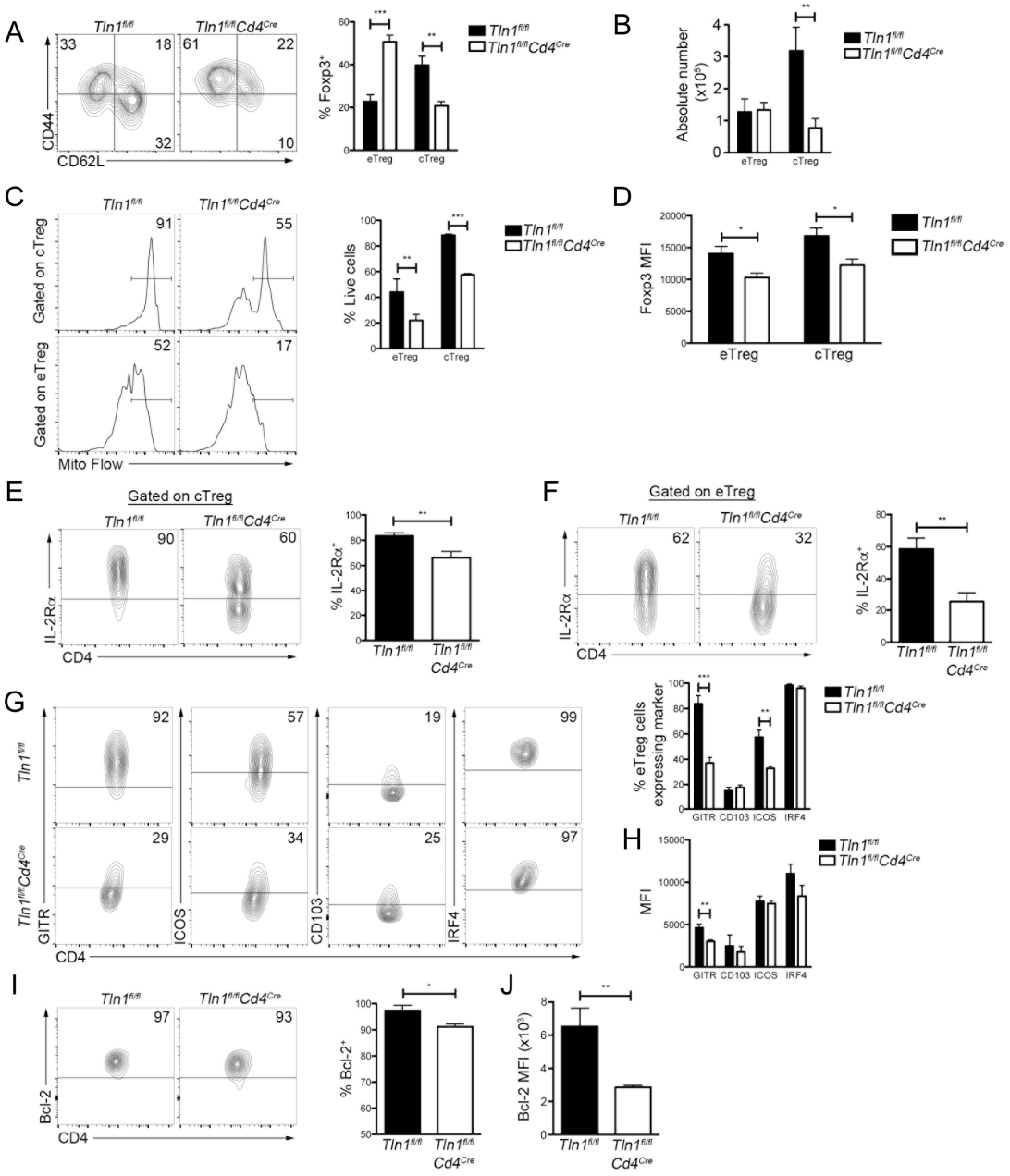

Figure 2.14 Talin is required for the homeostasis of cTreg and eTreg cells. Frequency (**A**) and absolute number (**B**) of eTreg cells ($CD62L^{lo}CD44^{hi}$) and cTreg cells ($CD62^{hi}CD44^{lo}$) isolated from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice (n=6). (**C**) Frequency of live $CD4^{+}Foxp3^{+}$ eTreg and cTreg cells assessed by Mito Flow staining for mitochondrial membrane potential (n=3). (**D**) Foxp3 expression on a per cell basis (MFI) in eTreg and cTreg cells (n=6). IL-2Rα expression by cTreg (**E**) and eTreg (**F**) cells (n=6). Frequencies of cells expressing GITR, ICOS, CD103, and IRF4 (**G**) and per cell expression (MFI) of these molecules (**H**) in eTreg cells isolated from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice (n=6). Frequencies of cells expressing Bcl-2 (**I**) and per cell expression (MFI) of Bcl-2 (**J**) in cTreg cells isolated from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice (n=6). Data shown are mean ± SEM and are representative of at least 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

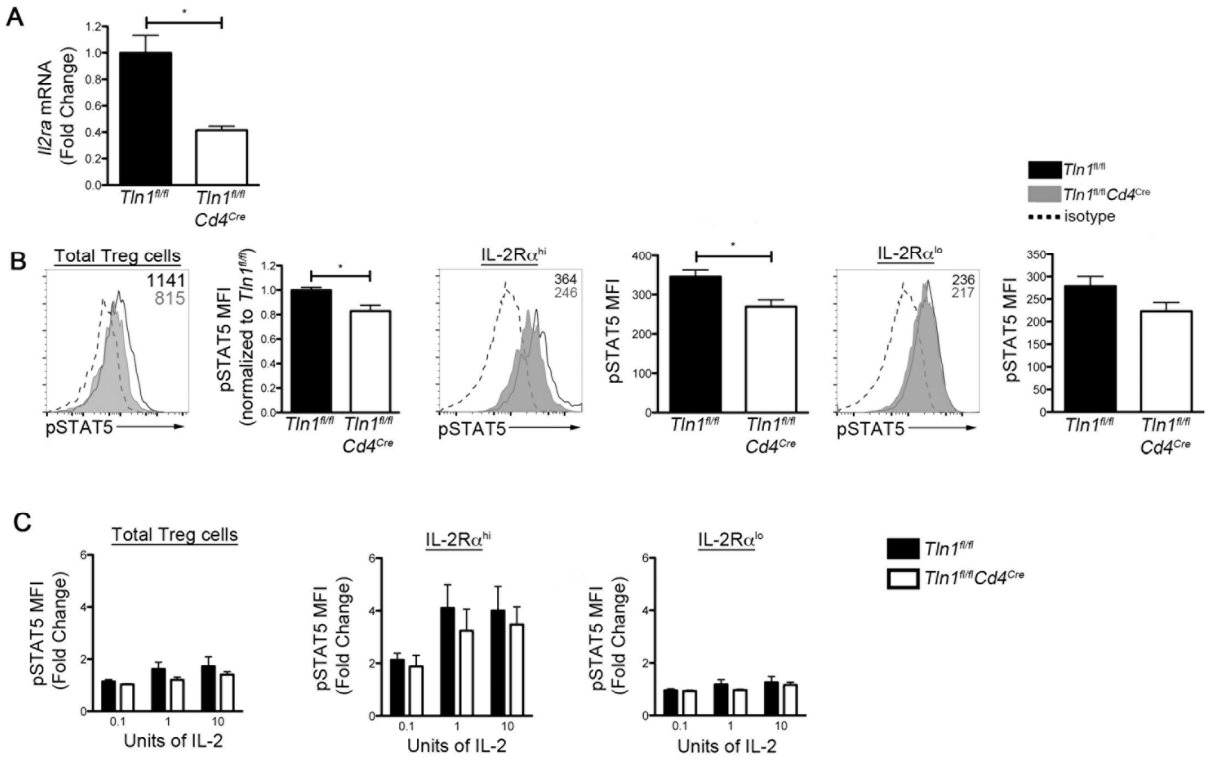

Figure 2.15 Talin-deficient Treg cells display defects in IL-2 signaling. (**A**) Quantitative real-time PCR of *Il2ra* transcript expression by GFP$^+$ Treg cells isolated from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$ *Cd4*^{Cre}*Foxp3*^{GFP} mice. mRNA expression was normalized to the abundance of *Rpl13* transcript and expressed relative to transcript abundance of control Treg cells, set to one (n=3). (**B**) *Ex vivo* pSTAT5 expression represented as histograms (left panel) and MFI (right panel) by total, IL-2Rα^{hi}, or IL-2Rα^{lo} Treg cells isolated from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} or *Tln1*$^{fl/fl}$ *Cd4*^{Cre}*Foxp3*^{GFP} mice (n=3). (**C**) MFI of pSTAT5 expression after *in vitro* IL-2 stimulation (0.1, 1, or 10 U) in Treg cells isolated from *Tln1*$^{fl/fl}$*Foxp3*^{GFP} (black line) or *Tln1*$^{fl/fl}$*Cd4*^{Cre}*Foxp3*^{GFP} mice (gray histogram) compared to isotype control (dotted line); displayed cells were gated on CD4$^+$Foxp3$^+$ cells (n=3). Data shown are mean ± SEM and are representative of at least 3 independent experiments. *, $P < 0.05$.

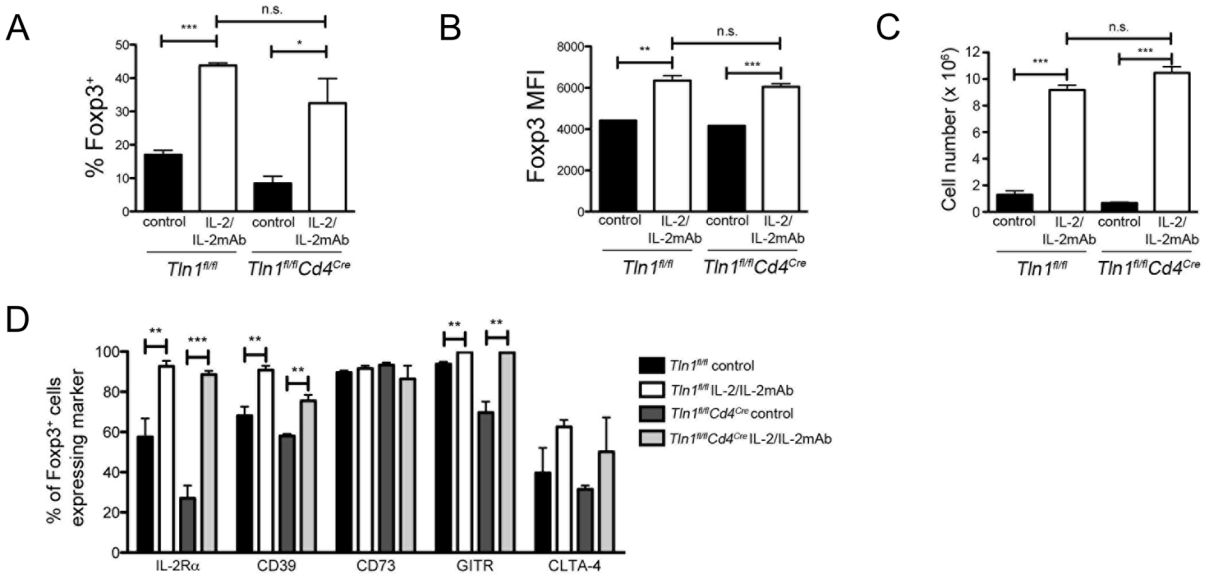

Figure 2.16 Talin-deficient Treg cells are rescued with IL-2/IL-2mAb complex treatment. Frequency (**A**), Foxp3 MFI (**B**) and absolute number (**C**) of Foxp3$^+$ Treg cells derived from *Tln1$^{fl/fl}$Foxp3^{GFP}* and *Tln1$^{fl/fl}$Cd4^{Cre}Foxp3^{GFP}* mice given isotype or IL-2/IL-2 mAbs complexes (n=3). (**D**) Expression of suppressive molecules IL-2Rα, CD39, CD73, GITR and CTLA4 by Foxp3$^+$ Treg cells derived from *Tln1$^{fl/fl}$Foxp3^{GFP}* and *Tln1$^{fl/fl}$Cd4^{Cre}Foxp3^{GFP}* mice given isotype- or IL-2/IL-2 mAbs complexes; displayed cells were gated on CD4$^+$Foxp3$^+$ cells (n=3). Data shown are mean ± SEM and are representative of at least 3 independent experiments. *, P < 0.05; **, P <0.01; ***, P<.001

CHAPTER III: INTEGRIN ACTIVATION IS REQUIRED FOR TREG CELL-MEDIATED MAINTENANCE OF IMMUNE HOMEOSTASIS

3.1 Introduction

Because we had previously shown that talin is essential for Treg cell survival and function in a T cell-specific deletion model, we wanted to more specifically explore how talin is essential for Treg cell homeostasis utilizing a mouse model in which talin is deleted from Treg cells. Using this more specific model, we worked to understand the exact role for talin in Treg cells, minimizing the effects of the deletion of the protein in other cell types. Because talin is known to have multiple functions, including facilitating the final stages of integrin activation, tethering integrins to the actin cytoskeleton, and recruiting signaling molecules to facilitate integrin signaling, we also aimed to understand what function of talin is required for Treg cells.

3.2 Results

3.2.1 Spontaneous lethal inflammation in mice with a Treg cell-specific deletion of talin.

To investigate the possibility that talin plays a role in Treg cell function, we generated mice in which talin was deleted selectively in Treg cells, using a previously described *Foxp3*^{Cre} mouse strain (61). Strikingly, male *Tln1*$^{fl/fl}$*Foxp3*^{Cre} hemizygous mice developed systemic autoimmunity that manifested as runting, failure to thrive, dermatitis, lymphadenopathy, splenomegaly, and lymphocytic infiltration into multiple organs, ultimately resulting in death by 2-3 months of age (**Fig. 3.1**). These mice exhibited increased percentages of $CD4^+$ and $CD8^+$ T cells that displayed an activated ($CD62L^{lo}CD44^{hi}$) phenotype, were highly proliferative based on Ki67 expression, and were capable of high levels of IFNγ and TNFα production (**Figure 3.2**).

Autoimmune and inflammatory disorders can result from either a deficiency in the number or function of Treg cells. However, male *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice harbored elevated numbers of Foxp3^{+} cells in the spleen (**Fig. 3.3A, 3.3B**), suggesting that deletion of talin did not decrease the size of the Treg cell population. As integrin signaling is required for T cell trafficking throughout the body, we hypothesized that deletion of talin might prevent Treg cells from homing to organs, resulting in a numerical deficiency that could account for the severe autoimmunity observed in these mice. However, Treg cells were readily detectable in the thymus, blood, liver and lung (**Fig. 3.3C**).

We next assessed the phenotype and suppressive function of talin-deficient Treg cells in male *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice. Notably, talin-deficient Treg cells expressed less Foxp3 on a per cell basis than wild-type Treg cells (**Fig. 3.2D**). Analysis of the expression of putative Treg cell suppressive molecules revealed that with the exception of IL-2Rα, other suppressive molecules including CD103, CTLA4, CD39, CD73, and GITR remained at wild-type levels (**Fig. 3.4A**). Nonetheless, despite normal expression of most suppressive molecules, talin-deficient Treg cells isolated from male *Tln1*$^{fl/fl}$*Foxp3*^{Cre} animals exhibited significantly impaired suppressive capacity *in vitro* (**Fig. 3.4B**). In addition, talin-deficient Treg cells produced significantly higher levels of IFNγ compared to wild-type Treg cells (**Fig. 3.4C**), suggesting the possibility that a subset of Foxp3^{+} Treg cells may have acquired an inflammatory phenotype as a consequence of talin deficiency. Taken together, these results reveal that expression of talin is essential for Treg cells to maintain immune homeostasis by facilitating their suppressive function.

3.2.2 Activated integrins in Treg cells are required to maintain peripheral tolerance.

We next sought to investigate the mechanisms by which talin influences Treg cell function. Talin has many binding partners and exerts multiple functions, including binding to and activating integrins, linking integrins to the cytoskeleton, and recruiting signaling molecules such as phosphatidylinositol phosphate kinase (10) and TIAM1 (11) to focal adhesions. The talin(L325R) mutation has been previously shown to inhibit the capacity of talin to activate integrins without disrupting the binding of talin to integrins or affecting the interaction of talin with other known binding partners (12, 52, 53). We therefore generated *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} hemizygous male mice to selectively abolish the capacity of talin to activate integrins in Treg cells. Strikingly, *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice developed systemic autoimmunity resembling that exhibited by male hemizygous *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice (**Fig. 3.5A-C**). These mice exhibited increased numbers of activated, proliferating $CD4^+$ and $CD8^+$ T cells (**Fig. 3.5D, 3.5F**). Moreover, like talin-deficient Treg cells, talin(L325R) Treg cells were not numerically deficient (**Fig. 3.6A, 3.6B**), and were detectable at high numbers in the blood, thymus and peripheral organs (**Fig. 3.6C**). Additionally, Treg cells isolated from *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice exhibited lower Foxp3 expression on a per cell basis (**Fig. 3.6D**). With respect to suppressive molecules, talin(L325R) Treg cells only exhibited impaired IL-2Rα expression (**Fig. 3.7A**), similar to talin-deficient Treg cells. Moreover, talin(L325R) Treg cells isolated from *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice exhibited impaired *in vitro* suppressive capacity compared to control Treg cell counterparts (**Fig. 3.7B**), similar to talin-deficient Treg cells. Together these data indicate that integrin activation, controlled by talin, plays a critical role in mediating Treg cell suppressive function and immune homeostasis.

3.3 Discussion

We suggested in Chapter II that expression of talin is required for Treg cell function and homeostasis using a model of T cell-specific deletion. Here we definitely show that talin is required for Treg cell-mediated maintenance of immune homeostasis, as its deletion leads to lethal systemic inflammation in $Tln1^{fl/fl}Foxp3^{Cre}$ male mice. We go on to show that it is specifically talin's role in integrins activation that is required in Treg cells, as $Tln1^{L325R/fl}Foxp3^{Cre}$ male mice, also succumb to systemic inflammation and exhibit similar pathology to $Tln1^{fl/fl}Foxp3^{Cre}$ male mice. The $Tln1^{fl/fl}Cd4^{Cre}$ animals described in Chapter II most likely did not develop systemic autoimmunity because talin is deleted in all $CD4^+$ and $CD8^+$ T cells, perhaps impairing the ability of these cells to mediate tissue damage. Together these models provide novel evidence that integrin signaling is specifically required in Treg cells for effective maintenance of immune tolerance.

Chapter III, in full, is an adapted version of the material that has been submitted for publication. **Klann JE**, Kim SH, Remedios KA, He Z, Metz PJ, Lopez J, Tysl T, Olvera JG, Ablack JN, Cantor JM, Boland BS, Yeo G, Zheng Y, Lu L, Bui JD, Ginsberg MH, Petrich BG, Chang JT. 2018. Integrin activation controls regulatory T cell-mediated peripheral tolerance. The dissertation author was the primary author of all material.

