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Integrin activation controls regulatory T cell-mediated peripheral tolerance

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Abstract

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-to-cell and cell-to-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 murine Treg cell function. Treg cell-specific loss of talin, a β integrin-binding protein, or expression of talin(L325R), a mutant that selectively abrogates integrin activation, resulted in lethal systemic autoimmunity. This dysfunction could be attributed, in part, to a global dysregulation of the Treg cell transcriptome. Activation of integrin α4β1 led to increased suppressive capacity of the Treg cell pool, 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 function.

INTRODUCTION

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).

The affinity of integrins for extracellular ligands is tightly regulated and critical for normal hematopoietic cell adhesion and function (2). 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) (3, 4). 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 (5), and for outside-in integrin signaling, which is initiated by the binding of extracellular ligands (6). In addition to facilitating integrin activation, talin functions to link integrins to the actin cytoskeleton (7) and recruits numerous other integrin-associated proteins (2).

In T cells, integrin signaling is essential for facilitating trafficking throughout the body during homeostasis and infection. For instance, integrin α4β1 (VLA-4) facilitates cell trafficking to sites of inflammation and integrin αLβ2 (LFA-1) facilitates trafficking to lymph node. Importantly, LFA-1, along with talin, is recruited to the immunological synapse (IS) that forms between T cells and antigen presenting cells (APCs) and is thought to stabilize this cell-to-cell interaction to facilitate T cell activation (8). Using a CD4[Cre] conditional mouse model in which talin was deleted in all T cells, 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 (9). We utilized the same mouse model to demonstrate that T-cell specific deletion of talin resulted in spontaneous T cell activation that appeared to be due, in part, to defects in Treg cell function, homeostasis and survival, indicating that there may be a specific requirement for talin-mediated integrin signaling in Treg cells (10). However, because this model results in loss of talin in all CD4^{+} and CD8^{+} T cells during thymic development, we were unable to exclude the possibility that talin-dependent functions in non-Treg T cells might be involved in the maintenance of immune homeostasis.

Regulatory T (Treg) cells are a distinct subset of CD4^{+} T cells that maintain immune homeostasis (11). Deficiency in the number or function of Treg cells results in inadequate immune suppression, which can result in autoimmune and inflammatory disorders (12–14). The role of talin in integrin activation may thus represent a previously unexplored

mechanism contributing to Treg cell function, identity and homeostasis. The integrin LFA-1 is 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 (15–17), cytolysis through the production of granzyme B in Treg cells (18), conversion of ATP to adenosine through the expression of both CD39 and CD73 on the surface of Treg cells (19) and clustering around activated dendritic cells to provide a physical barrier to prevent activation of naïve T cells (20). Whether integrins play a role in Treg cell homeostasis beyond simply facilitating adhesion to mediate these contact-dependent suppressive mechanisms, however, remains largely unexplored.

Here we sought to investigate whether integrin activation is required to maintain Treg cell identity and function. Treg cell-specific deletion of talin resulted in 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 (21–23), 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 β1 integrins led to increased expression of IL-2Rα on Treg cells, and increased the suppressive capacity of talin-deficient Treg cells. 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.

MATERIALS AND METHODS

Mice

All mice were bred and housed in specific pathogen-free conditions prior to use. $Tln1^{fl/fl}$, $Tln1^{L325R/fl}$, $Foxp3^{GFP}$, and $Foxp3^{Cre}$ mice have been described previously (22, 24–26). 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.

Antibodies and flow cytometry

Murine antibodies against the following proteins were obtained from Biolegend: CD4 (RM4-5), CD8 (53-6.7), Foxp3 (150D), CD44 (1M7), CD62L (MEL-14), IFNγ (XMG1.2), TNFα (MP6-XT22), IL-17A (TC11-18H10.1), IL-2Rα (PC61), CD103 (2E7), CTLA4 (UC10-4B9), CD39 (24DMS1), CD73 (TY/11.8), GITR (DTA-1), GFP (FM264G), and Ki67 (16AB). For intracellular detection of cytokines, splenocytes were stimulated *ex vivo* with PMA (Sigma) and ionomycin (Sigma) in the presence of Brefeldin A (Sigma) for 4 hours at 37°C; cells were fixed and permeabilized with the Foxp3 Transcription Factor Fixation/Permeabilization kit (eBioscience) prior to intracellular staining. All samples were analyzed on an Accuri C6 or LSRFortessa (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$^-$ conventional T (Tconv) cells were isolated from spleens of wild-type mice using the CD4$^+$ T cell negative isolation kit (Miltenyi Biotec) with a biotin-conjugated anti-CD25 (PC61, Biolegend) antibody included to deplete pre-activated cells. Tconv cells were then labeled with CFSE as previously described (27). Wild-type or talin-deficient CD4$^+$YFP$^+$ 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). 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 250ng/mL soluble anti-CD3 (2C11) for 96 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.

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 $Tln1^{\text{wt/wt}}Foxp3^{\text{Cre/wt}}$ and $Tln1^{\text{fl/fl}}Foxp3^{\text{Cre/wt}}$ 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.

Statistics

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). P values are denoted in figures by * P<0.05, **P<0.01, *** P<0.005.