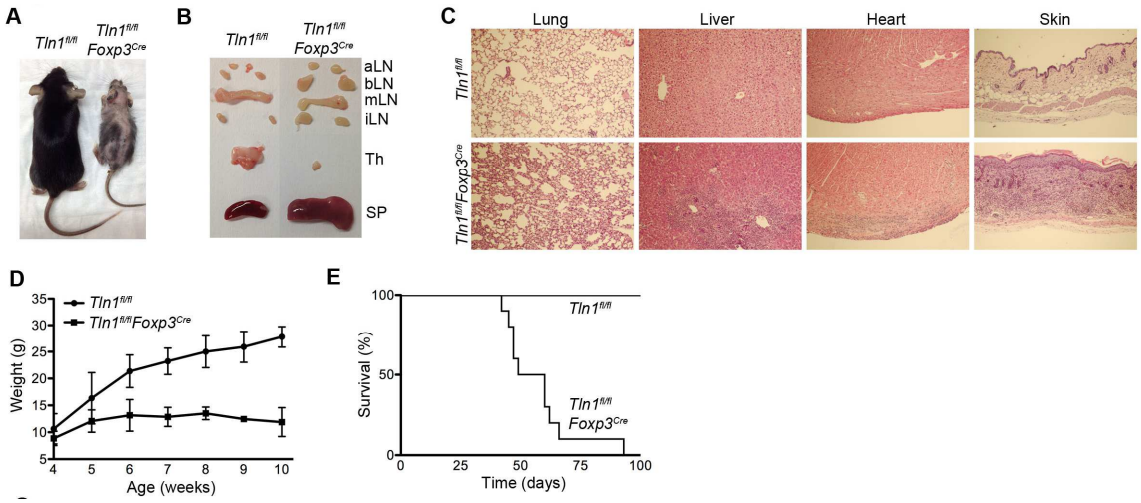

Figure 3.1 Treg cell-specific deletion of talin leads to lethal systemic inflammation. (A and B) Morphology (A) or organs (B) (spleen, lymph nodes (aortic, brachial, mesenteric, inguinal), and thymi) of male *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice. (C) H&E stains of lung, liver, heart, and skin tissue from *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice. (D and E) Body weight from 4-10 weeks of age (D) and survival (E) of *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice; n=10. Data are mean ± SEM and representative of at least 2 independent experiments.

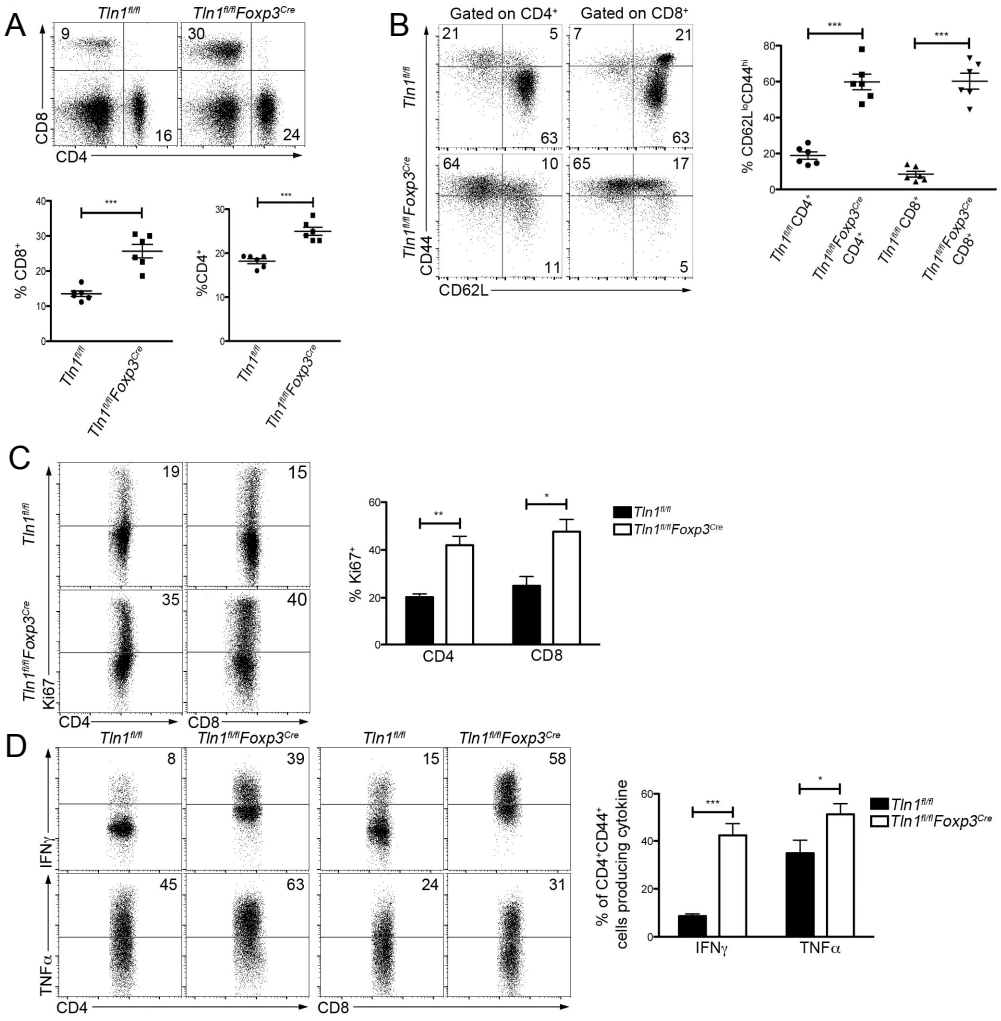

Figure 3.2 Treg cell-specfic deletion of talin leads to hyper-activation of T cells. (A-C) Percentages of splenic $CD4^+$ or $CD8^+$ T cells (A) expressing CD44, CD62L (B), or Ki-67 (C) from *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice; displayed cells gated on $CD4^+$ or $CD8^+$ events; n=6. (D) IFNγ and TNFα expression by splenic $CD4^+$ (left) and $CD8^+$ (right) T cells from *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice; displayed cells gated on $CD4^+CD44^{hi}$ or $CD8^+CD44^{hi}$ events; n=6. Data are mean ± SEM and representative of at least 2 independent experiments. *, $P < 0.05$; **, $P < 0.01$, *** $P < 0.005$.

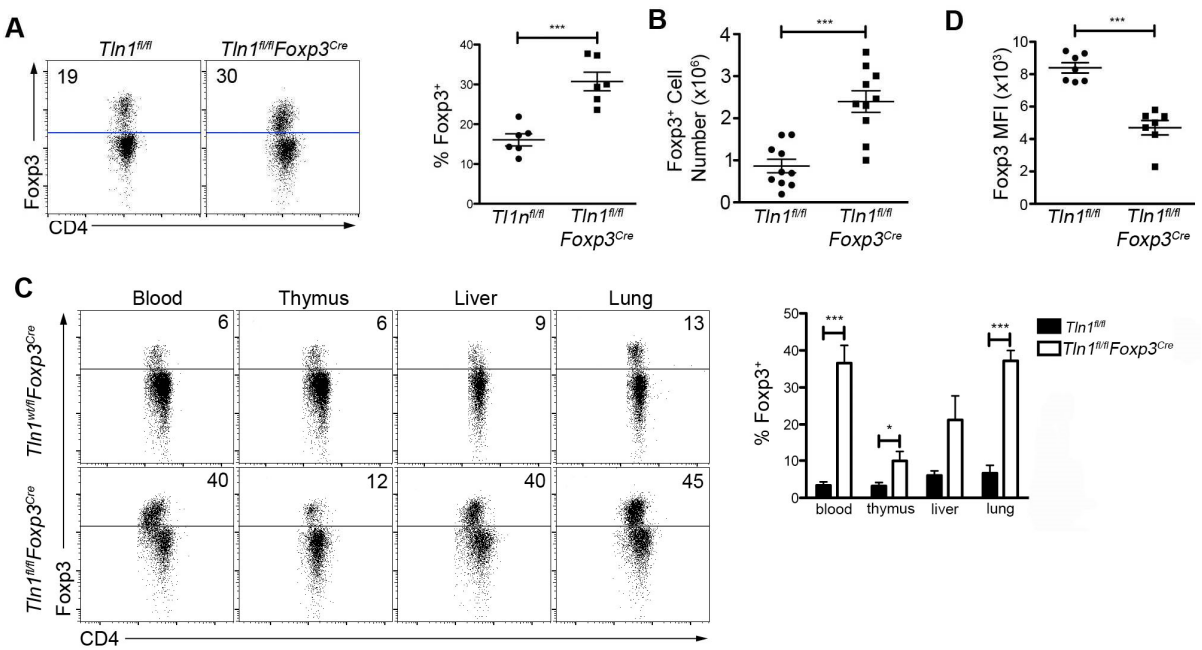

Figure 3.3 Treg cell-specific deletion of talin does not lead to a numerical defect in the Treg cell pool. (A-B) Percentages (n=6) (A) and numbers (n=10) (B) of splenic $CD4^+$ T cells from *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice expressing Foxp3. (C) $Foxp3^+$ cells as a percentage of $CD4^+$ T cells within tissues (blood, thymus, liver, lung and lymph node) derived from *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice; n=6. (D) Mean fluorescence intensity of Foxp3 from talin-deficient and wild-type Treg cells; n=7. Data are mean ± SEM and representative of at least 2 independent experiments. *, $P < 0.05$; **, *** $P < 0.005$.

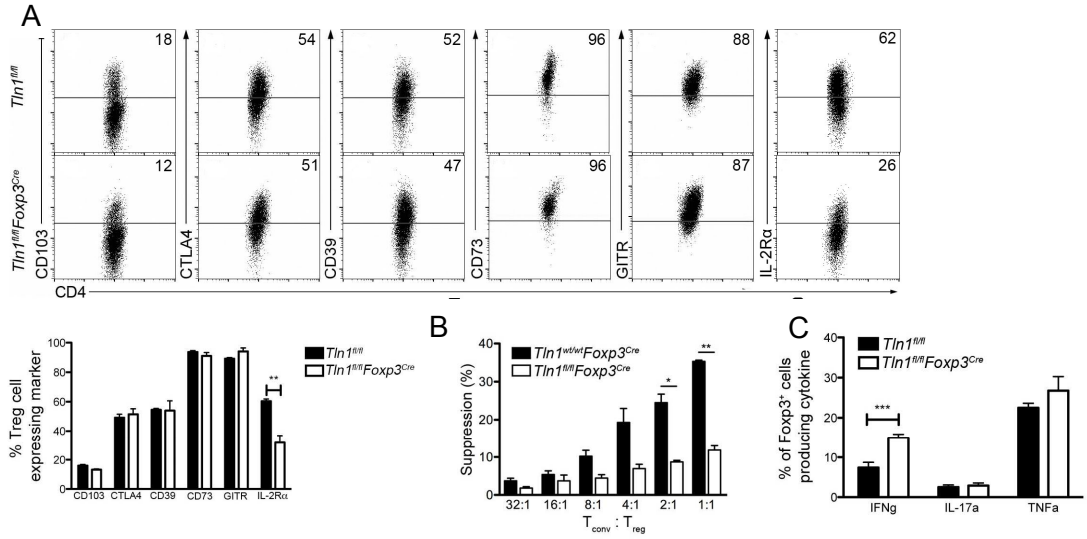

Figure 3.4 Talin deficiency decreases Treg cell suppressive capacity. (A) Expression of putative suppressor molecules on Treg cells from *Tln1*$^{fl/fl}$ or *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice; displayed cells gated on CD4^{+}Foxp3^{+} cells; n=6. (B) Suppression by sorted YFP^{+} Treg cells from *Foxp3*^{Cre} or *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice at decreasing T_{conv}:T_{reg} cell ratios, measured at 72 hours. (cC) Percentages of splenic Treg CD4^{+}YFP^{+} cells expressing IFNγ, IL-17A and TNFα; n=6. Data are mean ± SEM and representative of at least 2 independent experiments. *, P < 0.05; **, P <0.01, *** P <0.005.

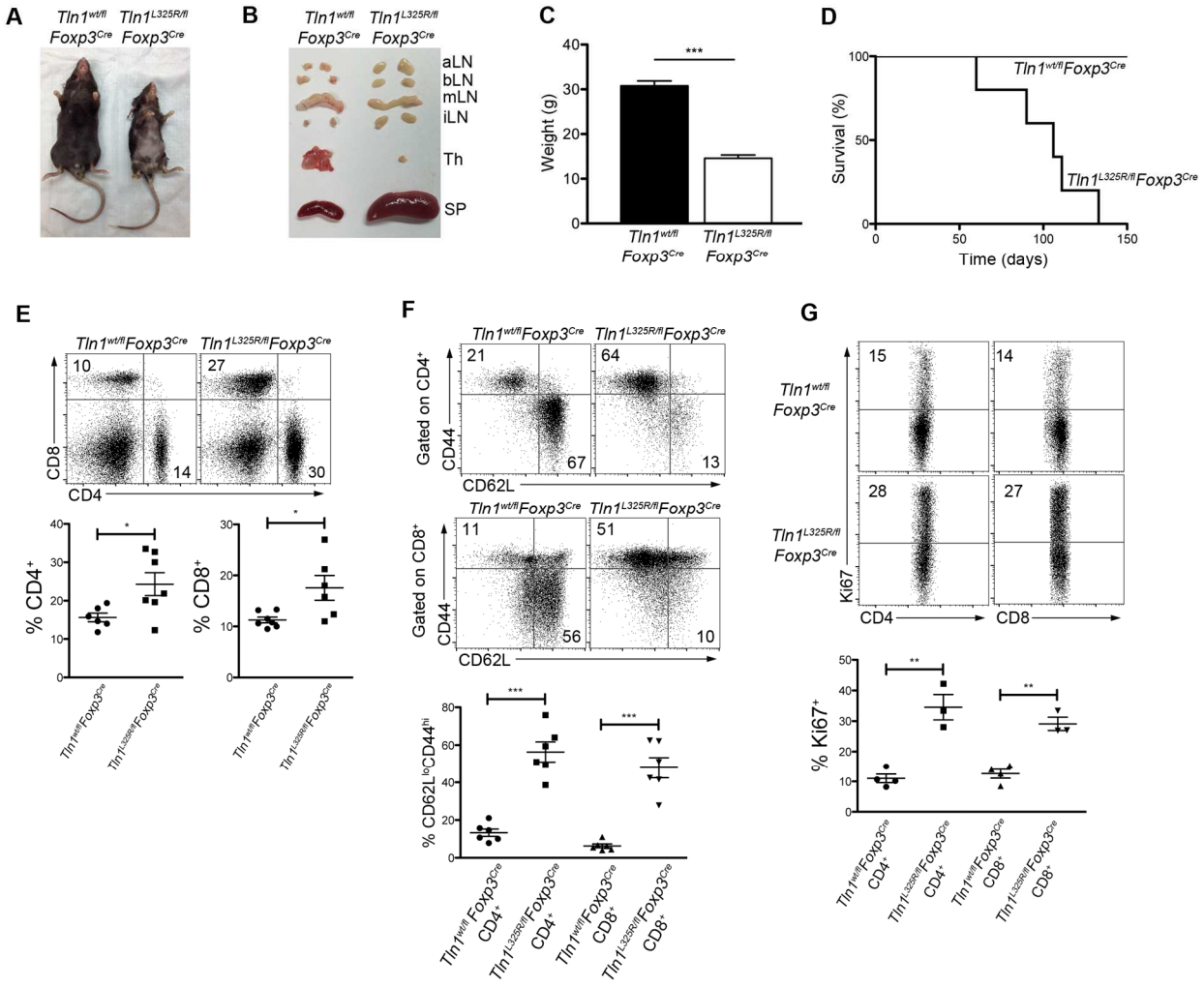

Figure 3.5 Expression of mutant talin1(L325R) in Treg cells results in lethal systemic inflammation. (A and B) Morphology (A) or organs (B), spleen, lymph nodes (aortic, brachial, mesenteric, inguinal), and thymi) of male *Tln1*$^{wt/fl}$*Foxp3*^{Cre} or *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice. (C) Body weight of *Tln1*$^{wt/fl}$*Foxp3*^{Cre} or *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice at 8 weeks of age; n=10. (D) Survival of *Tln1*$^{wt/fl}$*Foxp3*^{Cre} (n=5) or *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} (n=5) mice. (E) Percentages of splenic CD4^{+}and CD8^{+} T cells; n=6. (F) Expression of activation markers CD44 and CD62L from splenic CD4^{+}and CD8^{+} T cells; n=6. (G) Expression of proliferation marker Ki-67 from splenic CD4^{+}and CD8^{+} T cells; n=3. Data are mean ± SEM and representative of at least 2 independent experiments. *, P < 0.05; **, P <0.01, *** P <0.005.

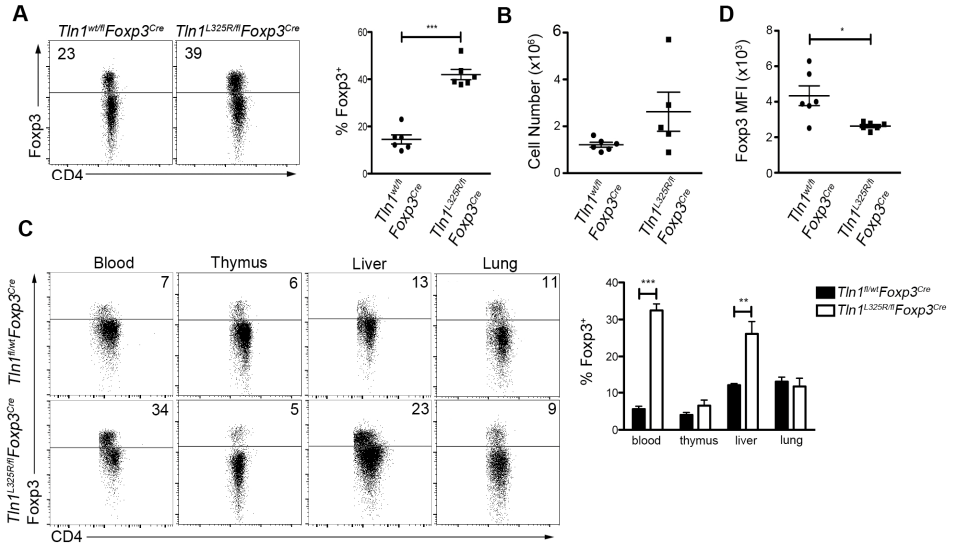

Figure 3.6 Integrin activation is not required to maintain the size of the Treg cell pool. (A-B) Percentage (A) and by number (B) of splenic CD4$^+$ T cells from *Tln1*$^{wt/fl}$*Foxp3*^{Cre} or *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice expressing intracellular Foxp3; n=6. (C) Foxp3$^+$ cells as a percentage of CD4$^+$ T cells in various tissues (blood, thymus, liver, lung, and lymph node) from *Tln1*$^{wt/fl}$*Foxp3*^{Cre} or *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice; n=6. (D) MFI of Foxp3 from CD4$^+$Foxp3$^+$ Treg cells; n=6. Data are mean ± SEM and representative of at least 2 independent experiments. *, P < 0.05; **, P <0.01, *** P <0.005.

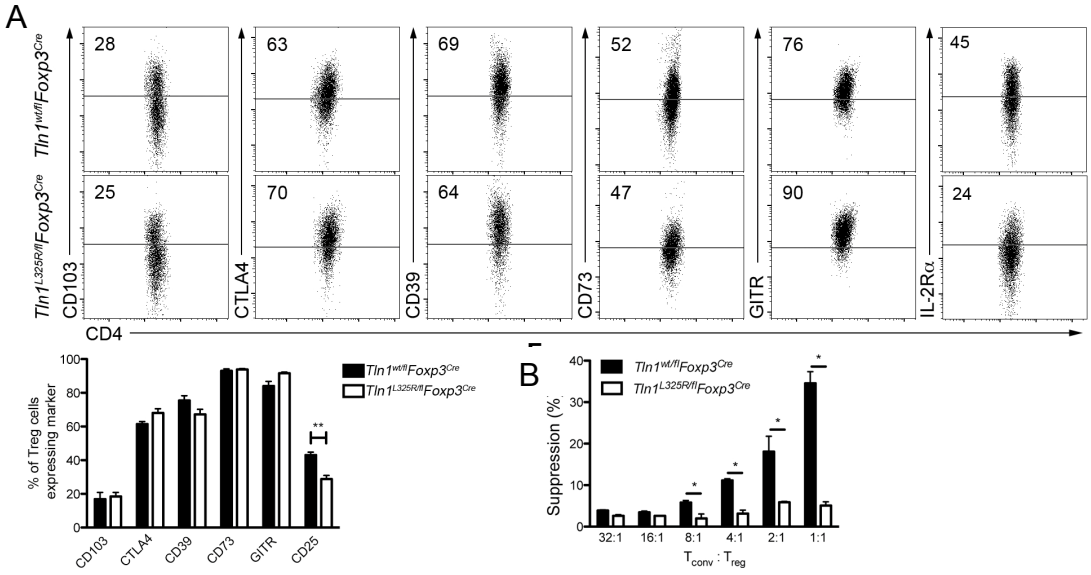

Figure 3.7 Integrin activation is critical for Treg cell phenotype and function. (A) Expression of putative suppressor molecules on Treg cells from *Tln1*$^{wt/fl}$*Foxp3*^{Cre} or *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice; displayed cells gated on CD4^{+}Foxp3^{+}; n=4. (B) Suppression by sorted YFP^{+} Treg cells from *Tln*$^{wt/fl}$*Foxp3*^{Cre} and *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice at decreasing T_{conv}:T_{reg} cell ratios, measured at 72 hours. Data are mean ± SEM and representative of at least 2 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

CHAPTER IV: TALIN'S ROLE IN INTEGRIN ACTIVATION IS REQUIRED FOR THE MAINTENANCE OF TREG CELL PHENOTYPE AND TRANSCRIPTIONAL SIGNATURE IN THE PERIPHERY

4.1 Introduction

Because the inflammatory environment within diseased male *Tln1*$^{fl/fl}$*Foxp3*^{Cre} hemizygous mice may have altered Treg cell phenotype and function, we next examined female *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ heterozygous mice which harbor both *Foxp3*^{Cre} talin-deficient (YFP^{+}) and *Foxp3*^{wt} talin-sufficient (YFP^{-}) Treg cell populations as a result of random X chromosome inactivation (**Fig. 4.1A**) (61, 62). Because these mice do not exhibit spontaneous T cell activation, due to a population of wild-type Treg cells, we can work to understand the mechanism by which talin intrinsically influences Treg cell phenotype and function, and thus understand mechanistically how integrin signaling is required for Treg cell-mediated maintenance of immune homeostasis.

4.2 Results

4.2.1 Talin influences Treg cell phenotype and function.

Owing to the presence of wild-type Treg cells, *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ heterozygous female mice appeared healthy without evidence of systemic T lymphocyte activation (**Fig. 4.1B**), enabling us to examine talin-deficient Treg cells in the absence of inflammation. Like their counterparts in diseased male mice, peripheral *Foxp3*^{Cre} talin-deficient (YFP^{+}) Treg cells derived from the spleens of healthy female heterozygous mice were present in numbers comparable to control Treg cells (**Fig. 4.1C**). To exclude the possibility that Cre expression may adversely reduce Foxp3 expression, we normalized the Foxp3 mean fluorescence intensity (MFI) of talin-deficient

Treg cells to the Foxp3 MFI of talin-sufficient Treg cells isolated from the same individual animals (63).

Consistent with our observations in inflamed *Tln1*$^{fl/fl}$*Foxp3*^{Cre} male mice, we observed a reduction in Foxp3 expression on a per cell basis in talin-deficient Treg cells isolated from uninflamed female mice (**Fig. 4.1D**). In addition, consistent with our hypothesis that talin is required for Treg cell function, talin-deficient Treg cells were unable to suppress T cell proliferation *in vitro* (**Fig. 4.1E**). In contrast to our observations in inflamed male mice, however, talin-deficient Treg cells isolated from female animals exhibited impaired expression of many putative suppressive molecules, including CD103, CD39, CD73, GITR and IL-2Rα (**Fig. 4.2A, 4.2B**). These data indicate that the inflammatory environment within male *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice alters the phenotype of Treg cells. Notably, only talin-deficient Treg cells isolated from the spleen, but not thymi, of healthy female *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice exhibited these defects. Talin-deficient Treg cells isolated from the thymus were present at normal numbers, expressed comparable amounts of Foxp3 to wild-type Treg cells, and did not exhibit defects in expression of any suppressive molecules, indicating that talin is dispensable for Treg cell development in the thymus (**Fig. 4.3**). Taken together, examination of uninflamed *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ female mice revealed that talin is required for maintaining high expression of many important Treg cell suppressive molecules in the periphery.

4.2.2 Talin is not required for Treg cell stability

Since we observed a consistent decrease in the expression of Foxp3 on a per cell basis when Treg cell lack functional talin, we hypothesized that Treg cells may rely on integrin signaling to maintain their stability in the periphery. Even after exposure to inflammatory signals

capable of activating naïve T cells, Treg cells are known to remain stable. Therefore, it remains an outstanding question what signals allow Treg cells to maintain expression of Foxp3 and their potent suppressive capacity, and prevent the acquisition of effector T cell function phenotypes (Vignali review). To test whether Treg cells require integrin signals in the periphery to maintain high levels of Foxp3 expression, enabling them to remain a stable suppressive population, we first examined Foxp3 gene methylation patterns. Foxp3 expression is maintained, in part, in Treg cells, through unique methylation patterns. Several regions, of the Treg cell genome, including the Treg cell-specific demethylated region (TSDR), also known as the CNS2, remain hypomethylated throughout the lifespan of a thymic-derived Treg cell. Demethylation of the TSDR, is thought to play a role in fine-tuning the stability of Foxp3 expression, and as such, we hypothesized that functional integrin signals may be required for Treg cells to maintain their hypomethylated signature in this region. Analysis of the methylation patterns of Foxp3 intron 1, a site within the TSDR, revealed that talin-deficient Treg cells, isolated from $Tln1^{fl/fl}Foxp3^{Cre/wt}$ uninflamed female mice did not exhibit increased methylation at this genetic locus (**Fig. 4.4A**). We also tested talin-deficient Treg cells isolated from $Tln1^{fl/fl}Foxp3^{Cre}$ and $Tln1^{L325R/fl}Foxp3^{Cre}$ four- to six-week-old inflamed male mice, to determine if exposure to inflammation as these mice develop induces changes to this epigenetic locus (**Fig. 4.4B**). However, talin-deficient Treg cells from male animals retained a hypomethylated signature at Foxp3 intron 1, indicating that integrin signaling is not required to maintain the hypomethylated epigenetic signature of the TSDR.

To further understand if Treg cells require peripheral integrin signal to remain stable, we then tested the expression of Foxp3 in talin-deficient Treg cells after *in vitro* activation, which has been previously shown to induce progressive loss of Foxp3 over time. Expectedly talin-

deficient Treg cells expressed lower levels of Foxp3 on a per cell basis at baseline, however talin-deficient Treg cells lost Foxp3 at a similar rate to wild-type Treg cells at 96 hrs after activation (**Fig. 4.4C**). Furthermore, we tested the stability of Foxp3 expression in talin-deficient Treg cells *in vivo*, using an established adoptive transfer model. Wild-type and talin-deficient Treg cells were transferred in RAG1 deficient hosts, along with naïve conventional T cells. Three weeks post-transfer, there were no significant differences in the Foxp3 expression of wild-type or talin-deficient Treg cells (**Fig. 4.4D**). Taken together these results reveal that peripheral integrin signals are not required to maintain stable expression of Foxp3.

4.2.3 Talin influences key aspects of the Treg cell transcriptional program.

The observation that peripheral but not thymic derived talin-deficient Treg cells from uninflamed female *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice exhibited reduced expression of suppressive molecules raised the possibility that talin might be required for the maintenance of the Treg cell identity and transcriptional programming. To test this hypothesis, we performed RNA-sequencing analysis on YFP^{+} Treg cells isolated from heterozygous *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ or *Tln1*$^{wt/wt}$ *Foxp3*$^{Cre/wt}$ mice. 1,914 genes (454 upregulated and 1460 downregulated) were differentially expressed (adjusted p value < 0.05) in talin-deficient Treg cells compared to control cells (**Fig. 4.5**), Supplemental Table 1). Among these differentially expressed genes were several downregulated transcripts that have been previously reported to influence Treg differentiation and function. These included Treg cell 'signature' genes *Itgae* (CD103) and *Nrp1*, 'suppressor' genes *Nt5e* (CD73*), Il10,* and *Icos* (28, 54, 64, 65)*,* and transcriptional regulators such as *Id2* (31, 66, 67). Gene Ontology (GO) analysis identified differentially expressed transcripts involved in a number of critical Treg cell pathways, including cytokine and chemokine production and signaling, apoptosis, and cell

survival (**Fig. 4.6**). Moreover, there were significant changes in diverse groups of transcription factors and molecules involved in integrin signaling (Figure 6, E and F). Notably, we observed downregulation of multiple integrins including *Itgav, Itgae, Itgb1,* and *Itgb8,* indicating that talin may be required for adequate expression of multiple integrin subunits (Figure 6E). Taken together, these findings suggest that talin is required for the maintenance of diverse aspects of the Treg cell transcriptional program.

4.3 Discussion

Because inflammation can have a serve impact on cell phenotype and function, we developed a model in which we could examine talin-deficient Treg cells that have developed in the absence of inflammation by taking advantage of the fact that Foxp3 is expressed on the X chromosome. Analysis of $Tln1^{fl/fl}Foxp3^{Cre/wt}$ heterozygous female mice allowed us to dissect the mechanism by which talin is required for Treg cell mediated immune suppression. We show here that talin is definitively required for Treg cell function, partly due to impaired expression of multiple Treg cell suppressive molecules in the periphery, and partly due to global changes in the genetic programming of the Treg cell gene signature.

Chapter IV, in part, is an adapted version of the material that has been submitted for publication. **Klann JE**, Kim SH, Remedios KA, He Z, Metz PJ, Lopez J, Tysl T, Olvera JG, Ablack JN, Cantor JM, Boland BS, Yeo G, Zheng Y, Lu L, Bui JD, Ginsberg MH, Petrich BG, Chang JT. 2018. Integrin activation controls regulatory T cell-mediated peripheral tolerance. The dissertation author was the primary author of all material.

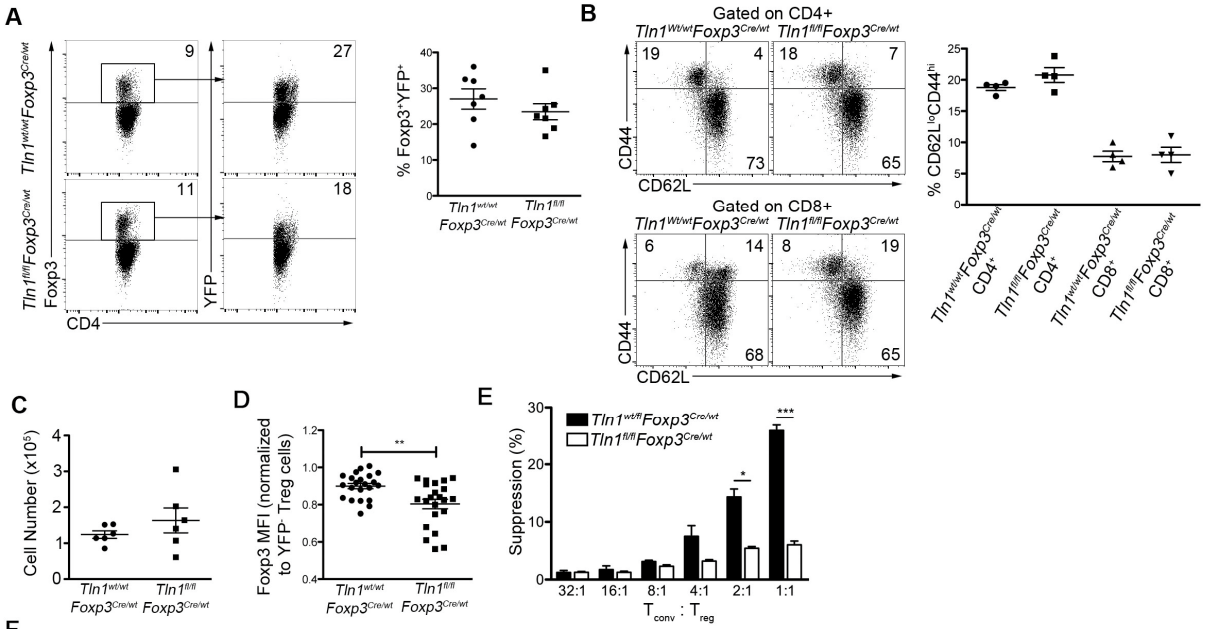

Figure 4.1 Talin controls the suppressive function of peripheral Treg cells. (A) Percentages of talin-deficient Foxp3$^+$ and YFP$^+$ Treg cells from *Tln1*$^{wt/fl}$*Foxp3*$^{Cre/wt}$ or *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice, displayed cells gated on CD4$^+$ events; n=7. (B) Percentages of splenic CD4$^+$ (top) and CD8$^+$ (bottom) T cells expressing CD44 and/or CD62L from female *Tln1*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ and *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ heterozygous mice; n=4. (C) Absolute number of Foxp3$^+$YFP$^+$ cells isolated from *Tln1*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ and *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ heterozygous mice; n=6. (D) Foxp3 MFI of Foxp3$^+$YFP$^+$ Treg cells from *Tln1*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ and *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ heterozygous mice, normalized to the Foxp3 MFI of Foxp3$^+$YFP$^-$ wild-type Treg cells from each individual animal; n=22. (E) Suppression by sorted YFP$^+$ Treg cells from *Tln1*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ or *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice at decreasing T_{conv}:T_{reg} cell ratios, measured at 72 hours. Data are mean ± SEM and representative of at least 2 independent experiments. *, P < 0.05; **, P <0.01, *** P <0.005.

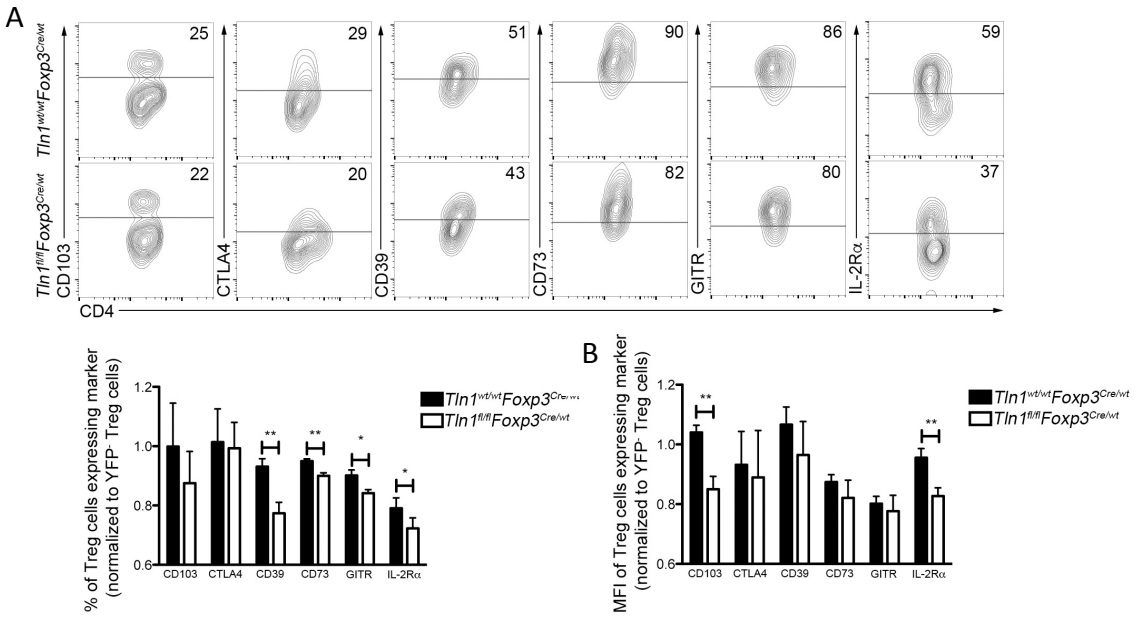

Figure 4.2 Deletion of talin alters the phenotype of Treg cells. Expression (A) and MFI (B) of putative suppressive molecules by splenic Treg cells from *Tln1*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ or *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice; displayed cells gated on CD4^{+}Foxp3^{+}YFP^{+} cells; n=5. Data are mean ± SEM and representative of at least 2 independent experiments. *, P < 0.05; **, P <0.01.

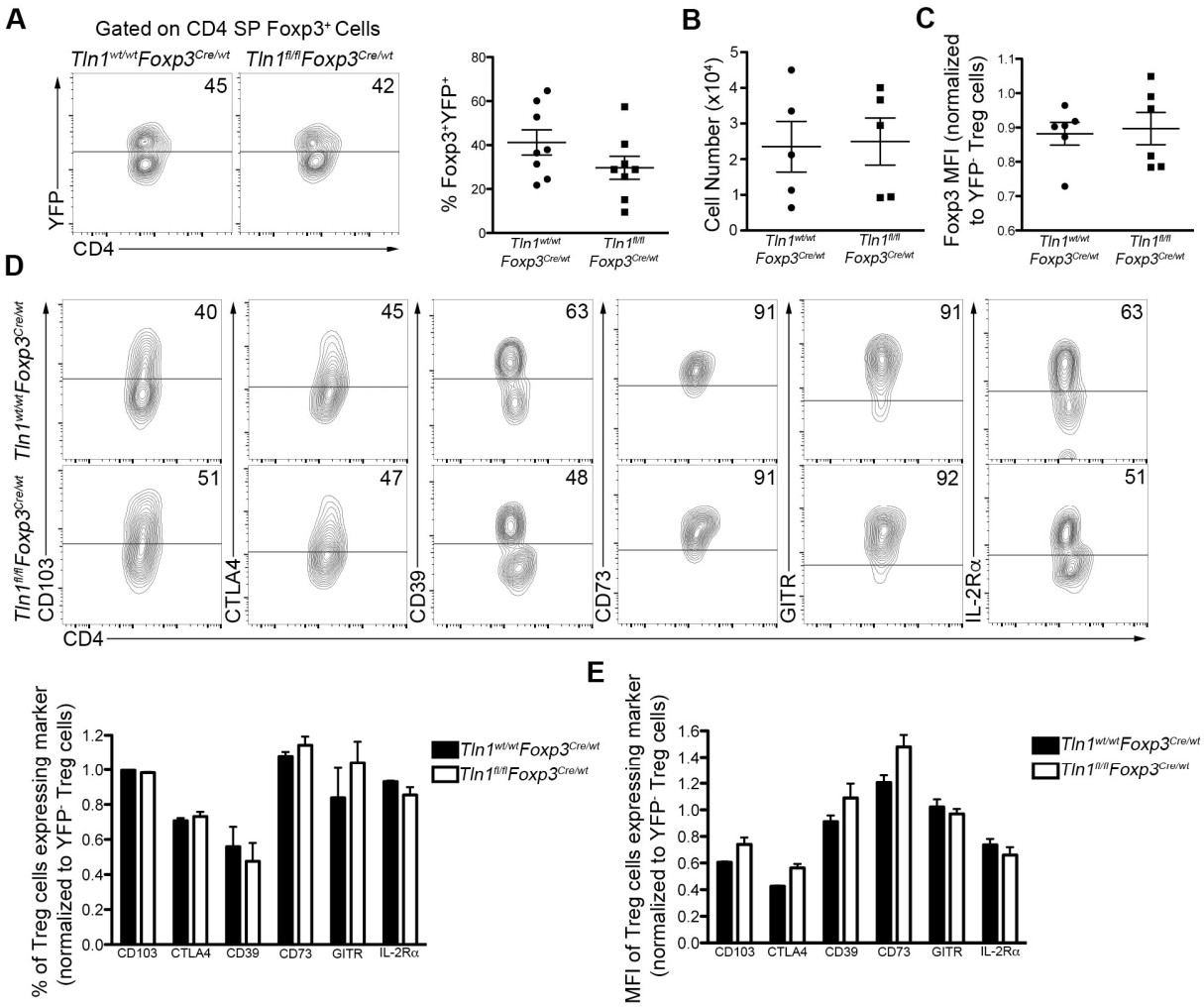

Figure 4.3 Talin is dispensable for Treg cell development in the thymus. (A) Expression of YFP in CD4 single-positive (SP) and Foxp3$^+$ thymic Treg cells; n=8. (B) Absolute number of CD4 SP Foxp3$^+$YFP$^+$ thymic Treg cells; n=5. (C) Foxp3 MFI in CD4 SP Foxp3$^+$YFP$^+$ thymic Treg cells; n=6. Expression (D) and MFI (E) of putative suppressive molecules by CD4 SP Foxp3$^+$YFP$^+$ thymic Treg cells from *Tln1$^{fl/fl}$Foxp3$^{Cre/wt}$* or *Tln1$^{wt/fl}$Foxp3$^{Cre/wt}$* mice; n=6. Data are mean ± SEM and representative of at least 2 independent experiments.