Study Approval

All animal studies were approved by the Institutional Animal Care and Use Guidelines of the University of California, San Diego.

RESULTS

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 (26). 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. 1A–E). 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 (Fig. 1F–I).

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. 2A, 2B), 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. 2C).

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. 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. 2E). 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. 2F). In addition, talin-deficient Treg cells produced significantly higher levels of IFNγ compared to wild-type Treg cells (Fig. 2G), 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.

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 (28) and TIAM1 (29) 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 (21–23, 30). The mutation in talin blocks the conformational change that leads to integrin activation, as it specifically blocks the interaction between the talin head and the membrane proximal region of the β-integrin cytoplasmic tail, but does not disrupt the interaction between the talin head and the distal regions of the β-integrin cytoplasmic tail (21). We therefore generated $Tln1^{L325R/fl}Foxp3^{Cre}$ hemizygous male mice to selectively abolish the capacity of talin to activate integrins in Treg cells. Treg cells from $Tln1^{L325R/fl}Foxp3^{Cre}$ male animals express half the wild-type level of talin, all of which is the mutant form, and were compared to $Tln1^{wt/fl}Foxp3^{Cre}$ control animals, which express half the wild-type level of talin, all of which is in the wild-type form. Importantly, $Tln1^{wt/fl}Foxp3^{Cre}$ mice exhibited no evidence of systemic disease. By contrast, $Tln1^{L325R/fl}Foxp3^{Cre}$ mice developed systemic autoimmunity resembling that exhibited by male hemizygous $Tln1^{fl/fl}Foxp3^{Cre}$ mice (Fig. 3A–C). This inflammation resulted in death of these animals at 3–4 months of ages (Fig. 3D). These mice exhibited increased numbers of activated, proliferating $CD4^+$ and $CD8^+$ T cells (Fig. 3E–G). Moreover, like talin-deficient Treg cells, talin(L325R) Treg cells were not numerically deficient (Fig. 4A, 4B), and were detectable at high numbers in the blood, thymus and peripheral organs (Fig. 4C). Additionally, Treg cells isolated from $Tln1^{L325R/fl}Foxp3^{Cre}$ mice exhibited lower Foxp3 expression on a per cell basis (Fig. 4D). With respect to suppressive molecules, talin(L325R) Treg cells were impaired only in IL-2Rα expression (Fig. 4E), 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. 4F), 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.

Talin influences Treg cell phenotype and function

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. 5A) (26, 31). Owing to the presence of wild-type Treg cells, these female heterozygous mice appeared healthy without evidence of systemic T lymphocyte activation (Fig. 5B), 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. 5C). 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 (32).

Consistent with our observations in inflamed $Tln1^{fl/fl}Foxp3^{Cre}$ male mice, we observed a slight reduction in Foxp3 expression on a per cell basis in talin-deficient Treg cells isolated from uninflamed female mice (Fig. 5D). 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. 5E). 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. 5F, 5G). These data indicate that the inflammatory environment within male *Tln1*fl/fl*Foxp3*Cre mice alters the phenotype of talin-deficient Treg cells. However, because talin-deficient Treg cells isolated from inflammatory environments were able to maintain expression of each of these suppressive markers, the reduced suppressive capacity we observed in talin-deficient Treg cells was not likely due to the reduced expression of CD103, CD39, CD73, and GITR.

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 (Supplemental Fig. 1). 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.

To further explore how integrin signaling may mediate Treg cell suppression, we investigated if talin is required for Treg cell differentiation. Recent work has suggested that Treg cells can be subdivided into central Treg (cTreg) cells and effector (eTreg) cells, based on phenotypic and functional characteristics (33). cTregs, characterized by high expression of CD62L and low expression of CD44, express high levels of the chemokine receptor CCR7, allowing localization and trafficking to lymphoid tissues. cTreg cells are thought to depend on IL-2 signaling for survival, and as such, express high levels of IL-2Rα. eTreg cells are also capable of responding to IL-2 signaling, but are thought to depend more highly on ICOS signaling for survival. eTreg cells are concentrated in peripheral organs, express low levels of CD62L and high levels of CD44, and are thought to be more potently suppressive than cTreg cells. Both populations can be detected in the spleen (11). Our previous work, in which talin was deleted in all T cells revealed a numerical defect in cTreg cells (10), and as such we hypothesized that integrin signaling may be required to maintain the cTreg cell population. However, based on the expression of CD62L and CD44, deletion of talin did not affect the percentage or numbers of cTreg or eTreg cells in the spleen (Fig. 5H, 5I). Thus, integrin signaling is most likely dispensable for Treg cell differentiation. Additionally, talin-deficient cTreg cells expressed significantly less IL-2Rα than control cTreg cells, while talin-deficient eTreg cells express significantly less GITR than control eTreg cells, indicating that talin is required for both subsets (Supplemental Fig. 2).

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 suppressive capacity raised the possibility that talin might be required for the maintenance of the Treg cell transcriptional program. 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. 6A, Supplemental Table I). 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* (25, 34–36), and transcriptional regulators such as *Id2* (37–39). 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. 6B–D). Moreover, there were significant changes in diverse groups of transcription factors and molecules involved in integrin signaling (Fig. 6E, 6F). 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 (Fig. 6E). Taken together, these findings suggest that talin is required for the maintenance of diverse aspects of the Treg cell transcriptional program.