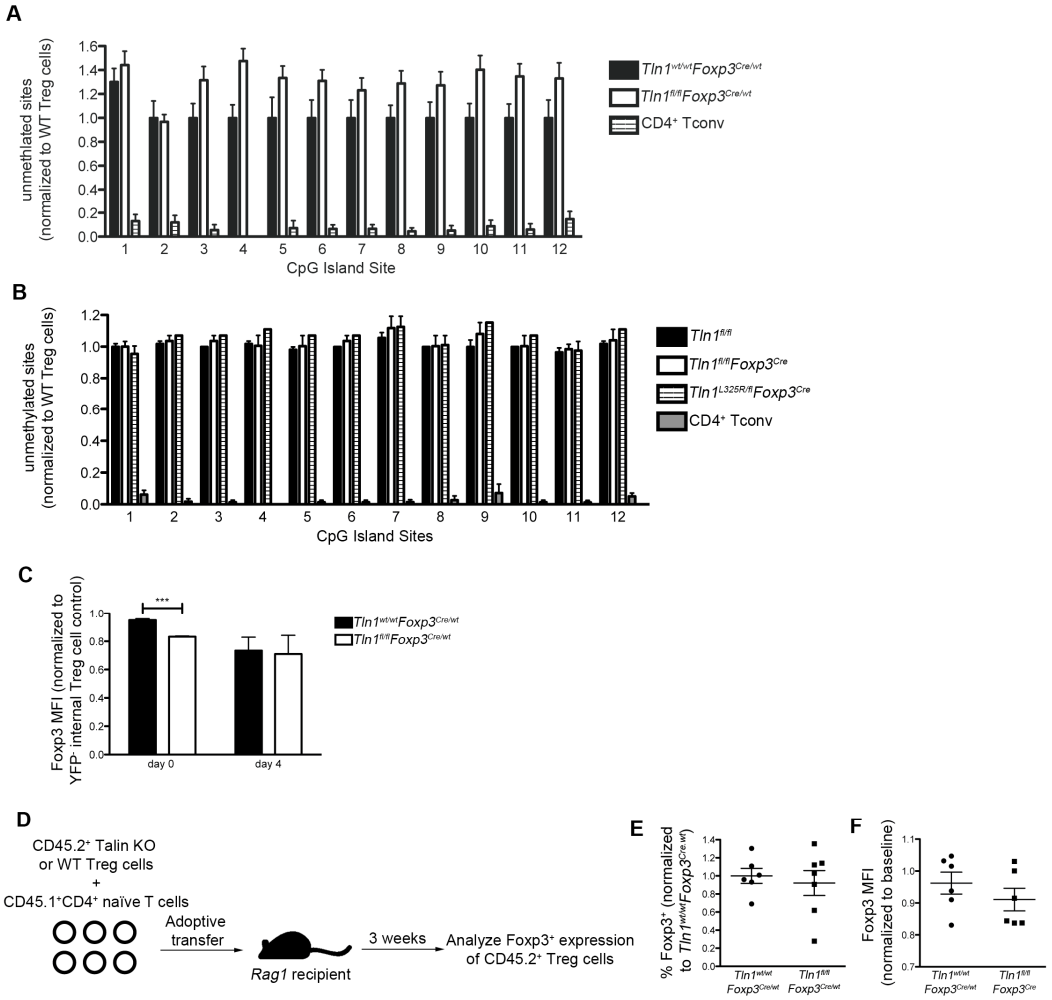

Figure 4.4 Talin is dispensable Treg cell stability in the periphery. (A and B) CpG methylation status of the 12 CpG islands within Foxp3 intron 1 in CD4$^+$YFP$^+$ Treg cells isolated from *Tln1*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ and *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice (A) and from *Tln1*$^{fl/fl}$*Foxp3*^{Cre}, *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} and *Tln1*$^{fl/fl}$ (B) compared to CD4$^+$Foxp3$^-$ conventional T cells. (C) Foxp3 MFI of CD4$^+$Foxp3$^+$YFP$^+$ cells from *Tln1*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ and *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ heterozygous mice 96 hours post *in vitro* activation with anti-CD3 and anti-CD28. (D) Experimental approach for the adoptive transfer of sorted CD45.2$^+$CD4$^+$YFP$^+$ Treg cells from *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice or *Tln1*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ mice, along with CD45.1$^+$ naïve conventional T cells into RAG1 deficient hosts. Treg cell phenotype was assessed three weeks post-transfer. (E) Percentage of CD45.2$^+$CD4$^+$ cells that maintained expression of Foxp3 after transfer; normalized to wild-type Treg cells. (F) Foxp3 MFI of Foxp3$^+$ Treg cells isolated after transfer; normalized to the MFI of respective Treg cell populations prior to transfer. Data are mean ± SEM and representative of at least 2 independent experiments. *** P <0.005.

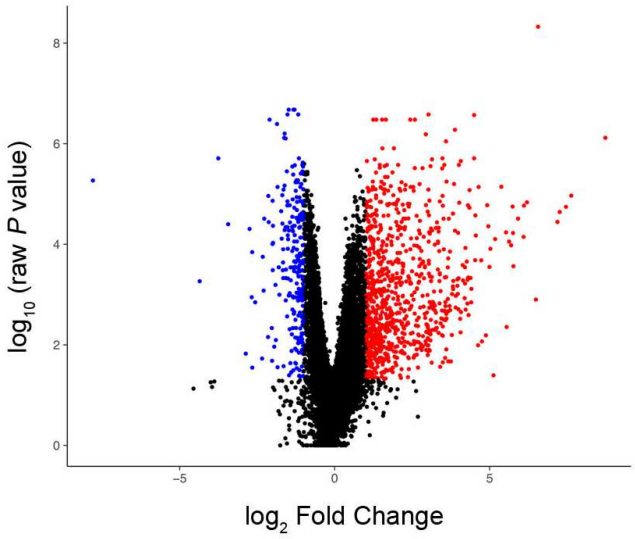

Figure 4.5 Deletion of talin causes global dysregulation in the Treg cell transcriptome. A) RNA-seq analysis of mRNA expression in sorted Treg cells isolated from $Tln^{wt/wt}Foxp3^{Cre/wt}$ (n=4) or $Tln^{fl/fl}Foxp3^{Cre/wt}$ mice (n=3), expressed as $\log_2$ normalized counts. Individual points represent genes upregulated (red), downregulated (blue), or not significantly changed (black) in talin-deficient Treg cells (adjusted P-value < 0.05) compared to controls.

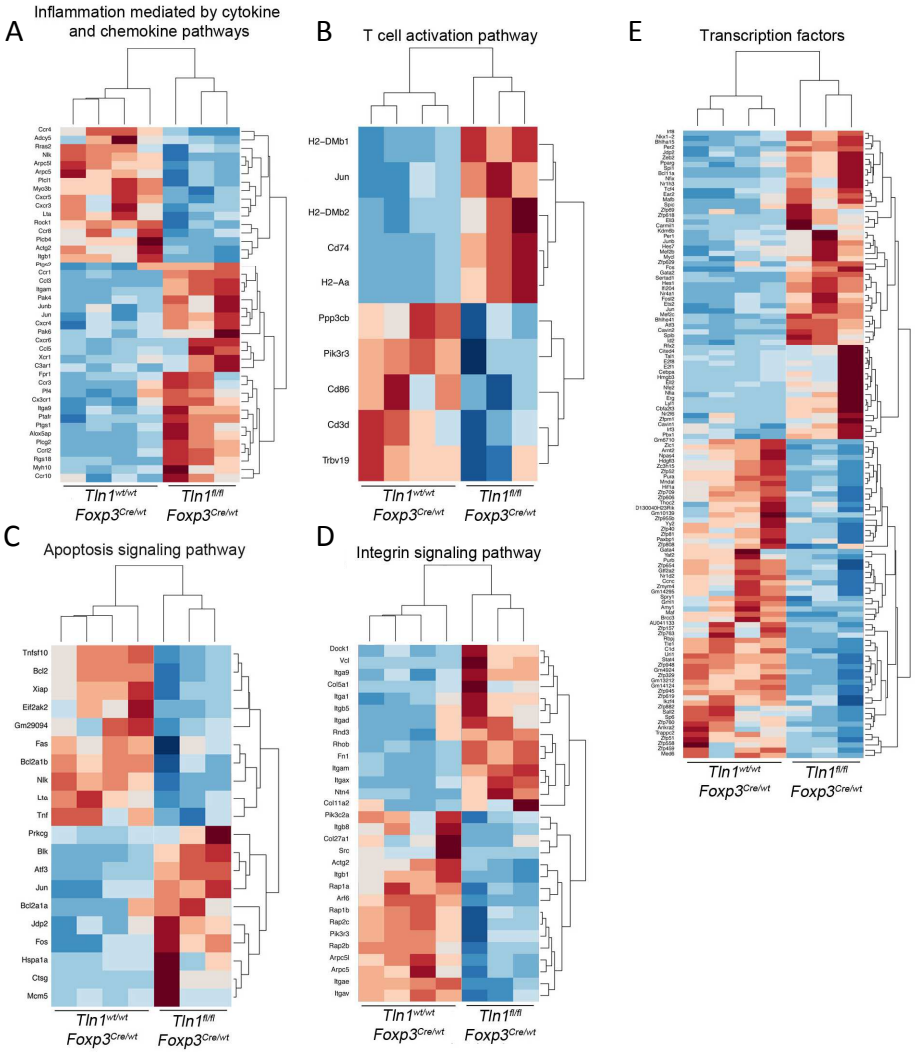

Figure 4.6 Talin modulates multiple aspects of the Treg cell transcriptome. (A-E) Heatmaps showing fold change of differentially expressed genes within selected significantly enriched GO categories from *Tln*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ (n=4) or *Tln*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice (n=3).

CHAPTER V: β2 INTEGRINS ARE REQUIRED TO MAINTAIN TREG CELL PHENOTYPE

5.1 Introduction

We demonstrated that deletion of talin has a global impact on Treg cell function, phenotype and transcriptional programming. Therefore to further understand how integrin signaling impacts Treg cells, we sought to understand the role of individual integrins in Treg cell homeostasis. Talin regulates the activation of β1, β2 and β3 integrins, therefore we aimed to understand how deletion or blockade of individual integrins may impact Treg cell function or phenotype. β1 and β2 integrins are highly expressed on T cells and are well known to facilitate trafficking during infection and to lymph nodes, respectively. To understand the role of β1 integrins in Treg cells, we created a mouse model with a Treg cell-specific deletion of β1 integrins. To understand the role of β2 integrins, we relied on *in vivo* blockade of integrin signaling as there is currently no *Itgb2*$^{fl/fl}$ mouse available.

5.2 Results

5.2.1 Deletion of β1 integrins does not disrupt Treg cell identity or function.

To investigate the role of β1 integrins in Treg cells, we generated *Itgb1*$^{fl/fl}$*Foxp3*^{Cre} hemizygous male mice. Unlike *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice, deletion of β1 integrins does not lead to spontaneous, lethal inflammation. These mice harbor normal levels of $CD4^+$ and $CD8^+$ T cells, which maintain a naïve ($CD62L^{hi}CD44^{lo}$) phenotype (**Fig. 5.1**). β1 integrin-deficient Treg cells were present at similar numbers to wild-type Treg cells, and there were no observed defects in the expression of Foxp3 or any putative suppressive markers (**Fig 5.2**). Taken together, these results reveal that singular deletion of β1 integrins does not alter the identify or function of Treg cells.

5.2.2 Blockade of LFA-1 interactions with ICAM-1 reduces the expression of Foxp3 and other putative Treg cell suppressive molecules

To understand if β2 integrin signaling is required for the maintenance of Treg cells function or phenotype, we administered isotype or blocking anti-LFA-1 and anti-ICAM-1 mAbs to Foxp3 GFP reporter mice for three weeks. LFA-1/ICAM-1 blockade led to a significantly decreased frequency of Treg cells in the spleen (**Fig. 5.3A**) without changing the absolute number of Treg cells (**Fig. 5.3B**), presumably due to altered T cell trafficking to the lymph nodes. However, Treg cells isolated from LFA-1/ICAM-1 mAb-treated mice exhibited a reduction in Foxp3 expression on a per cell basis (**Fig. 5.3C**). Additionally, LFA-1/ICAM-1 blockade resulted in a decrease in the frequency of Treg cells expressing high levels of IL-2Rα (**Fig 5.3D**), as well as a reduction in the expression of IL-2Rα on a per cell basis (**Fig. 5.3E**). Additionally, LFA-1/ICAM-1 blockade led to a downregulation of Treg cells expressing CD39, CD73, GITR, and defects in the per cell expression of CD103, CTLA4, CD73 and GITR (**Fig. 5.4**). This downregulation mirrored the defects observed in talin-deficient Treg cells isolated from $Tln1^{fl/fl}Foxp3^{Cre/wt}$ heterozygous female mice. Taken together these results provide evidence that functional signaling through LFA-1 is required for the maintenance of Treg cell identity.

We also addressed this question using a reductionist *in vitro* system in which we incubated sorted wild-type Treg cells alone or the presence of $CD11c^+$ dendritic cells (DCs). When Treg cells are left alone for 18 hours, the expression of IL-2Rα is significantly reduced when compared to baseline levels. Incubation with DCs rescued this decrease in IL-2Rα. Notably, blockade of LFA-1 and ICAM-1 with mAbs prevented this upregulation of IL-2Rα (**Fig. 5.5**), providing specific evidence that ICAM-1 expressed on DCs may be required to activate integrins on Treg cells in order for them to maintain their phenotype in the periphery.

5.3 Discussion

Here we set out to understand how individual integrin subunits may play a role in Treg cell homeostasis. Our results reveal that deletion of β1 integrins alone does not disrupt Treg cell phenotype or function, and does not result in spontaneous lethal inflammation. It is an interesting possibility that *Itgb1*$^{fl/fl}$*Foxp3*^{Cre} mice may be able to compensate for the lack of β1 integrins, through the upregulation or increased functionality of other integrin subunits, and this may warrant further study of these animals. Our data also indicate that β2 integrins are essential for the maintenance of Treg cell phenotype. Transient blocking of LFA-1 expressed on Treg cells and ICAM-1 expressed on DCs, endothelial or other presenting cell types leads to a change in Foxp3 expression, as well as changes to many of the putative suppressive molecules expressed by Treg cells. Overall, our work shows a novel role for β2 integrin signaling in maintaining Treg cell phenotype and homeostasis in the periphery.

Chapter V, in part, is an adapted version of the material published in the *Journal of Immunology*. **Klann JE**, Remedios KA, Kim SH, Metz PJ, Lopez J, Mack LA, Zheng Y, Ginsberg MH, Petrich BG, Chang JT. Talin Plays a Critical Role in the Maintenance of the Regulatory T Cell Pool. *J Immunol.* 2017;198(12):4639-51. The dissertation author was the primary author of all material.

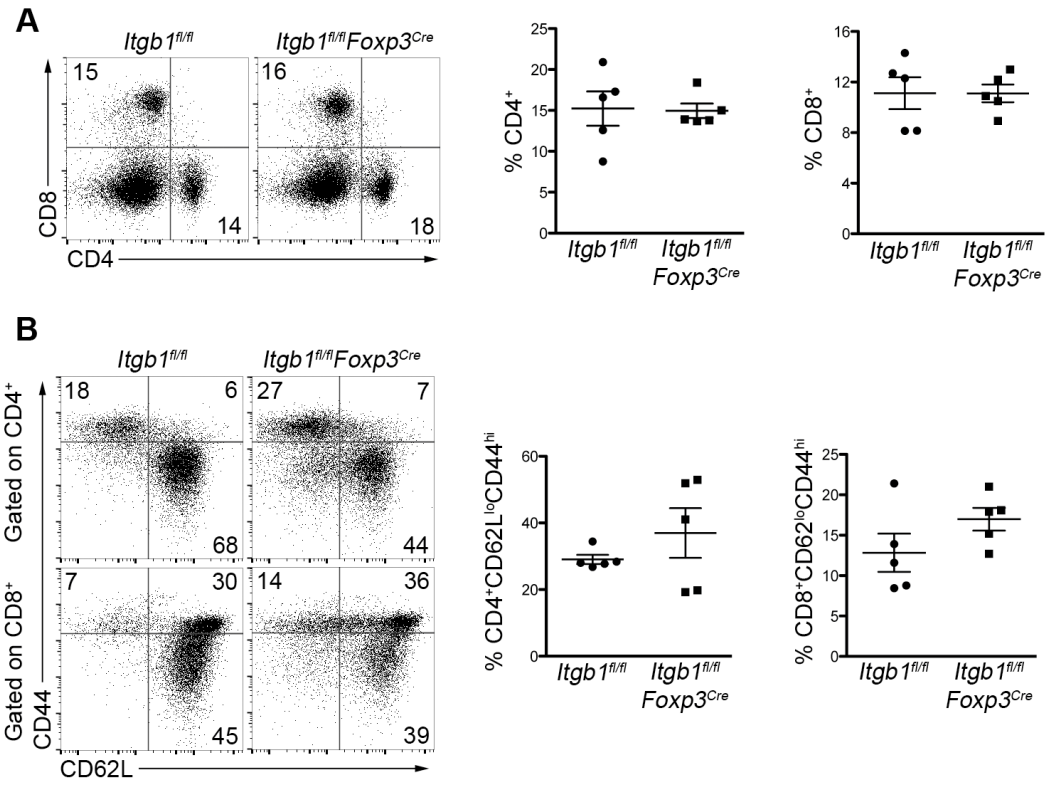

Figure 5.1 Treg cell-specific deletion of integrin β1 does not alter the activation state of T cells. Analysis of *Itgb1*$^{fl/fl}$*Foxp3*^{Cre} and *Itgb1*$^{fl/fl}$ male mice. (A) Percentages of CD4$^+$ and CD8$^+$ T cells. (B) Percentages of activated CD62L^{lo}CD44^{hi} CD4$^+$ and CD8$^+$ T cells. Data are representative of at least 2 independent experiments.