Activation of β integrins enhances Treg cell function and phenotype

Having observed that disrupting integrin activation resulted in reduced Treg cell function, 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 integrins in their active conformation (40, 41), has been previously shown to exert beneficial effects on cell-matrix interactions *in vivo* (42). 9EG7 can be used as a reporter of β1 integrin activation and to increase the function of β1 integrins; for example, 9EG7 stimulates and increases the adhesion of multiple cell lines (43, 44), stabilizes the extension of active β1 integrins (45), and increases the maximum ligand binding of β1 integrins by two-fold (46). Treatment of $Tln1^{fl/fl}Foxp3^{Cre}$ mice with β1aAb did not alter the proportions of CD4^{+}, CD8^{+}, or Treg cells (Fig. 7A, 7B), and did not prolong the survival of mice (data not shown). Nonetheless, treatment 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. 7C, 7D). 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. 7E, 7F), thus confirming a role for talin and activated integrins in the maintenance of IL-2Rα expression. However, β1aAb treatment in $Tln^{fl/fl}Foxp3^{Cre}$ mice did not result in improvements to Treg cell suppressive capacity (Fig. 7G). Because $Tln1^{fl/fl}Foxp3^{Cre}$ male mice exhibit signs of disease even at weaning at 3 weeks of age, it is likely that Treg cells from these inflamed mice may already be irreversibly impaired.

Thus, to investigate whether β1aAb treatment might improve suppressive function in talin-deficient Treg cells in the setting of an uninflamed environment, we treated female $Tln1^{fl/fl}Foxp3^{Cre/wt}$ mice; Treg cells derived from these mice exhibited a significant increase in their *in vitro* suppressive capacity (Fig. 7H). However, treatment could not rescue suppression to wild-type levels, suggesting that other integrins may also need to be involved

in mediating Treg cell suppressive capacity. To further test the role of with β1 integrin activation in Treg cell function, we treated wild-type animals with β1aAb. β1aAb therapy significantly enhanced the suppressive function of wild-type Treg cells *in vitro* (Fig. 7I) without altering the proliferation of conventional T cells (Fig. 7J). Taken together, these results raise the possibility that agents that activate integrins in Treg cells may be useful in the treatment of autoimmune disease.

DISCUSSION

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 and function. Here we have shown that mice with a 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 IL-2Rα, and exhibited global transcriptional dysregulation.

This work has built on our previous study that identified a specific role for talin in Treg cell homeostasis by using *Tln1*^{fl/fl}*Cd4*^{Cre} mice in which talin is deleted in all T cells (10). In the present study, by using a mouse model with a Treg cell-specific deletion in talin or expression of a mutant form of talin, we have been able to determine that it is talin's role in integrin activation that is required for Treg cell function. There are two notable differences observed between *Tln1*^{fl/fl}*Cd4*^{Cre} mice, described in our prior study, and *Tln1*^{fl/fl}*Foxp3*^{Cre} mice, which we used in the current study. First, *Tln1*^{fl/fl}*Cd4*^{Cre} mice do not develop systemic autoimmunity, most likely due to defects in talin-deficient non-Treg CD4⁺ and CD8⁺ T cells. It has been previously shown that talin-deficient T cells have defects in contact-dependent proliferation (9), which may explain why they are unable to cause disease. Second, talin-deficient Treg cells were numerically deficient in *Tln1*^{fl/fl}*Cd4*^{Cre} mice, but not in *Tln1*^{fl/fl}*Foxp3*^{Cre} mice. We hypothesize that this difference may be due to the timing at which the deletion of talin occurs in the two mouse strains. In *Tln1*^{fl/fl}*Cd4*^{Cre} mice, talin is deleted during the double-positive stage of thymic development, while in *Tln1*^{fl/fl}*Foxp3*^{Cre/wt} mice, talin is deleted only after Foxp3 is expressed by developing thymic Treg cells.

Our data indicate that talin plays a critical role in maintaining Treg cell function and phenotype in the periphery, suggesting a specific role for activated integrins in the maintenance of Treg cell identity after thymic development. Phenotypically, we observed downregulation of CD103, CD39, CD73, and GITR on the surface of peripheral Treg cells, which was apparently masked by the presence of inflammation in diseased male *Tln1*^{fl/fl}*Foxp3*^{Cre} and *Tln1*^{L325R/fl}*Foxp3*^{Cre} mice, indicating that the upregulation of these molecules is not capable of rescuing talin-deficient Treg cell function. IL-2Rα expression by talin-deficient Treg cells was reduced in both inflamed and uninflamed mice. This finding is intriguing in light of the fact that IL-2 signaling is a central regulator of Treg cell development, homeostasis and suppressive function (11, 38, 47, 48). Moreover, treatment of inflamed *Tln1*^{fl/fl}*Foxp3*^{Cre} mice with β1aAb partially rescued IL-2Rα expression, indicating a possible role for direct signaling through β1 integrins in maintaining high IL-2Rα

expression on Treg cells. 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 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 (49, 50). Germline deletion of β2 integrins reduced the splenic and thymic Treg cell population size and blunted the suppressive capacity of Treg cells both *in vitro* and *in vivo* in an adoptive transfer model of colitis (51). 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 (52, 53). 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.

All currently available integrin-targeting therapies for autoimmune disease function to block integrin signaling and are thought to function by preventing trafficking of activated effector T cells to sites of inflammation, thereby ameliorating disease (54). 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 (54). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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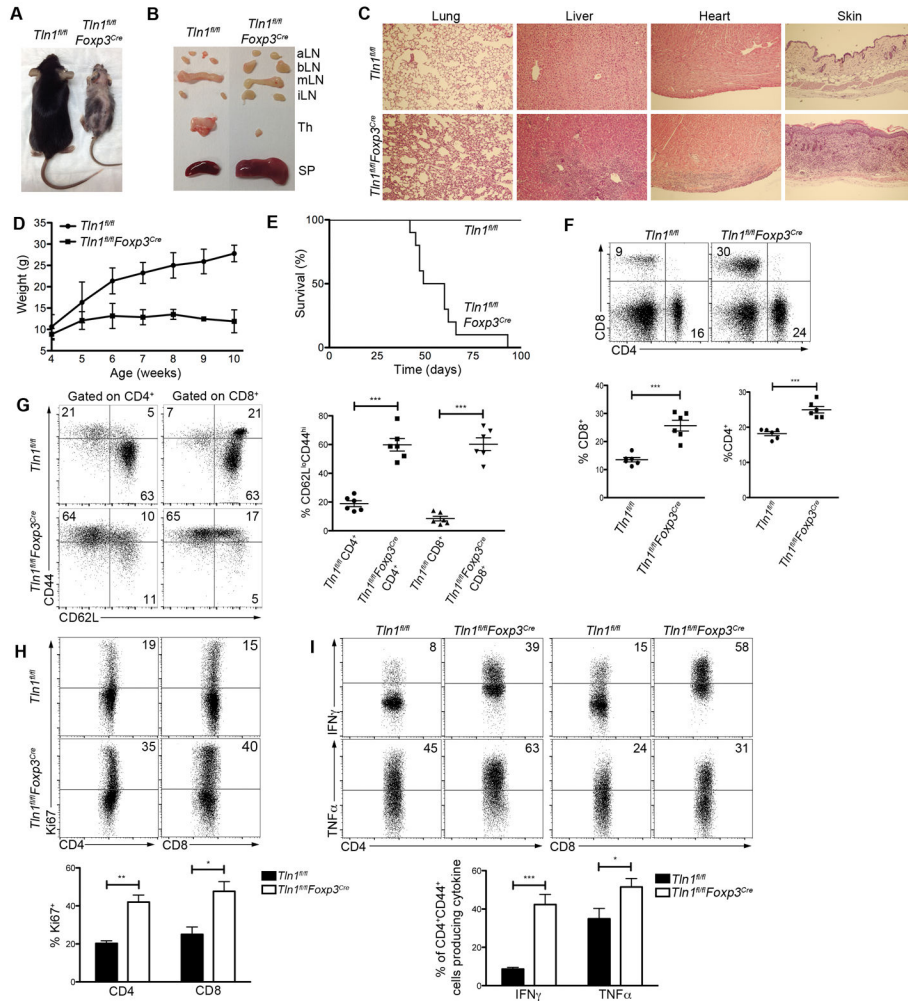
Figure 1. Spontaneous lethal inflammation in mice with a Treg cell-specific deletion of talin
(A and B) Morphology (A) or organs (B) (spleen, lymph nodes (aortic, brachial, mesenteric, inguinal), and thymi) of male $Tln1^{fl/fl}$ or $Tln1^{fl/fl}Foxp3^{Cre}$ mice. (C) H&E stains of lung, liver, heart, 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. (F–H) Percentages of splenic CD4+ or CD8+ T cells (F) expressing CD44, CD62L (G), or Ki-67 (H) from $Tln1^{fl/fl}$ or $Tln1^{fl/fl}Foxp3^{Cre}$ mice; displayed cells gated on CD4+ or CD8+ events; n=6. (I) 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+CD44hi or CD8+CD44hi events; n=6. 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.