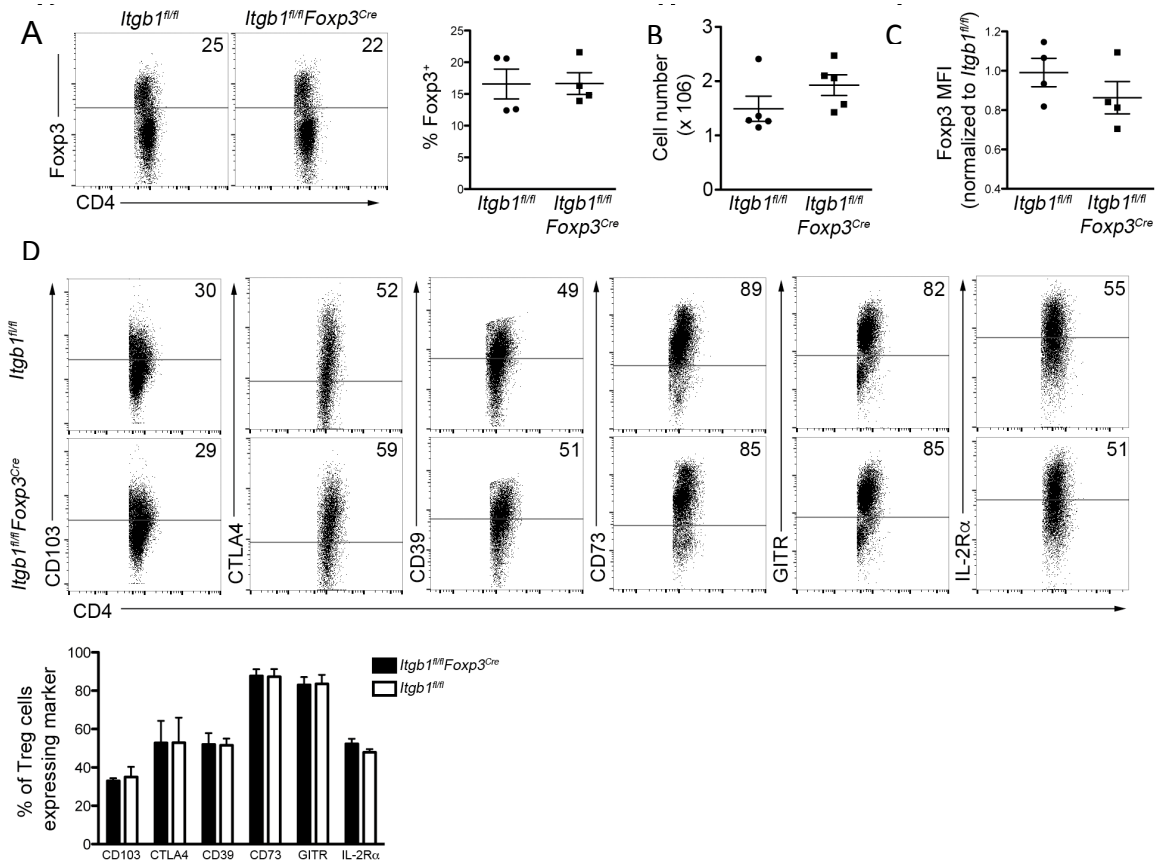

Figure 5.2 Treg cell-specific deletion of integrin β1 the phenotype of Treg cells. Percentage (C) and absolute number (D) of $CD4^+$ cells expressing Foxp3. (E) Foxp3 MFI of $CD4^+Foxp3^+$ Treg cells. (F) Percentage of $CD4^+Foxp3^+$ Treg cells expressing putative suppressive molecules, CD103, CTLA4, CD39, CD73, GITR and IL-2Rα. Data are representative of at least 2 independent experiments.

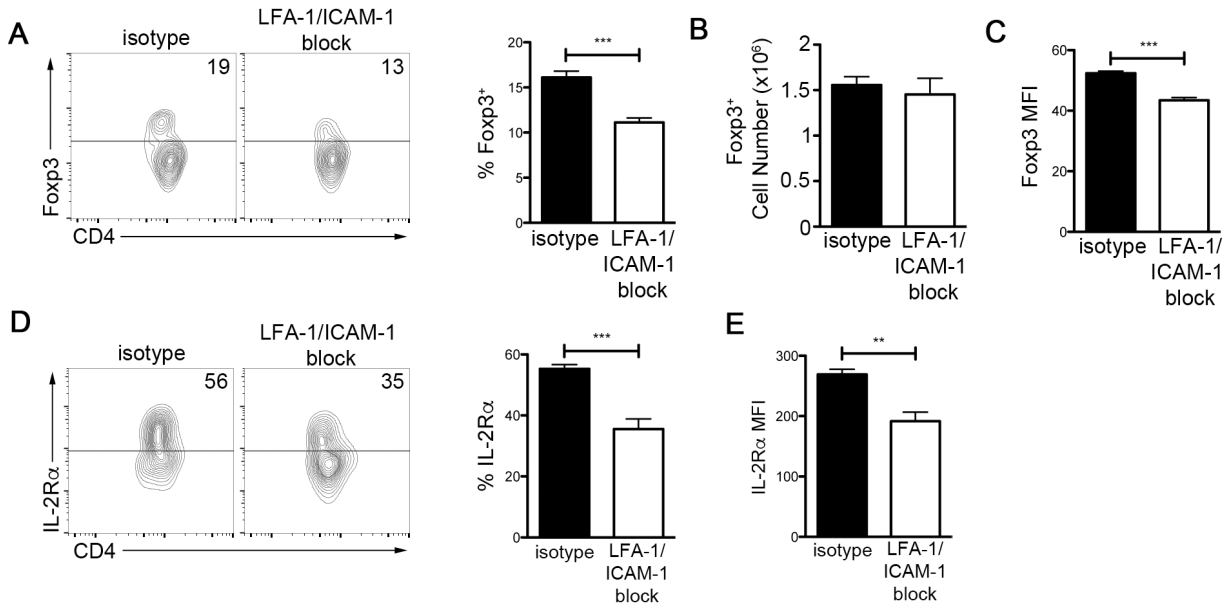

Figure 5.3 β2 blockade alters the phenotype of Treg cells. Frequency (**A**), absolute number (**B**) and MFI (**C**) of $CD4^+Foxp3^+$ cells isolated from mice treated with isotype or LFA-1/ICAM-1 mAbs for 3 weeks (n=5). Frequency (**D**) and MFI (**E**) of $CD4^+Foxp3^+$ cells with high IL-2R□ expression isolated from mice treated with isotype or LFA-1/ICAM-1 mAbs for 3 weeks (n=5). Data shown are mean ± SEM and are representative of at least 2 independent experiments. **, P <0.01; ***, P<.001.

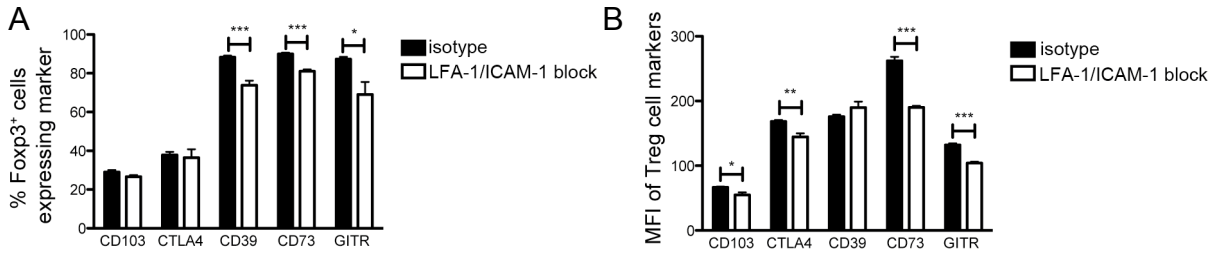

Figure 5.4 β2 blockade leads to downregulation of Treg cell putative suppressive molecules. Percentage (A) and MFI (B) or Treg cell suppressive molecules, displayed cells gated on $CD4^{+}Foxp3^{+}$ Treg cells from mice treated with isotype or LFA-1/ICAM-1 mAbs for 3 weeks (n=5). Data shown are mean ± SEM and are representative of at least 2 independent experiments. **, P <0.01; ***, P<.001.

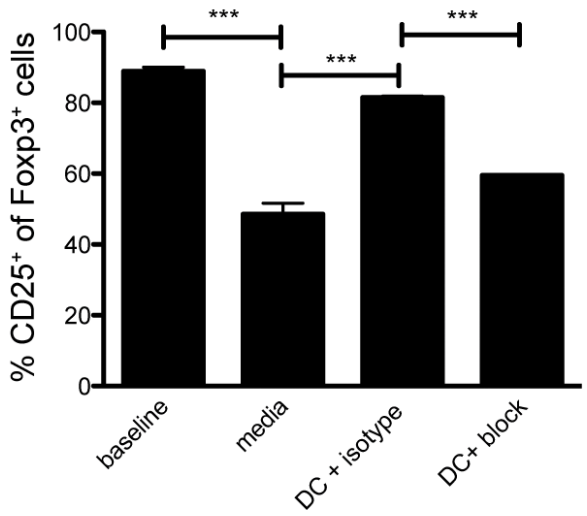

Figure 5.5 LFA-1 signaling to Treg cells is required for maintained expression of IL-2Rα. Frequency of cells with high IL-2R expression by sorted $CD4^+Foxp3^+$ cells directly *ex vivo* ('baseline') or cultured for 18 hours in serum-free media alone ('media'), with purified $CD11c^+$ DCs and isotype control mAb ('DC + isotype'), or with purified $CD11c^+$ DCs and blocking LFA-1 and ICAM-1 mAbs ('DC + block') (n=3). Data shown are mean ± SEM and are representative of at least 2 independent experiments. **, P <0.01; ***, P<.001.

CHAPTER VI: ACTIVATION OF β INTEGRINS ENHANCES TREG CELL FUNCTION AND PHENOTYPE

6.1 Introduction

Having observed that specifically disrupting β2 integrin activation resulted in altered Treg cell phenotype, we sought to test the hypothesis that integrin activation might augment Treg cell function. Integrin-modulating therapy with a β1 integrin-activating antibody (β1aAb), 9EG7, which maintains β1 integrin in its active conformation (68, 69), has been shown to exert beneficial effects on cell-matrix interactions *in vivo,* in a mouse model of scleroderma (70). Although, specific deletion of β1 integrins from Treg cells did not alter Treg cell phenotype, we hypothesized that activation of this integrin was worth exploring, as it may stabilize expression of Foxp3 or other Treg cell suppressive molecules.

9EG7 is the only currently integrin activating antibody that is reactive against mouse integrins, while there are many available integrin activating antibodies specific to human cells. We therefore worked to create a humanized mouse model in which we could test the efficacy of β2 integrin activation in enhancing the phenotype and function of Treg cells. We utilized the β2 integrin-activating antibody (β2aAb), KIM127, to explore this possibility.

6.2 Results

6.2.1 β1 integrin activating antibody treatment partially ameliorates inflammation in $Tln1^{fl/fl}Foxp3^{Cre}$ hemizygous male animals

To understand if activation of β1 integrins can rescue the spontaneous inflammation that develops in $Tln1^{fl/fl}Foxp3^{Cre}$ male animals, we started treatment at three weeks of age with a β1 activating antibody (β1aAb) known as 9EG7 and continued treatment for six weeks. Although

treatment of *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice with β1aAb did not alter the proportions of CD4$^+$, CD8$^+$, or Treg cells (**Fig. 6.1A, 6.1B**), it nonetheless yielded improvement in several immunologic parameters. Percentages of recently activated (CD69$^+$) T lymphocytes and inflammatory cytokine production were reduced in mice treated with β1aAb compared to control-treated mice (**Fig. 6.1C, 6.1D**). Improvements in these parameters were associated with increased expression of IL-2Rα by talin-deficient Treg cells, both on a per cell basis and as a percentage of Foxp3$^+$ cells (**Fig. 6.1E, 6.1F**), which is particularly striking in that this is the only Treg cell suppressive molecule that is significantly downregulated in *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice.

6.2.2 β1 integrin activating antibody treatment increases the suppressive capacity of wild-type Treg cells

Because the administration of β1 integrin-activating antibody improved certain immunologic parameters in *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice, we next tested whether β1aAb therapy might be useful in the setting of autoimmune disease in wild-type mice. Strikingly, β1aAb therapy attenuated the severity of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, as reflected by clinical score and body weight (**Fig. 6.2**). Moreover, β1aAb therapy significantly enhanced the suppressive function of wild-type T_{reg} cells *in vitro* (**Fig. 6.3A**) without altering the proliferation of conventional T cells (**Fig. 6.3B**), raising the possibility that agents that activate β1 integrins in Treg cells may be useful in the treatment of autoimmune disease.

6.2.3 β2 integrin activating antibody treatment increases the size of the Treg cell population

We next investigated the effects of activating β2 integrins in Treg cells. Because there are currently no antibodies that can activate murine β2 integrins, we utilized a previously described humanized mouse model in which we could test the effects of β2 integrin activation on human cells (71, 72). Immunodeficient NOD-SCID-gamma (NSG) mice were reconstituted with human peripheral blood mononuclear cells (PBMCs) and subsequently treated with KIM127, a β2 integrin-activating antibody (β2aAb) that maintains β2 integrins in their active confirmation (71). This system can be used to model Graft-versus-host disease (GVHD). Strikingly, β2aAb-treated mice harbored increased frequencies of Treg cells, compared to Treg cells from control-treated mice (**Fig. 6.4A**). There were no changes to the frequency and number of non-Treg $CD4^+$ or $CD8^+$ T cells, suggesting a preferential effect of β2aAb treatment on Treg cells (**Fig. 6.4B, 6.4C**). Additionally, animals treated with β2aAb outlived animals treated only with isotype, indicating that activating β2 integrins may provide some protection in an inflammatory setting. Taken together, these results raise the possibility that agents that activate beta integrins in Treg cells may be useful in the treatment of autoimmune and inflammatory disorders.

6.3 Discussion

Our data demonstrate that integrin activation may enhance Treg cell function and reduce inflammation. Treatment of inflamed male $Tln1^{fl/fl}Foxp3^{Cre}$ male mice with β1aAb partially reduced T cell activation and cytokine production and lead to an upregulation of IL-2Rα on Treg cells, indicating that signaling through β1 may be required on peripheral Treg cells to maintain expression of this essential surface molecule. Strikingly, prophylactic treatment of wildtype mice with β1aAb prior to induction of EAE, protected the animals from developing severe disease. This finding is particularly intriguing in light of the fact that there are current therapies available

for multiple sclerosis that function to block α4β1, thus preventing the trafficking of activated, potentially pathogenic T cells into the CNS. Our data indicate that integrins on different T cell subsets may have unique functions, and thus calls for a more thorough understanding of the influence of integrins over T cell function in general. Utilization of our humanized mouse model, also showed therapeutic potential of a β2aAb, as treatment of mice with KIM127 leads to an increase in Treg cells in the blood and prolonged the survival of animals, who will succumb to GVHD in this model. Thus, our findings raise the intriguing possibility that activating integrins on Treg cells may represent a new therapeutic strategy in the treatment of autoimmune and inflammatory disorders.

Chapter VI, in part, is an adapted version of the material that has been submitted for publication. **Klann JE**, Kim SH, Remedios KA, He Z, Metz PJ, Lopez J, Tysl T, Olvera JG, Ablack JN, Cantor JM, Boland BS, Yeo G, Zheng Y, Lu L, Bui JD, Ginsberg MH, Petrich BG, Chang JT. 2018. Integrin activation controls regulatory T cell-mediated peripheral tolerance. The dissertation author was the primary author of all material.

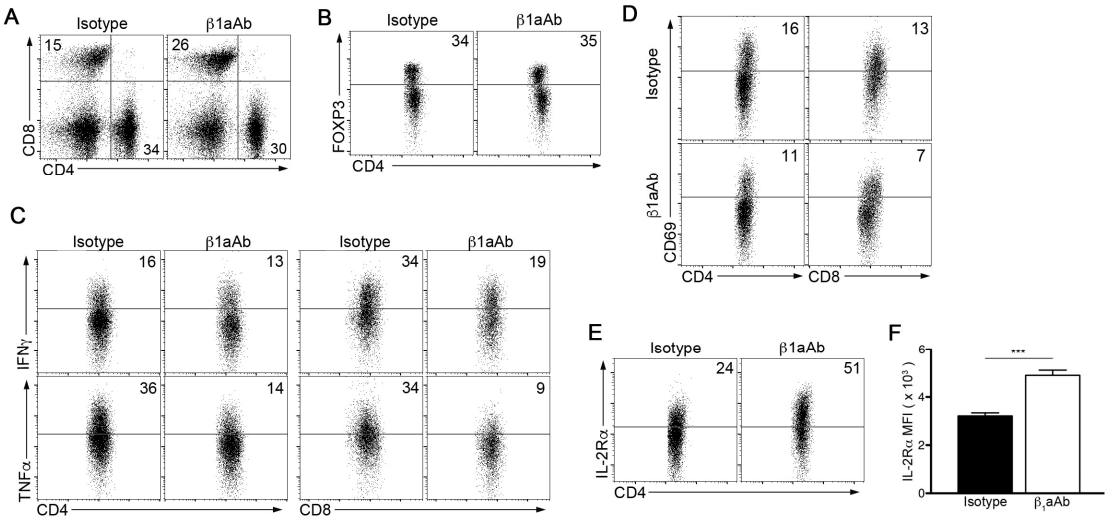

Figure 6.1 β1aAb treatment ameliorates inflammation in Tln1fl/flFoxp3Cre male mice. (A and B) Percentages of splenic $CD4^+$, $CD8^+$ (A), and Treg cells (B) from male *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice treated with β1aAb (9EG7) or isotype control administered every 5 days for 6 weeks. (C) IFNγ and TNFα expression by splenic $CD4^+$ (left) and $CD8^+$ (right) T cells in β1aAb- or isotype-treated *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice; displayed cells were gated on $CD4^+CD44^{hi}$ or $CD8^+CD44^{hi}$ events. (D) Percentages of $CD4^+$ and $CD8^+$ T cells expressing CD69 in β1aAb- or isotype-treated *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice; displayed cells were gated on $CD4^+$ or $CD8^+$ events. (E and F) IL-2Rα expression in gated Treg cells from β1aAb- or isotype-treated *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice as a percentage of $Foxp3^+$ cells (E) and on a per cell basis (F).

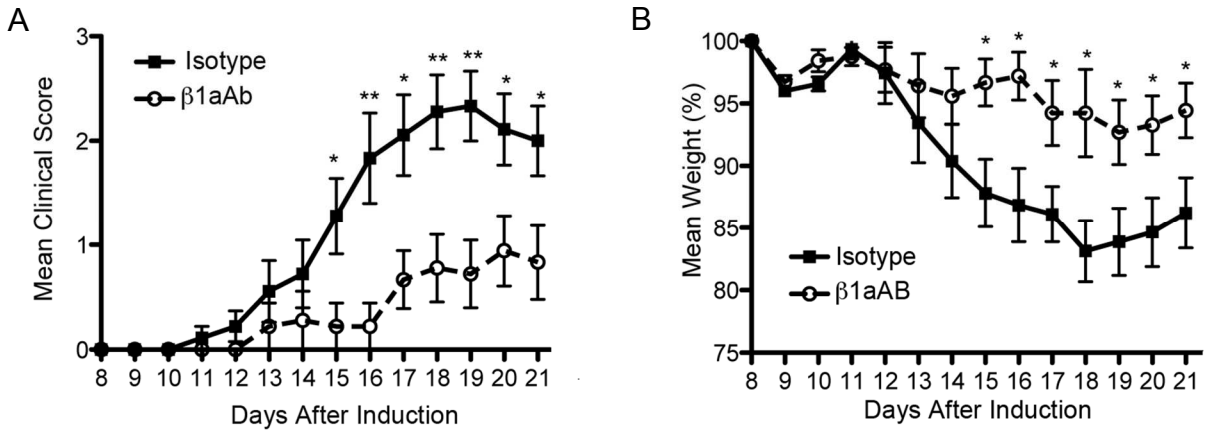

Figure 6.2 β1aAb treatment protects mice from Experimental autoimmune encephalomyelitis. Experimental autoimmune encephalomyelitis (EAE) disease scores (A) and weights (B) from wild-type adult C57BL/6 mice immunized with MOG_{35-55} peptide in CFA and pertussis toxin and treated with β1aAb or isotype control antibody (n=9 mice per group).

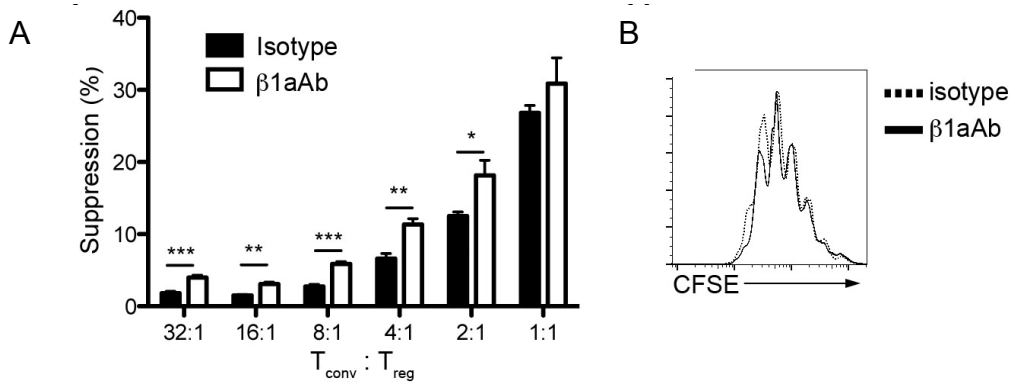

Figure 6.3 β1aAb treatment increases the suppressive capacity of wild-type Treg cells. (A) Suppression by sorted GFP$^+$ Treg cells from β1aAb- or isotype-treated *Foxp3*^{GFP} mice at decreasing T_{conv}:Treg cell ratios, measured at 72 hours; cultures were treated daily with β1aAb or isotype control. (B) Proliferation of non-Treg Tconv cells isolated from β1aAb- or isotype-treated *Foxp3*^{GFP} mice based on CFSE dilution. Data are mean ± SEM and representative of at least 2 independent experiments. *, $P < 0.05$; **, $P < 0.01$, *** $P < 0.005$.

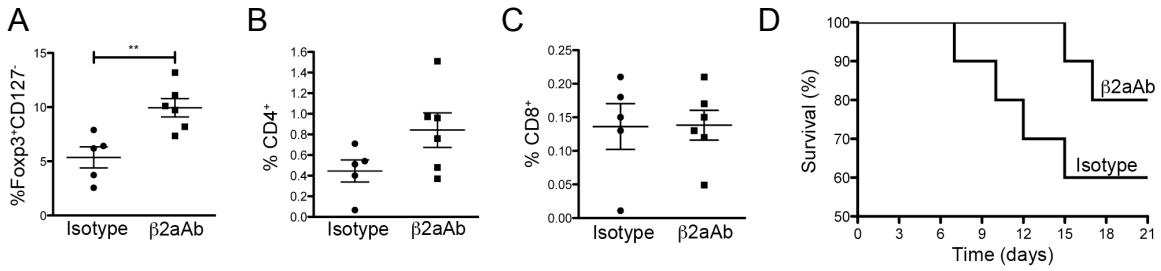

Figure 6.4 β2aAb increases the size of the Treg cell population. (A) Percentages of $Foxp3^{+}CD127^{-}$ Treg cells isolated from the blood of humanized mice treated with isotype (n=5) or β2aAb (KIM127) (n=6), displayed cells gated on $CD4^{+}$ cells. Percentages of $CD4^{+}$ (B) and $CD8^{+}$ (C) isolated from th e blood of humanized mice treated with isotype (n=5) or β2aAb (KIM127) (n=6). (D) Survival or isotype (n=10) and β2aAb (KIM127) (n=10) treated mice post injection with human PBMCS. Data are mean ± SEM and representative of at least 2 independent experiments. *, $P < 0.05$; **, $P < 0.01$, *** $P < 0.005$, unpaired Student's t-test.

CHAPTER VII: CONCLUSIONS

Peripheral tolerance mediated by Treg cells is governed by multiple diverse mechanisms, including regulation by transcription factors, microRNAs, cytokines, and TCR signaling. Our findings suggest that talin, through activation of integrins, may be another key mediator of peripheral Treg cell homeostasis, function and identity. T cell specific deletion of talin led to a numerical and functional deficiency in Treg cells, resulting in a comprised activation threshold of effector T cells. Further investigation into the role of talin in Treg cells revealed that mice with Treg cell-specific deficiency in talin succumbed to fatal systemic autoimmunity. Furthermore, talin-deficient Treg cells isolated from the periphery were functionally impaired, unable to maintain high expression of Treg cell signature molecules, and exhibited global transcriptional dysregulation. Taken together our data reveal a novel role for talin, by virture of its role in integrin activation, as a key mediator of Treg cell identity and function, and as such points to new therapeutic avenues for autoimmune and inflammatory disorders.

Studies across the animal models investigated in this dissertation pointed to a role for talin in the maintenance of IL-2Rα expression on Treg cells. IL-2 signaling is a well-known mediator of Treg cell homeostasis and survival. IL-2 is produced primarily by activated $CD4^+$ T cells in secondary lymphoid organs and can be consumed by cells expressing the high-affinity IL-2R; IL-2Rβ (CD122) and the common γ chain (CD132), which comprise the IL-2R, and IL-2Rα (73). The association of IL-2Rα with the IL-2R increases the affinity of the receptor by 10- to 100-fold and Treg cells express constitutively high levels of IL-2Rα (31). IL-2 signaling reinforces the expression of Foxp3 and IL-2Rα in Treg cells, and regulates the balance between apoptosis and proliferation (30, 55). Treg cells receiving IL-2 signal upregulate the survival factors Bcl-2 and Mcl-1; conversely, in the absence of IL-2, Treg cells upregulate apoptotic

factors such as Bim, Bak and Bax (30). In the thymus of *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice, talin-deficient Treg cells expressed levels of Foxp3 and IL-2Rα comparable to that of wild-type Treg cells. In the periphery, however, talin-deficient Treg cells failed to maintain high levels of IL-2Rα and consequently exhibited impaired STAT5 phosphorylation and IL-2 signaling, resulting in reduced Foxp3 expression. Impaired IL-2 signaling also led to reduced Bcl-2 and Mcl-1 expression and a two-fold reduction in total peripheral Treg cell numbers in *Tln1*$^{fl/fl}$*Cd4*^{Cre} mice. Taken together, our data suggest that talin, when deleted in conjunction with CD4 expression is required for maintenance of Treg cell numbers owing to its role in IL-2 signaling and its downstream effects on the expression of Foxp3 and regulators that control balance between apoptosis and survival.

IL-2 signaling is known to be specifically required for the homeostasis and survival of quiescent, long-lived cTreg cells, which consequently express high levels of IL-2Rα. Conversely, highly proliferative and apoptotic eTreg cells rely on ICOS signaling to regulate their survival (31). Our data suggest that talin influences the function of both eTreg and cTreg cell subsets, although it may be more critical in the maintenance of the cTreg cell pool. Deletion of talin from T lymphocytes resulted in a loss of splenic cTreg cells, likely due to their inability to maintain high surface expression of IL-2Rα, thereby reducing the amount of IL-2 signal these cells can receive, impairing the expression of Bcl-2 and ultimately leading to increased cell death. By contrast, the absolute number of eTreg cells was not reduced in the absence of talin, perhaps because eTreg cell survival does not depend on IL-2 signaling (31). Furthermore, the observation of normal numbers of eTreg cells suggests that talin may be dispensable for the conversion of cTreg cells into eTreg cells. However, talin-deficient eTreg cells were not able to maintain wild-type expression levels of Foxp3, ICOS or GITR, which may influence the apoptotic rates and

suppressive function of these cells and raises the possibility that expression of IL-2Rα may be required to maintain the expression of these molecules by eTreg cells. Thus, our data suggest that talin is required for various aspects of the homeostasis and function of the cTreg and eTreg cell pools.

Examination of Treg cells isolated from inflamed male *Tln1*$^{fl/fl}$*Foxp3*^{Cre} and *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice, as well as uninflamed female *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice, also revealed a downregulation of IL-2Rα. Systemic blockade of LFA-1 signaling with antibody treatment lead to a downregulation of IL-2Rα, and treatment of inflamed *Tln1*$^{fl/fl}$*Foxp3*^{Cre} mice with β1aAb partially rescued IL-2Rα expression, indicating a possible role for both β1and β2 integrins in maintaining high IL-2Rα expression on Treg cells. It is possible that Treg cells require integrin signaling in order to traffic to microenvironments within the spleen or lymph nodes where IL-2 signal is released (74); alternatively, direct signaling through β1 or β2 integrins may be required for the maintenance of IL-2Rα. However, it's important to note that there is no numerical deficiency when talin is deleted in conjunction with Foxp3 expression in Treg cells, thus revealing differences between the models utilized in this dissertation. This difference can possibility be attributed to influences from other dysregulated T cells that have also lost expression of talin in our *CD4*^{Cre} model, or genetic/phenotypic differences arising from the timing of talin deletion during thymic development, as Treg cells from our *CD4*^{Cre} model will lose expression of talin earlier in development than Treg cells from our *Foxp3*^{Cre} models.

Taken together, our studies suggest an essential role for integrin activation in maintaining high expression of IL-2Rα by Treg cells in the periphery and thereby influencing Treg cell homeostasis and survival. These findings are particularly intriguing in light of prior evidence linking defective Treg cell function and polymorphisms in IL-2Rα with a variety of human

immune-mediated disorders, including systemic lupus erythematosis, rheumatoid arthritis, type 1 diabetes, and inflammatory bowel disease (75-79). Targeting the IL-2 pathway with low dose IL-2 or IL-2/IL-2 mAbs has been proposed as one approach to enhance Treg cell numbers and suppressive function (31). Our findings raise the possibility that therapies targeting integrins in Treg cells may help to reinforce high expression of IL-2Rα, thereby potentially enhancing their numbers and suppressive capacity.

Our data indicate that talin plays a critical role in maintaining expression of multiple additional Treg cell signature molecules including CD103, GITR, CD39, CD73, and IL-10 in uninflamed female heterozygous *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ mice. This downregulation was observed only in Treg cells isolated from the periphery, but not the thymus, suggesting a specific role for activated integrins in the maintenance of Treg cell identity after thymic development. Although this downregulation was masked by the presence of inflammation in diseased male *Tln1*$^{fl/fl}$*Foxp3*^{Cre} and *Tln1*$^{L325R/fl}$*Foxp3*^{Cre} mice. The finding that talin-deficient Treg cells expressed significantly less *Il10* at the mRNA level compared to wild-type Treg cells is also notable, as the exact mechanisms which control IL-10 production in Treg cells have not yet fully been elucidated. Our findings suggest the possibility that activation of β1 or β2 integrins on Treg cells may be required for these cells to produce IL-10.

It has become increasingly appreciated that in addition to their known role in adhesion in migration, integrins play a role in the reprogramming and function of immune cells. Engagement of both VLA-4 and LFA-1 contributes to T cell activation and differentiation, as well as the production of cytokines (80, 81). The importance of LFA-1 is further highlighted in observations from *Itgb2*$^{-/-}$ (CD18$^{-/-}$) mice, which lack expression of β2 integrins. These mice develop autoimmune dermatitis, even when housed in germfree environments (39, 82). Moreover, *Itgb2*$^{-/-}$

mice harbor lower frequencies of Treg cells in the spleen and mesenteric lymph nodes and β2-deficient Treg cells display a reduced suppressive capacity both *in vitro* and *in vivo* (83). Lastly, β2 integrin-deficient Treg cells exhibit reduced expression of IL-2Rα (83), which mirrors the deficiencies we observed in talin-deficient Treg cells. Our finding that LFA-1/ICAM-1 interactions *in vivo* are required for high expression of IL-2Rα and Foxp3 by Treg cells provides a possible explanation for these prior observations in *Itgb2*$^{-/-}$ mice. Intriguingly, recent studies revealed that LFA-1 expressed by Treg cells exhibits a stronger intrinsic adhesiveness compared to that expressed by conventional T cells, which can be attributed to reduced calpain levels in Treg cells that effectively slows the recycling of integrins from the cell surface (39, 40). Thus, these prior studies suggest that integrin expression and function may be regulated by different mechanisms in Treg cells compared to conventional T cells. Here we extend these prior findings by demonstrating that defective integrin signaling owing to talin deficiency alters Treg phenotype, function, and transcriptional identity. Moreover, activation of VLA-4 with β1aAb increases the expression of IL-2Rα and the suppressive capacity of Treg cells, whereas LFA-1 activation with β2aAb can increase the size of the Treg cell pool.

All currently available therapies for autoimmune disease that target integrins function to block integrin signaling, and are thought to function by prevent trafficking of activated effector T cells to sites of inflammation, thereby ameliorating disease (84). The α4 integrin antagonist natalizumab blocks α4β1 (VLA-4) and α4β7 integrins and is approved for the treatment of inflammatory bowel disease (IBD) and multiple sclerosis (MS); the β7 integrin antagonist vedolizumab blocks α4β7 integrin and is approved for treatment of IBD (84). Our data demonstrate that integrin activation may enhance Treg cell function and reduce inflammation, suggesting that the role of integrins may be more nuanced than previously appreciated. Thus, our

findings raise the intriguing possibility that activating integrins on Treg cells may represent a new therapeutic strategy in the treatment of autoimmune and inflammatory disorders.

Chapter VII, in part, is an adapted version of the material published in the *Journal of Immunology*. **Klann JE**, Remedios KA, Kim SH, Metz PJ, Lopez J, Mack LA, Zheng Y, Ginsberg MH, Petrich BG, Chang JT. Talin Plays a Critical Role in the Maintenance of the Regulatory T Cell Pool. *J Immunol.* 2017;198(12):4639-51. The dissertation author was the primary author of all material.

Chapter VII, in part, is an adapted version of the material that has been submitted for publication. **Klann JE**, Kim SH, Remedios KA, He Z, Metz PJ, Lopez J, Tysl T, Olvera JG, Ablack JN, Cantor JM, Boland BS, Yeo G, Zheng Y, Lu L, Bui JD, Ginsberg MH, Petrich BG, Chang JT. 2018. Integrin activation controls regulatory T cell-mediated peripheral tolerance. The dissertation author was the primary author of all material.

APPENDIX A: MATERIALS AND METHODS

Mice

All animal work was approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. All mice were housed in specific pathogen-free conditions prior to use. *Cd4*^{Cre} and NSG mice were purchased from Jackson Laboratories, and *Tln1*$^{fl/fl}$, *Tln1*$^{L325R/fl}$, *Foxp3*^{Cre}, and *Foxp3*^{GFP} mice have been described previously (12, 54, 61, 85) (12, 54, 61, 85). For administration of IL-2/IL-2mAb complexes, 2μg of recombinant murine IL-2 (Biolegend) was combined with 10μg IL-2 monoclonal antibody JES6-1 (Bio X Cell), diluted to a volume of 200μL in PBS and incubated for 30 minutes at 37°C before intraperitoneal (i.p.) injection, as previously described (59, 60). To block LFA-1 signaling, mice were treated with 100μg anti-CD11a (M17/4) (Bio X Cell), 100μg anti-CD18 (M18/2) (Bio X Cell) and 200μg anti-ICAM-1 (YN1/1.7.4) or isotype control in PBS via i.p. injection twice weekly for three weeks. In some experiments, mice were injected intraperitoneally with β1aAb (9EG7, BioXCell) or an isotype control (Rat IgG2a, BioXCell) in PBS at 2mg/kg, every 5 days for 4-6 weeks or with β2aAb (KIM127, BioXCell) or isotype control (mouse IgG1, BioXCell) every other day for 1-3 weeks.

Antibodies and flow cytometry

The following antibodies were purchased from Biolegend: CD4 (RM4-5), CD8 (53-6.7), Foxp3 (FJK-16s), CD44 (1M7), CD62L (MEL-14), IFNγ (XMG1.2), TNFα (MP6-XT22), IL-2 (JES6-5H4), IL-17A (TC11-18H10.1), IL-2Rα (PC61), CTLA4 (UC10-4B9), CD39 (24DMS1), CD73 (TY/11.8), GITR (DTA-1), OX40 (OX-86), PD1 (J43), CD28 (37.51), IRF4 (IRF4.3E4), Bcl-2 (BCL/10C4), ICOS (15F9), Annexin V and fixable viability dye. Anti-human Ki67 (B56)

was purchased from BD Biosciences. Anti-GFP rabbit IgG was purchased from Life Technologies. For intracellular detection of cytokines, splenocytes were stimulated *ex vivo* with PMA (Sigma) and ionomycin (Sigma) in the presence of Brefeldin A (Sigma) for 3 hours at 37°C; cells were fixed in 4% paraformaldehyde (Electron Microscopy Services) and permeabilized with the Foxp3 Transcription Factor Fixation/Permeabilization kit (eBioscience) prior to staining. To assess pSTAT5 levels directly *ex vivo*, cells were fixed in Lyse/Fix buffer (BD) for 20 minutes at room temperature. Cells were then resuspended in 90% methanol and incubated for 30 minutes on ice. After washing, cells were resuspended in Perm/Wash buffer (BD) and surface and intracellular antigens were stained, including pSTAT5 (pY694; BD). To assess pSTAT5 levels *in vitro*, bulk splenocytes were stimulated with 0U/mL, 0.1U/mL, 1U/mL or 10U/mL of IL-2 (Peprotech) at 37°C for 30 min and the same staining protocol was used. To measure apoptosis, cells were stained with Mito Flow (Cell Technology) according to the manufacturer's instructions. Human antibodies against the following proteins were obtained from Biolegend: CD4 (RPA-T4), CD8 (SK1), Foxp3 (206D), CD127(A019D5). All samples were analyzed on an Accuri C6, FACS Canto, or LSR FortessaX-20 (BD Biosciences).

Isolation of T cells from liver and lung

Mice were euthanized with CO_2 and perfused through the left ventricle of the heart with a heparin (Sigma) solution in PBS to remove all blood. Lungs were treated with 1.3mM EDTA (Fisher), digested with 75U/mL collagenase solution (Sigma), and T cells were isolated with a Percoll (Fisher) gradient. Livers were mechanically disassociated and T cells were isolated using a Percoll (Fisher) gradient.