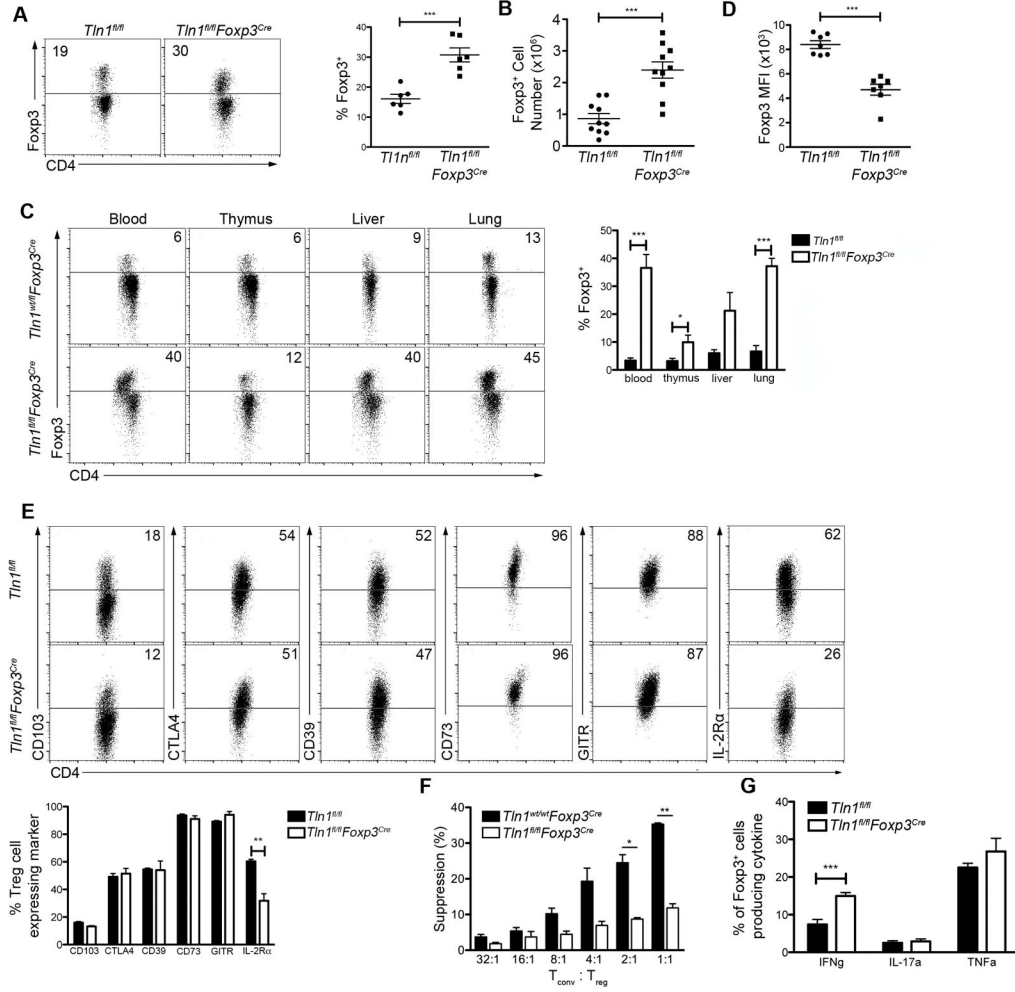
Figure 2. Talin deficiency decreases Treg cell suppressive capacity
(A–B) Percentages (n=6) (A) and numbers (n=10) (B) of splenic CD4$^+$ T cells from $Tln1^{fl/fl}$ or $Tln1^{fl/fl}Foxp3^{Cre}$ mice expressing Foxp3. (C) Foxp3$^+$ cells as a percentage of CD4$^+$ T cells within tissues (blood, thymus, liver, lung and lymph node) derived from $Tln1^{fl/fl}$ or $Tln1^{fl/fl}Foxp3^{Cre}$ mice; n=6. Mice were perfused prior to isolation of cells from liver and lung. (D) Mean fluorescence intensity of Foxp3 from talin-deficient and wild-type Treg cells; n=7. (E) 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. (F) 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. (G) 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, unpaired Student's t-test.
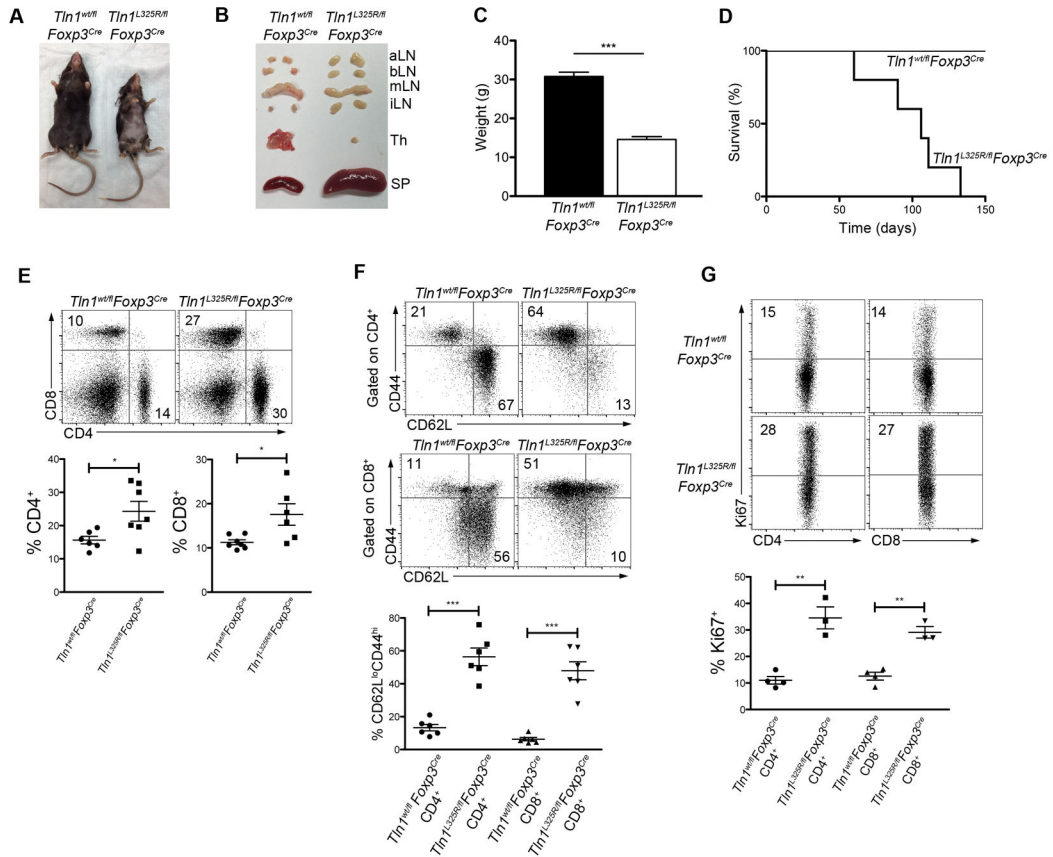
Figure 3. Expression of mutant talin(L325R) in Treg cells results in lethal systemic inflammation
(A and B) Morphology (A) or organs (B), spleen, lymph nodes (aortic, brachial, mesenteric, inguinal), and thymi) of male $Tln1^{wt/fl}Foxp3^{Cre}$ or $Tln1^{L325R/fl}Foxp3^{Cre}$ mice. (C) Body weight of $Tln1^{wt/fl}Foxp3^{Cre}$ or $Tln1^{L325R/fl}Foxp3^{Cre}$ mice at 8 weeks of age; n=10. (D) Survival of $Tln1^{wt/fl}Foxp3^{Cre}$ or $Tln1^{L325R/fl}Foxp3^{Cre}$; n=5. (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, unpaired Student's t-test.
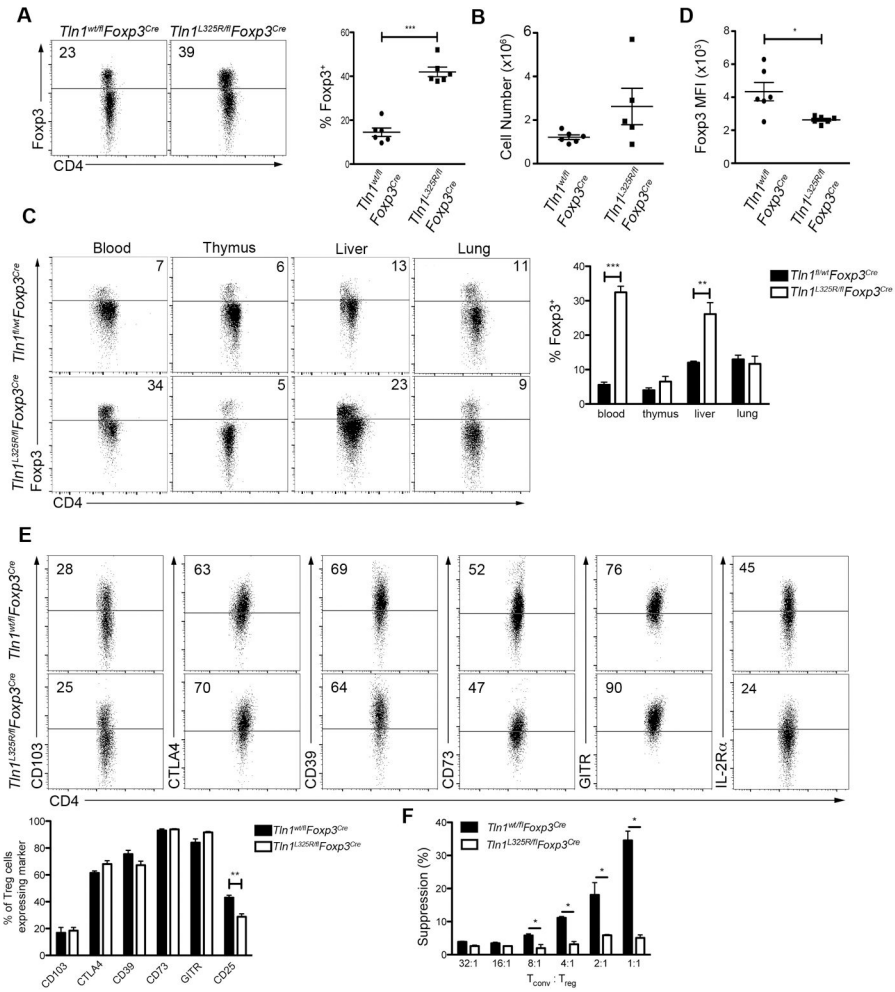
Figure 4. Integrin activation is critical for T$_{reg}$ cell homeostasis and function
(A–B) Percentage (A) and by number (B) of splenic CD4$^+$ T cells from $Tln1^{wt/fl}Foxp3^{Cre}$ or $Tln1^{L325R/fl}Foxp3^{Cre}$ mice expressing intracellular Foxp3; n=6. (C) Foxp3$^+$ cells as a percentage of CD4$^+$ 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. Mice were perfused prior to isolation of cells from liver and lung. (D) MFI of Foxp3 from CD4$^+$Foxp3$^+$ Treg cells; n=6. (E) 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. (F) Suppression by sorted YFP$^+$ Treg cells from $Tln^{wt/fl}Foxp3^{Cre}$ and $Tln1^{L325R/fl}Foxp3^{Cre}$ mice at decreasing Tconv:Treg 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, unpaired Student's t-test.
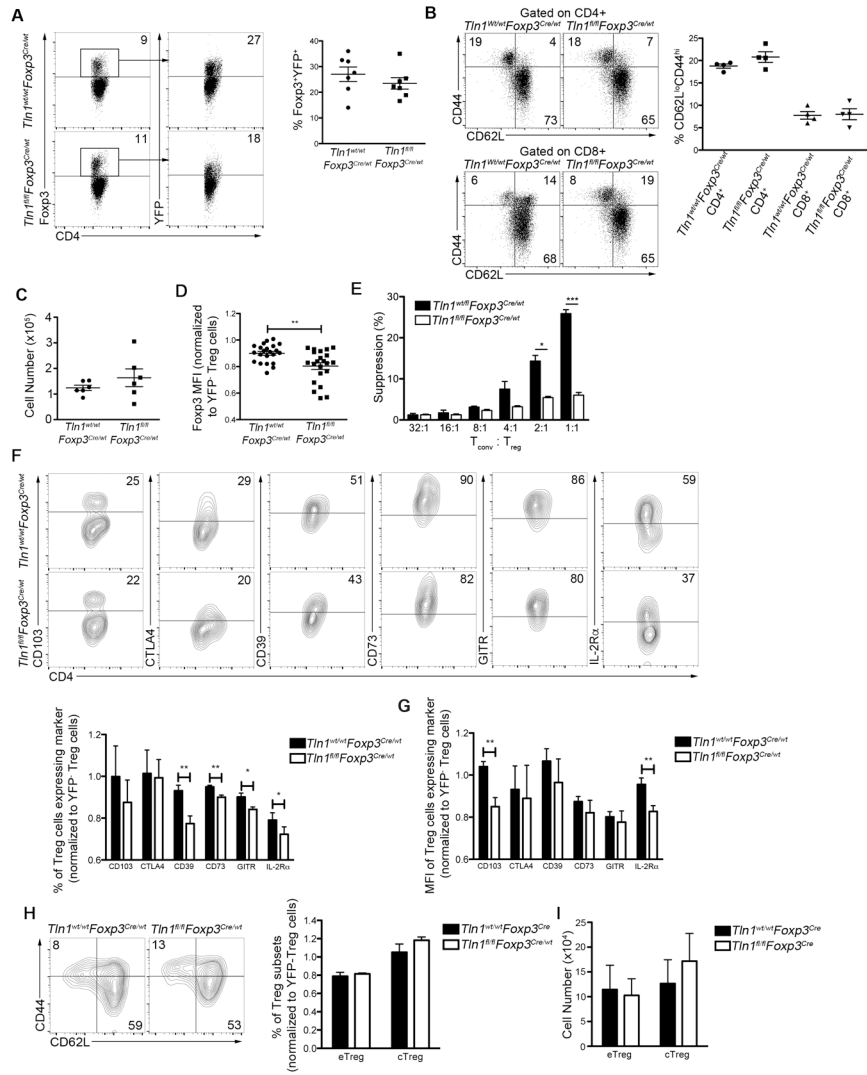
Figure 5. Talin controls the suppressive function of peripheral Treg cells