Treg cell suppression assays

$CD4^+CD25^-$ T conventional (Tconv) cells were isolated from spleens and lymph nodes of wild-type mice by magnetic separation using the $CD4^+$ T cell negative isolation kit (Miltenyi Biotec); a biotin-conjugated anti-CD25 (PC61, Biolegend) antibody was included to deplete Treg cells. $CD4^+GFP^+$ Treg cells were sorted with a FACS Aria 2 (BD Biosciences). Antigen-presenting cells were isolated from spleens of wild-type mice and depleted of $CD3^+$ T cells using CD3 microbeads (Miltenyi Biotec). Tconv cells were labeled with CFSE as previously described (86). CSFE-labeled Tconv cells were co-cultured with antigen presenting cells (1:3 ratio) and Treg cells (32:1, 16:1, 8:1, 4:1, 2:1 and 1:1 ratios) in the presence of 250 ng/mL soluble anti-CD3 (2C11) for 72 hours at 37°C. Percentage suppression was calculated as: [(divided Tconv cells without Treg cells) – (divided Tconv cells with Treg cells for a given experimental condition)] / (divided Tconv cells without Treg cells) * 100.

Generation of bone marrow chimeras

Bone marrow cells were depleted of $CD3^+$ cells using CD3 microbeads (Miltenyi Biotec). Lethally irradiated (1000 rads) RAG1-deficient mice were injected intravenously with bone marrow cells from $Tln1^{fl/fl}Cd4^{Cre}$ mice alone or in combination with bone marrow cells from wild-type or Foxp3-deficient mice. Spleens and thymi from recipient mice were harvested for analysis 8-10 weeks after reconstitution.

Adoptive Transfer Experiments

Total $CD4^+$ T cells were isolated from spleens of $Tln1^{fl/fl}Cd4^{Cre}$ mice by magnetic separation using the $CD4^+$ T cell negative isolation kit (Miltenyi Biotec) and $3x10^6$ cells were

injected into CD45.1$^+$ wild-type recipients. Spleens were harvested from recipient mice 4 days after transfer.

Quantitative real-time PCR

Total RNA was isolated from cells with TRIzol (Life Technologies) according to manufacturer's protocol and was converted to cDNA using an iScript Advanced cDNA synthesis Kit (Bio-Rad) according to manufacturer's protocol. 100ng cDNA was combined with 250nM forward and reverse primers for the indicated genes and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Samples were run on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Samples were normalized based on expression of *Rpl13a* reference gene. Relative gene expression was determined based on three biological replicates and figures show one representative experiment. The following primer sequences were utilized: *Rpl13a* 5'GGGCAGGTTCTGGTATTGGAT, *Rpl13a* 3'GGCTCGGAAATGGTAGGGG, *Il10* 5'ATCGATTTCTCCCCTGTGAA, *Il10* 3'TGTCAAATTCATTCATGGCCT, *Il2ra* 5'CTCCCATGACAAATCGAGAAAGC, *Il2ra* 3'TCTCTTGGTGCATAGACTGTGT, *Casp7* 5'CGGAATGGGACGGACAAAGAT, *Casp7* 3'CTTTCCCGTAAATCAGGTCCTC, *Mcl1* 5'TAACAAACTGGGGCAGGATT, *Mcl1* 3'GTCCCGTTTCGTCCTTACAA, *Bcl2* 5'TCGCAGAGATGTCCAGTCAG, *Bcl2* 3'CCTGAAGAGTTCCTCCACCA.

Histology

Organs were fixed overnight at 25°C in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Images were acquired on a Nikon Eclipse TE300 microscope.

RNA Sequencing

Total RNA from FACS-sorted YFP^{+} Treg cells isolated from *Tln1*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ and *Tln1*$^{fl/fl}$*Foxp3*$^{Cre/wt}$ from 1-2 mice was extracted using TRIzol reagent (Life Technologies) according to the manufacterer's protocol. cDNA libraries were prepared using TruSeq non-Stranded Total RNA Sample Prep Kit (Illumina) according to manufacturer's instructions. cDNA libraries were sequenced with a HiSeq2500 (Illumina). FASTQ files were processed with kallisto 0.42.4 with following commands: kallisto quant -b 8 –i kallisto_GRCm38.rel79.cdna.all.idx -l 200 -s 20 -t 4. Differential expressed genes were identified by identifying genes that had an absolute log2 fold change > 1 and q-value < 0.05. Log2 fold change was calculated by log2(TPM1+1/TPM2+1) and q-value was calculated by sleuth using the default setting for two-condition comparisons. Genes with less than 1 TPM standard deviation across samples were removed before PCA analysis. PCA was performed using R.

Isolation of naïve human T cells and generation of humanized mice

Leucocyte reduction system (LRS) cones were obtained from the San Diego Blood Bank, and white blood cells were further isolated using a Ficoll gradient. $20x10^6$ cells were injected i.v. into male NSG mice. 3-5 days later, mice were bled to determine human T cell reconstitution, and mice exhibiting greater than 10% human chimerism, based on CD45RA expression, were treated with β2aAb (KIM127) or isotype (mouse IgG1).

Statistical analysis

An unpaired Student's t-test (two-tailed) was used for statistical evaluation of the data between two groups, using a statistical software package (Graph Pad Prism). EAE data was analyzed using a Mann-Whitney statistical test. P values are denoted in figures as; * $P<0.05$, ** $P<0.01$, *** $P<0.005$.

APPENDIX B: SUPPLEMENTAL TABLE I

gene_id	*Tln1wt/wtFoxp3Cre/wt* TPM	*Tln1fl/flFoxp3Cre/wt* TPM	fold change	p value
Gm4963	471.87025	1.121915	420.5935833	5.40E-06
Gm5752	4.67084069	0.118857833	39.2977102	0.018757124
Gm8935	1.720884438	0.060356549	28.51197559	5.23E-05
Gm15526	1773.72375	85.7224	20.69148496	0.000544444
Gm3164	1.474598	0.079509067	18.54628738	0.000204976
Ces2c	5.638233875	0.329096283	17.13247509	0.000177199
Bloc1s2-ps	8.635677888	0.524935409	16.45093424	0.028395198
Ces2d-ps	1.114324125	0.069372	16.06302435	0.000543189
Rpl17-ps5	619.190875	44.96483333	13.77055866	1.95E-06
Slc7a10	3.6701365	0.336161278	10.91778482	0.000286962
Gm15516	5618.65	518.3193333	10.84013202	3.99E-05
Gm3252	2.548329375	0.243260433	10.47572488	0.0001228
Gm7332	14.81432125	1.472651667	10.05962346	0.001132468
Gm4540	34.89018888	3.912204	8.918294873	0.014989188
Zfp988	0.340506375	0.039601833	8.598247766	0.013446621
3830408C21Rik	0.606243	0.07140635	8.490043252	0.000422814
D830030K20Rik	3.61160883	0.459507167	7.859744292	0.000470763
Eps8l1	56.0160395	7.498928607	7.469872356	4.96E-05
Gm8281	5.852332525	0.824525	7.097823019	0.000644112
Gm10076	34.19358375	4.951681667	6.905448704	0.001426225
Gm14741	0.929373625	0.144156483	6.446977642	0.012535632
Gm12013	15143.48125	2392.886667	6.328540946	0.000143445
Rex2	0.306184926	0.048592089	6.301127043	0.030110181
Gm11483	20.20468	3.332845	6.062292126	0.000837245
Gm18752	8.883760625	1.466495667	6.057815803	1.36E-05
Gm3373	13.897706	2.35852316	5.89254591	0.007007245
E330021D16Rik	1.5259965	0.259623167	5.877736257	0.000336054
Gm3650	10.10667375	1.722143333	5.868660032	0.004641813
Noxa1	1.124571379	0.193367333	5.815725747	0.000290315
Vmn2r97	1.22977475	0.213979067	5.747173166	0.001031405
Trbv23	25.2649875	4.438443336	5.692308223	3.09E-05
Clec2e	0.348849125	0.063262667	5.514296873	0.005559401
Gata4	2.307242688	0.434623667	5.308598828	0.007274873
Gm17764	21.01577625	4.038245	5.204185543	3.62E-05
Smim10l2a	1.13240225	0.218086	5.192457333	5.81E-05

Spata9	0.759125	0.1497031	5.070870276	0.040736485
Amy1	2.7060265	0.540870857	5.00309171	0.003185386
Gm3411	5.08129875	1.0300595	4.933014792	4.55E-05
Rec8	12.73948188	2.58595825	4.926406633	0.000160398
Gm13241	2.31691625	0.474794167	4.879833015	0.002615309
Gm12978	3.69422875	0.789983333	4.676337581	0.01325321
Zfp985	1.717335125	0.36995055	4.64206669	0.003311629
Atp6v0d2	3.190315625	0.6887299	4.632172387	0.002388426
Sostdc1	0.66635975	0.1439069	4.630492006	0.003603964
Olfr288	1.593380375	0.3444372	4.62604032	0.01266216
Gm3173	25.88165013	5.632483333	4.595069101	0.020802479
Nrn1	13.28863175	2.9335625	4.529861474	4.04E-07
Tox2	4.39138875	0.972544933	4.515358211	1.67E-05
mt-Nd3	1881.97375	425.9765	4.418022473	1.09E-05
Gm3488	6.088274813	1.384564	4.397250551	0.000317627
Gm12105	1.68137525	0.383266617	4.386959826	0.048014018
Gtf3c2	4.141429689	0.944070173	4.386781624	0.035326135
Tmem254a	7.848827	1.7908695	4.382690643	0.000229245
Tmem254b	7.848827	1.7908695	4.382690643	0.000229245
Trbv26	152.207	34.73753167	4.381629687	3.32E-07
Gm8186	29.48280375	6.889716667	4.279247635	0.010906327
9230113P08Rik	0.608047375	0.144575917	4.205730726	0.003994654
Gm14542	0.681050125	0.162916617	4.180360107	0.010522423
Capn3	72.46152092	17.3911406	4.166576683	0.0003272
Tnfrsf8	1.482003794	0.3629685	4.08300939	8.64E-06
Gm6136	137.0255375	33.7255	4.062965338	4.29E-05
Gjc1	0.295452713	0.073556198	4.016693619	0.03773923
mt-Atp8	48711.35	12134.86667	4.014164419	9.89E-05
Mndal	656.962887	164.9253845	3.983394606	9.39E-05
Dnah7a	1.786054563	0.449751	3.97120754	3.72E-06
Gm16867	7.298408998	1.884237923	3.873400969	1.41E-05
Gpm6b	7.761679543	2.005123023	3.870924355	3.55E-06
Ntm	1.351611688	0.349331983	3.869132378	0.011328602
D930020B18Rik	0.317852741	0.082158417	3.868778807	0.013426105
Penk	16.50187875	4.289628892	3.846924563	2.20E-05
Npas4	2.677205	0.7020485	3.813418873	0.000225433
Cpe	0.770788625	0.20298805	3.797211831	0.00141913
Ephx4	2.857268875	0.753170333	3.793655629	0.008990552
Gucy1b3	2.792525962	0.737817614	3.784845889	2.72E-05

Arnt2	0.718394875	0.191730167	3.746905808	4.68E-05
Abhd14b	6.976860575	1.862921567	3.745117723	5.32E-05
Gm4613	1582.85	424.0717833	3.732504878	0.008051055
Camk2b	5.139500426	1.378063002	3.729510492	0.000136373
Trav6-7-dv9	18.4751	4.960111667	3.724734692	7.74E-05
Gm3194	16.350765	4.393270833	3.721774874	3.87E-05
Src	3.057424823	0.823231936	3.713928834	0.01431875
Naip3	0.717191875	0.193897833	3.69881325	0.000947504
Klrg1	4.51916	1.230041667	3.673989364	0.00021895
Gm10139	157.8829	43.31075	3.645351327	0.027924727
Smarca5-ps	1.16399275	0.320153283	3.635735788	0.007574959
Rps7	274.35	75.68633333	3.624828789	7.25E-06
Gm11212	2.95510625	0.816121333	3.620915334	0.002289824
Ifit3b	2.86331125	0.798589	3.585462923	0.00082431
Gm3752	14.04107168	3.917476667	3.584213225	5.22E-05
Ifit3	17.80715	4.991205	3.567705594	8.21E-06
Tnfsf11	16.9238875	4.79155	3.532027736	7.64E-07
Gm14412	0.559162375	0.16120245	3.468696506	0.019435542
Rab39b	0.69614025	0.200745367	3.467777422	0.000767246
Gm17711	469.227125	135.61615	3.459964945	0.001008921
Gm2897	4.70837125	1.361041333	3.459388877	0.011930219
Gm10409	5.09504375	1.493203333	3.412156694	4.29E-05
Gm3020	5.09504375	1.493203333	3.412156694	6.55E-05
Tle1	4.180726079	1.225988971	3.410084576	2.08E-05
Gm10406	5.814735	1.706642993	3.407118551	0.001146876
Trav6-3	26.8609375	7.885574939	3.406338499	0.0266072
Gm10478	31.45325	9.266065	3.394456007	0.000125231
Tmem158	3.0238375	0.8921425	3.389410884	3.06E-05
Gm8108	5.81428125	1.7174565	3.385402338	0.000287267
Zfp987	2.055551625	0.6073696	3.384350526	0.044760529
Dtx4	5.17484125	1.530908333	3.380242394	2.68E-06
Gm13642	6.00866	1.779926667	3.375790763	0.032121672
A730015C16Rik	1.2723675	0.379450833	3.353181462	0.005590803
Trav16	12.3246875	3.6806	3.348553904	0.000480298
Gm4951	3.57032875	1.067065167	3.345933183	0.001120447
Il1r2	5.693582126	1.702937167	3.343389432	0.000305032
Ccdc39	0.417756025	0.126050183	3.314204025	0.021661221
Slc15a2	82.3005295	24.84128472	3.313054475	1.06E-05
Zfp938	7.2409575	2.1959085	3.297476876	2.84E-05

Zfp972	11.2681405	3.428638333	3.286476847	0.04035024
Pdcd1lg2	7.39075625	2.256483333	3.275342716	2.83E-06
Akr1c12	2.537725	0.775040667	3.274312058	0.000445925
Il10	4.7678	1.460946333	3.263501123	0.000920152
Ifit1	15.18295	4.668348333	3.25231729	6.64E-05
Gm2974	1.913788172	0.5892235	3.247983443	0.011732091
Tigit	27.259575	8.432456667	3.232696719	7.85E-07
Fam160a1	1.257085925	0.389422267	3.228079215	9.43E-05
6430571L13Rik	1.69258675	0.5244125	3.227586585	0.000378003
Naip5	11.4193025	3.545193333	3.221066223	0.000942624
Gm10172	3.26357875	1.014107	3.218179886	0.006867655
Rgs9	4.800528615	1.496362	3.208133203	0.000170953
Gm13212	47.89109963	14.9549731	3.202352776	6.28E-07
Gm28900	27601.35	8655.908333	3.188729471	0.000328227
Sfrp2	3.1295558	0.985113333	3.176848484	0.012597708
Ccdc122	1.502600787	0.4732754	3.174897294	0.005639638
Gm4841	5.5884525	1.760616667	3.174144949	8.64E-06
Gm3696	8.221736375	2.6044945	3.156749371	0.001088227
Ifit1bl1	75.92043	24.08638333	3.152006217	6.82E-06
Insl6	6.04103875	1.917886333	3.149841909	0.000280978
Ms4a4d	1.065606125	0.338916333	3.144156891	0.003458245
Slc24a4	0.130325863	0.041455952	3.14371899	0.042458802
Dnah11	0.588984163	0.187987313	3.13310591	0.047385751
Casp4	40.01062863	12.7717655	3.132740623	0.000469873
Gm3248	0.378079513	0.121259333	3.11794154	0.04543149
4933408B17Rik	0.574228766	0.18443035	3.113526414	0.009361924
Orm1	1.453482125	0.4718778	3.080208743	0.033719314
Sh3gl3	8.912708561	2.905942695	3.067062739	5.50E-05
Gm5239	14.698825	4.842415	3.035432733	0.000288097
Trav16n	34.3414375	11.33083	3.030796288	4.54E-05
Gm3667	8.70421875	2.876233333	3.026256128	4.31E-05
Ifi213	161.82095	53.51023367	3.024112191	0.001311315
Naip6	6.421073038	2.12354025	3.023758574	0.004631134
Ndufa12	29.43522525	9.742039167	3.021464474	7.94E-05
Matn2	2.016975125	0.669844133	3.011111129	6.99E-05
Hsf2bp	1.894308875	0.630654217	3.003720303	0.013131259
1700019D03Rik	2.394871297	0.799271837	2.996316381	0.001499025
Hgfac	0.644750875	0.216102167	2.983546556	0.006988187
Trim34b	1.275919	0.428948667	2.974526089	0.014275712

Iigp1	58.5570475	19.73598417	2.96701938	2.63E-07
Bmp7	4.22764875	1.426953333	2.962709888	7.25E-06
Gm5970	2.12686225	0.724268667	2.936565322	0.025563743
Cnrip1	3.771703	1.286971283	2.930681554	2.41E-05
Rsad2	3.473074838	1.186633033	2.926831413	0.000494114
Ncoa7	40.9398675	14.00593833	2.923036395	2.10E-07
Zfp612	0.357548875	0.122742333	2.913003731	0.031161843
Mpp7	3.0483475	1.0484065	2.907600725	5.01E-05
Traj37	678.570875	233.6305	2.904461853	0.002724915
Gm9320	9.95929875	3.431806667	2.902057055	0.002204457
Gm8325	655.9422604	226.7760867	2.892466618	1.69E-05
Gm14325	21.48693625	7.428986667	2.892310515	0.000521599
Spaca6	5.999754513	2.085561883	2.876804836	0.001322914
Gucy1a3	6.234254625	2.184498933	2.853860228	0.000184103
Srcin1	0.19691625	0.069147467	2.847772442	0.01343792
Acot6	1.88036375	0.668799333	2.811551472	0.002317641
Gm11027	383.507875	136.6790167	2.805901625	0.001048787
Arrdc4	2.96396875	1.056769333	2.804745233	4.39E-05
Mettl5	46.66570588	16.69927633	2.794474739	0.023136963
Cnbd2	64.38962699	23.0709	2.790945607	3.68E-05
Itgb8	6.1825255	2.218130517	2.787268582	0.000965737
Ptgdr	2.8803	1.0361155	2.779902434	0.000278537
Zic1	0.897236625	0.323661833	2.772142195	0.000783415
Cdkl2	0.832897863	0.301341533	2.763966365	0.002660088
Slc4a10	0.4716054	0.171158596	2.755370811	0.014355655
Rpl22l1	581.1736985	211.7944458	2.744045984	0.004904156
Insm1	0.334558075	0.122173283	2.738389817	0.045808766
Maf	29.92287125	10.93887667	2.735461068	4.51E-05
Zfp947	2.806465	1.030953667	2.722202841	4.36E-05
Zfp551	3.61116375	1.3288565	2.717497149	0.02300845
Adcy5	0.14858415	0.054739667	2.714378056	0.044145463
Eea1	10.27801375	3.790581667	2.71146084	2.97E-05
Plcl1	5.138813625	1.896776383	2.709235348	0.001025089
Neb	12.45700374	4.612840237	2.700506218	5.83E-06
Trav6d-5	4.08140625	1.5140405	2.695704804	0.041497086
A230083G16Rik	1.63416175	0.6063835	2.694931096	0.009238937
Asb14	1.0797465	0.402085783	2.685363534	0.007437417
Spo11	3.436363375	1.285183517	2.673830881	0.0037551