(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 Tconv:Treg cell ratios, measured at 72 hours. Expression (F) and MFI (G) 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. (H and I) Percentage (H) and absolute number (I) of eTreg (CD62L^{lo}CD44^{hi}) and cTreg (CD62L^{hi}CD44^{lo}) in $Tln1^{fl/fl}Foxp3^{Cre/wt}$ and control mice; n=5. 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.
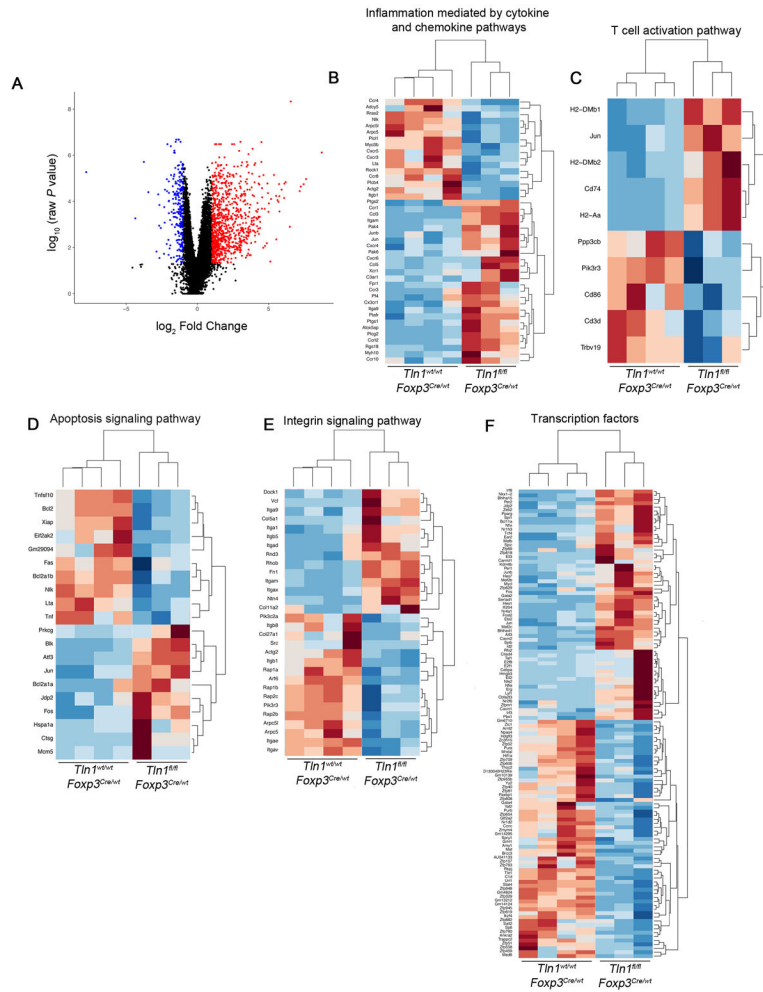
Figure 6. Talin modulates multiple aspects of the Treg cell transcriptome