Gm14421	5.04867875	1.893298167	2.666605207	0.005928302
6330408A02Rik	12.71677834	4.771359951	2.665231395	0.02362639
Rpl30	1329.810028	499.6428928	2.661520953	0.000286618
Casp1	25.80255	9.718186667	2.655078657	0.000117629
Nrbp2	2.006417	0.755774	2.654784367	0.003195117
Fap	1.828569469	0.689857238	2.650649103	0.03208049
Mapk8ip1	0.507785525	0.19166715	2.649309102	0.016571404
Syp	7.404519825	2.795188667	2.649023271	0.001281621
Mid2	0.561273588	0.21245767	2.641813723	0.023383507
Smim10l1	25.35935057	9.620199	2.636052598	0.000340271
Synb	0.591822875	0.224716167	2.633646185	0.022120989
Susd4	0.996024518	0.378377217	2.632358594	0.026430256
Pou4f1	0.313837863	0.1193178	2.630268598	0.024569338
D630039A03Rik	7.3563525	2.796913333	2.630168197	1.17E-05
Chic1	2.50491625	0.9546915	2.623796535	4.69E-05
Lipo3	10.32320786	3.938947667	2.620803506	5.83E-06
Nrp1	38.1900875	14.58526667	2.618401732	2.10E-07
Ddit4	55.698675	21.2754	2.617984856	0.004641813
Traj32	452.420625	173.00725	2.615038532	0.006182582
Mdfic	23.79496208	9.099908567	2.614857271	2.10E-07
Phf21b	5.373852538	2.062333283	2.605714887	0.026994563
Gm4631	28.044475	10.77171333	2.603529646	1.88E-05
Slc22a2	1.25837325	0.484477333	2.597383125	0.005377485
Gm8624	31.967775	12.33187667	2.592287927	8.80E-05
Zfp760	3.36330375	1.29778	2.591582356	0.000884799
Cdhr3	0.63478525	0.2450035	2.590923191	0.004144683
Mdm4-ps	0.437385125	0.168936817	2.589045619	0.021465613
Zfp991	10.36443875	4.019753333	2.578376803	0.009282734
Ddx43	6.48205375	2.516301667	2.576024105	0.000169363
Gm28439	840.2145	326.4235	2.574001259	0.001988533
Slc9b2	5.334403532	2.074630683	2.571254525	0.001311315
Trbv3	293.26325	114.07605	2.570769675	1.28E-05
Riiad1	8.478769013	3.302338	2.567504905	0.031667109
Art2b	106.6930875	41.615	2.563813228	4.11E-06
Zfp558	2.613462463	1.023304033	2.553945238	0.029189068
Fbxl21	0.862419763	0.338184667	2.550144485	0.006771233
Prex2	2.394252488	0.9439226	2.536492386	0.000152937
Dsel	2.470762959	0.976656201	2.529818535	0.000501863

Mtmr7	4.431323625	1.75165445	2.529793262	0.000839932
Car11	1.456758625	0.578801	2.51685575	0.004099084
Gm15047	4.21482625	1.680122333	2.508642476	0.019284244
3830406C13Rik	13.37655225	5.3324735	2.508507965	3.32E-05
Cox7b	319.8564375	127.51495	2.508383821	0.002402217
Gm28285	29.60588528	11.80529917	2.5078471	0.001130966
Ccdc73	4.8906225	1.95073945	2.507060848	0.033639071
Synpo	4.158067204	1.659393565	2.505775177	0.00016236
Zfp119b	6.019937625	2.408515167	2.499439368	0.000779387
Sardh	1.486713125	0.595700867	2.495737724	0.02934026
Cst7	38.6932375	15.57176667	2.48483286	0.000227231
Pura	13.7784875	5.547938333	2.483532922	3.60E-05
Apol10b	1.508872	0.610057833	2.473326163	0.00293933
Gm14122	1.140261875	0.461243333	2.472148197	0.040277627
Map3k19	0.887236067	0.35920157	2.470022798	0.004708877
Gm6710	5.771153375	2.337678333	2.468754273	0.00092464
Gm3383	23.59934625	9.566914167	2.466766801	0.000127148
Jazf1	1.759667118	0.715045745	2.460915445	0.000626354
Gm10563	69.9179625	28.43451667	2.458911587	0.022923622
Trav7-2	28.6704	11.70109333	2.450232571	0.000601225
Pdcd1	9.74571125	3.979613333	2.448909086	4.51E-05
Fam208a	34.2831625	14.032165	2.443184106	5.35E-06
F830016B08Rik	3.633955	1.487601667	2.442827997	0.021466672
Gm5127	0.741019563	0.303456067	2.441933591	0.047480809
Gm2237	15.6445375	6.4138215	2.439191284	0.000301569
Atp2b4	203.3381182	83.75775798	2.427692946	0.007179567
5730507C01Rik	6.06624875	2.508886667	2.417904655	0.000150789
Fgf13	14.52917263	6.016735267	2.414793402	0.041611221
Zfp433	19.3954875	8.037873333	2.413012335	3.26E-05
Zfp984	35.58187	14.76186667	2.410390962	1.44E-05
Itgae	7.3685925	3.066613667	2.40284343	3.57E-06
Atp6v1g2	7.119951654	2.964339817	2.401867564	0.000865485
Hoxb4	0.94480075	0.393535	2.400804884	0.005611083
Zfp932	56.56420226	23.63037502	2.393707346	1.16E-05
Gm14295	58.266484	24.3780225	2.390123481	0.029165343
Trav19	6.91652875	2.923395	2.365923438	0.015224049
Sema3d	0.49152925	0.207889	2.364383156	0.001466297
Lrif1	85.66373888	36.3165785	2.358805328	8.81E-05
Wdr17	4.762151125	2.020951333	2.356390798	0.027259473

Stra6	3.029011942	1.28683145	2.353852902	0.010085229
Mmd	22.60162588	9.603621	2.353448337	1.95E-06
Gm28053	28.1203875	11.94928167	2.353311963	0.000425649
Ccr4	15.082775	6.41186	2.352324443	0.001008921
Clip3	2.243883325	0.955322467	2.34882294	0.03208049
Tma7-ps	309.545625	131.9076833	2.346683811	0.012878952
Rdh16	4.907613379	2.096141933	2.341260055	0.000139853
Mblac2	5.59922625	2.399275	2.333715914	0.000329966
Lclat1	28.595263	12.25629083	2.333109045	0.000208399
Myo3b	3.636735305	1.559010372	2.332720405	0.000118459
Zfp759	2.61592625	1.123093333	2.329215366	0.000159219
Khdc1a	8.5487675	3.674353333	2.326604636	0.003475872
Apol9b	3.30853	1.424135	2.323185653	0.001157471
Cysltr2	4.68021025	2.016915316	2.320479304	0.000120259
Clec2d	271.78475	117.1473167	2.320025398	0.000364065
Me3	23.87033024	10.29815305	2.317923431	0.000264737
Chst2	1.2643075	0.545507667	2.317671368	5.14E-05
Gm45716	2.3517625	1.016294167	2.314056872	0.011732091
Nt5e	60.80269975	26.36432683	2.3062489	2.63E-07
Gm14410	8.150562625	3.536800333	2.304501769	0.041526281
Gimap7	94.2914625	40.93896667	2.303220383	5.35E-06
Trav12-2	18.1117875	7.866121667	2.302505385	0.002598099
Gm12573	28.7250225	12.47893667	2.301880622	0.0411208
AI182371	4.810257	2.09352725	2.297680625	0.027142487
Gm10705	51.5599875	22.45373333	2.296276826	0.008144456
Zfp948	5.15777625	2.250388333	2.291949427	6.53E-05
Rragd	4.631381381	2.022730567	2.289667965	0.009815238
Cd83	23.8270625	10.45158	2.279756984	3.09E-05
Gm20939	12.56031625	5.509518333	2.279748517	3.28E-05
Ifi44	2.201536688	0.968477583	2.273193232	0.017496884
Tex15	0.694303575	0.306072651	2.268427358	0.049982007
Art2a-ps	32.216675	14.20913667	2.267321073	4.54E-05
Gm9967	0.51038225	0.22548375	2.263499033	0.041620383
Zfp125	17.05844	7.538183333	2.262937799	0.000432688
Vmn2r84	3.2159	1.422133833	2.261320225	0.000125231
Park2	3.639860825	1.609764	2.261114564	0.02138107
Sumo1	156.5021684	69.38165283	2.2556708	0.000211716
Trim30d	26.54310625	11.78049017	2.253141073	1.39E-05
Atp5j	371.4885286	164.8897243	2.25295136	0.000436389

Prdm5	1.174531075	0.52137015	2.252777753	0.007800013
Ryr2	0.789852	0.350878815	2.251067794	0.036390265
Nlk	37.97519521	16.88203883	2.24944366	0.000152809
Zfp874b	8.92553	3.972306667	2.246938806	0.00092557
Trav4-3	15.8165625	7.042103333	2.245999775	0.014161269
Micu3	9.750185625	4.343675833	2.244685377	2.28E-05
Rtp4	23.7851875	10.61886333	2.239899578	3.92E-06
St8sia4	38.59341335	17.23765	2.238902249	2.59E-05
Ptger2	9.7154375	4.34774	2.23459487	4.03E-06
Zfc3h1	102.349835	45.826285	2.23343077	4.69E-05
Dusp19	9.347417963	4.186854833	2.232563185	0.000699995
Gm12693	68.9439375	30.88978333	2.231933347	0.000905278
Rps27	14368.1375	6442.015	2.230379392	0.00077106
Zfp931	7.582380292	3.40288395	2.228221827	0.000544857
Gm28551	0.7074335	0.318001717	2.224621639	0.042370566
Slc35g2	1.797587625	0.808399817	2.223636854	0.041444764
Ntrk2	0.324971875	0.14616289	2.223354197	0.028953443
AW146154	5.66968375	2.55072	2.222777784	4.81E-05
Trim5	12.32888375	5.557869333	2.218275209	0.000607597
Zfp966	20.87988254	9.427471667	2.214791333	0.007758039
Ptpn13	5.82114375	2.629108333	2.214113308	0.00527086
Myo1h	5.890108795	2.672232433	2.204190295	7.32E-05
Gm3468	13.6816425	6.21557	2.201188708	0.002851059
Zfp457	1.095865625	0.49786675	2.201122338	0.038335146
Tdgf1	1.421803875	0.646299667	2.199914294	0.003911247
Insig2	71.64470228	32.61723284	2.196529137	0.004726382
Pcdhgb6	0.645290375	0.293817333	2.196229772	0.035732831
Gm12669	15.21630625	6.935688333	2.193914363	0.009122738
Trbv12-1	125.549975	57.246	2.193165898	0.001036972
Magi2	0.37738025	0.172110317	2.192664898	0.016535386
Plcb4	11.7966649	5.387178846	2.189766711	0.00118025
Rps3a1	1873.2025	857.0088333	2.185744682	0.000346168
Gm12250	32.2946	14.79556667	2.182721401	6.33E-06
Gm14124	1.015041125	0.465346167	2.181260313	0.00266375
Ceacam19	0.29055825	0.133458683	2.177140091	0.045215559
Zfp455	4.201568875	1.930642333	2.176254401	0.000103585
Twsg1	20.4170875	9.382381667	2.176109247	2.64E-06
Zfp456	6.368478	2.933261667	2.17112509	0.000739899
Jrkl	14.40325	6.639675	2.169270333	7.30E-05

Slfn1	149.839375	69.08213333	2.169003298	7.36E-05
Mterf1a	10.13414488	4.681712833	2.164623341	0.000158277
Ccl25	12.69541488	5.869266067	2.163032776	0.013621756
Zfp97	15.1767375	7.018808333	2.162295475	8.04E-06
Gm5148	2.437251178	1.127551502	2.161543107	0.004361069
Gm4787	1.042608375	0.482463167	2.161011341	0.006691198
5330438D12Rik	1.74128625	0.807567333	2.156211845	0.0029305
Ugt3a1	10.7372257	4.981862	2.155263574	0.013820781
Zfp322a	11.36758561	5.284118333	2.151273854	0.001136203
Fam227a	1.53273975	0.71258285	2.150963569	0.004148707
Traj3	844.294375	393.1125	2.147716939	0.042713476
Cd200r1	15.1665475	7.064685	2.146811571	3.20E-05
Zfp992	8.269675	3.85424	2.145604581	4.11E-06
Fam124b	3.452065875	1.609356667	2.144997406	0.000273577
Galm	12.12786963	5.655532667	2.144425705	0.000103049
Tmem181b-ps	31.2889013	14.59201842	2.144247656	0.007755605
Ndufaf1	16.32596103	7.650881833	2.133866577	0.004020364
Gm9892	21.615675	10.15343167	2.128903381	0.010338617
Fam8a1	25.3859925	11.92572333	2.128675284	0.000104466
Alox8	1.765034	0.829407667	2.128065692	0.00594527
Sp6	6.003558298	2.827163667	2.123526971	0.000135835
1110032F04Rik	10.75036875	5.065941667	2.12208696	0.001236402
Bmt2	12.3850125	5.836838333	2.121870059	2.42E-05
Zfp54	6.2036445	2.923764945	2.121800014	0.000997718
Actg2	4.062542363	1.91933505	2.116640533	0.009194814
Gm10775	3.27579875	1.549253333	2.114437116	0.002077495
Zfp329	19.09053063	9.037640083	2.112335792	2.66E-05
Tmem254c	20.39662125	9.658916	2.111688439	0.003878034
Gm3739	25.4741	12.07247833	2.110096974	0.000210623
Trav3d-3	24.2931625	11.52616333	2.107653848	0.00595775
Trav3n-3	24.2931625	11.52616333	2.107653848	0.00595775
2210017I01Rik	3.248275	1.542353167	2.10605137	0.020222779
Zfp943	26.741095	12.69931362	2.105711837	1.53E-05
Zfp72	1.577054125	0.749460167	2.104253428	0.024994644
Trav7d-4	11.49077	5.477083333	2.097972461	0.010443317
Ildr1	2.484691572	1.18440485	2.097839748	0.00083135
Tktl1	0.719021375	0.342824833	2.097343323	0.038885843
Trim34a	32.28249267	15.42524683	2.092834754	2.83E-06
Ifi206	58.09973125	27.76749	2.092365253	0.004099084

D3Ertd751e	14.39732518	6.884489849	2.091269723	0.000836216
Serpinc1	4.3798735	2.094979767	2.090651934	0.01896917
Zfp748	10.18105913	4.870653833	2.090285919	0.000205936
Lpxn	44.4520875	21.28535	2.088388845	2.37E-06
Gm14681	305.113125	146.2878333	2.08570404	0.002802447
Dscam	0.211984125	0.101767533	2.083023122	0.043472211
Cysltr1	0.957432375	0.460046	2.081166612	0.033813723
Gm29094	18.4833125	8.88455	2.080388146	0.015264642
Cxcl15	0.616048875	0.296200167	2.079839731	0.03208049
Gm14388	12.80526375	6.158963333	2.079126479	0.004530003
Trbv12-2	153.427875	73.96053333	2.074456039	0.0003272
Spock2	5.234067791	2.523357717	2.074247245	7.59E-05
Trav10	29.2417375	14.10705667	2.07284469	0.002553739
Gpr34	19.18153363	9.257726833	2.071948543	4.13E-05
Slc25a40	15.12439388	7.30852	2.06941951	1.71E-05
Tmem65	15.890325	7.679365	2.069223823	7.74E-05
Trbv17	89.6296	43.32438333	2.068802672	0.000470763
Gm13081	0.504252125	0.2437963	2.068333789	0.007654366
Zfp160	11.9864775	5.798808333	2.067058749	5.25E-05
Zbtb6	17.19125725	8.326754833	2.064580691	5.35E-06
Nat2	13.01140788	6.311678833	2.061481298	0.002093609
Sorcs2	7.008874613	3.401897167	2.060284091	0.005329674
Zfp85	8.30489375	4.034771167	2.058330797	0.00062493
Tiam1	74.25339842	36.07565614	2.058268826	0.000240533
Eid2	1.76178375	0.856998667	2.0557602	0.021160213
Olfr536	0.794777	0.386648167	2.055556106	0.010741145
Ddc	2.661477375	1.29570185	2.054081636	0.020406793
Gm10778	5.67821875	2.764584333	2.053914103	0.000161705
Gm10177	1135.736575	553.6033333	2.051534929	0.001193487
Tm2d1	29.670885	14.465822	2.051102592	0.000141266
Cdk15	1.605663625	0.783398667	2.049612405	0.02641735
1110002L01Rik	9.506589875	4.639781483	2.048930517	0.000125231
Ifi203	278.4521178	136.1175577	2.045673772	2.70E-05
Nlrp1b	2.329449364	1.138912193	2.045328321	0.001621161
Prg4	23.99192342	11.7385125	2.043864026	0.000979353
Trav6-6	64.4181	31.5428	2.042244189	0.0123102
Gm7609	5.52450621	2.70707	2.040769618	0.031227273
Tnfsf8	32.146825	15.75853333	2.039963004	2.51E-06
Plagl1	8.702147165	4.266179776	2.039798513	0.008173712

Ttc32	7.54970125	3.701788333	2.039474052	0.00169729
Spopl	82.60943996	40.53437074	2.03800968	0.000728021
Btnl7-ps	3.425295	1.680788333	2.03790979	0.003553927
Rpl3	995.0384961	488.3694568	2.037470776	0.001673395
Usp27x	1.7184035	0.843692433	2.036765333	0.002049817
Ccdc148	0.923255538	0.4534924	2.035878744	0.034216656
Tox	33.70806783	16.57519358	2.033645499	1.36E-05
AW112010	197.66375	97.2856	2.031788363	0.000153141
Fam111a	37.29012663	18.36953272	2.02999865	6.08E-05
Scg5	5.271865	2.597835	2.029330192	0.004561199
Trav4-4-dv10	32.6078625	16.0706	2.029038275	0.004200679
Psme2	1675.721035	825.9574853	2.028822385	1.39E-05
Usp18	24.822625	12.25072167	2.026217367	0.000917982
Ly6a	123.4760036	60.94374617	2.026065206	0.003347231
Snrpf	13.76991875	6.800976667	2.024697249	0.010967102
Icos	42.43954738	20.961945	2.024599691	1.06E-05
Gm9493	456.682375	225.8051333	2.022462325	0.033551876
Hspa12a	0.578403875	0.286050333	2.022035312	0.015774218
Sgcb	5.39757125	2.673023333	2.01927577	3.07E-05
Gm20479	12.83451375	6.357816667	2.018698308	0.04433545
Ms4a6b	828.2622673	410.9896217	2.015287549	1.70E-05
Csprs	4.47076625	2.219005	2.014761684	0.011109214
Cxcr5	13.14954038	6.529810667	2.013770543	0.000110925
Adra2b	0.4792345	0.238044167	2.013216735	0.022953596
Kcnmb1	0.93097925	0.4632465	2.009684369	0.003574511
Msl3l2	4.09684625	2.038823333	2.009416992	0.009759385
Arxes2	2.63904875	1.313550833	2.009095258	0.020496898
Katnbl1	8.87994725	4.4207075	2.008716308	3.60E-05
Rps12-ps3	548.301625	273.0208333	2.008277604	0.003136131
Gnpda2	19.64791136	9.784556152	2.008053411	0.001922174
Mterf1b	6.31462375	3.149013333	2.005270566	0.002039034

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