(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. (B–F) Heatmaps showing fold-change of differentially expressed genes within selected significantly enriched GO categories.
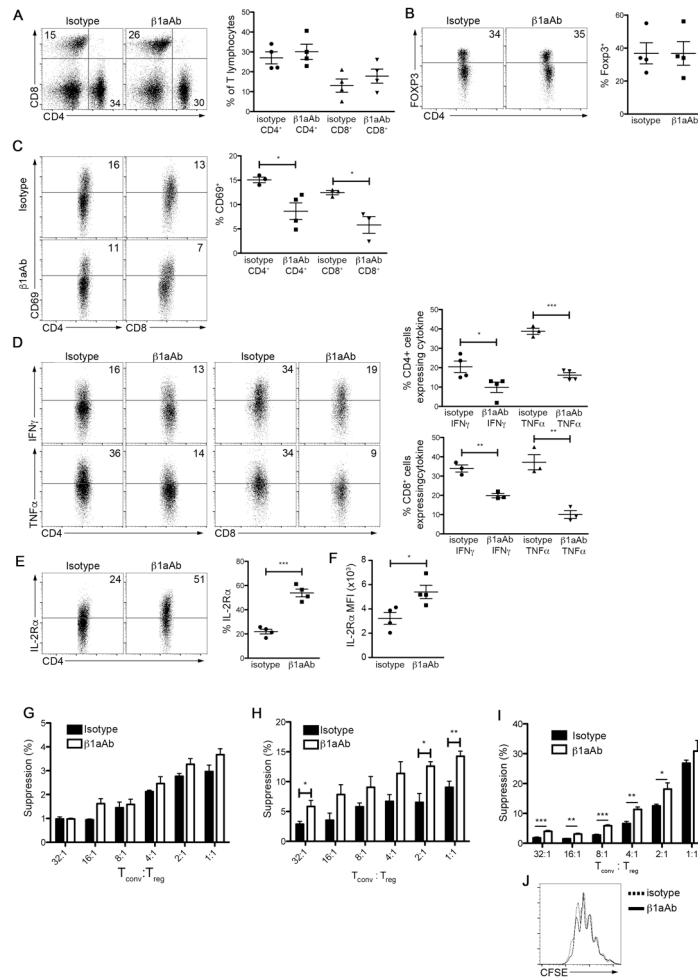
Figure 7. Integrin-modulating therapy improves Treg cell function and ameliorates inflammation
(A and B) Percentages of splenic CD4+, CD8+ (A), and Treg cells (B) from male
*Tln1*fl/fl*Foxp3*Cre mice treated with β1aAb (9EG7) or isotype control administered every 5
days for 6 weeks; n=4. (C) Percentages 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; n=4. (D) 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+CD44hi or CD8+CD44hi events; n=4. (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); n=4. (G) Suppression by sorted talin-deficent Treg cells from
β1aAb- or isotype-treated *Tln1*fl/fl*Foxp3*Cre mice at decreasing Tconv:Treg cell ratios,
measured at 72 hours; cultures were treated daily with β1aAb or isotype control. (H)
Suppression by sorted talin-deficient Treg cells from β1aAb- or isotype-treated
*Tln1*fl/fl*Foxp3*Cre/wt mice at decreasing Tconv:Treg cell ratios, measured at 72 hours;
cultures were treated daily with β1aAb or isotype control. (I) Suppression by sorted GFP+
Treg cells from β1aAb- or isotype-treated *Foxp3*GFP mice at decreasing Tconv:Treg cell
ratios, measured at 72 hours; cultures were treated daily with β1aAb or isotype control. (J)
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, unpaired Student's t-test.