UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Role for Talin in Immune Tolerance

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for the degree Master of Science

in

Biology

by

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The Thesis of Kelly Anne Remedios is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2011

DEDICATION

This thesis is dedicated to the memory of Marjorie Carroll, who will always be in my heart.

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ABSTRACT OF THE THESIS

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Professor John Chang, Chair

In T cells, Talin has been shown to be a potent activator of integrins which are known to regulate immune synapse formation and migration through the periphery. Outside of its role in mediating integrin adhesion, the role of Talin in T cells is not well understood. To explore the consequences of Talin deletion in T cells, we examined CD4-Cre positive Talin$^{fl/fl}$ mice. Here, we show that the majority of T cells in floxed Talin-deficient mice display a previously activated CD44hi phenotype. CD4+CD44hi T cells from floxed Talin-deficient mice produced IL-17 and IFNγ, while CD8+CD44+ T cells from floxed Talin-deficient mice produced less TNFα and IFNγ than their wild-type counterparts Furthermore, CD44hi cells from floxed Talin-deficient mice failed to express surface CTLA-4, a molecule known to inhibit T cell activation. This defect in

CTLA-4 expression, however, appeared to be limited to CD44hi T cells, as natural T regulatory cells expressed normal levels of CTLA-4. Together, these results suggest a previously unappreciated role for Talin in regulating immune tolerance through the expression of CTLA-4.

Introduction

In order to initiate an adaptive immune response, a T cell needs to be activated by an antigen-presenting cell (APC) bearing its cognate antigen. During this interaction, a cluster of T cell receptors (TCRs) surrounded by adhesion and signaling molecules along with the MTOC polarize at the interface between the T cell and APC (Grakoui, et al., 1999). This clustering of adhesion and signaling molecules is referred to as the immune synapse. Formation of the immune synapse has been hypothesized to play a critical role in the activation of naïve T cells and for mature T cells to carry out their effector functions.

Along with adhesion molecules such as LFA-1, one of the key molecules found to polarize to the immune synapse is Talin (Monks, et al., 1998). Talin is a 235-kDa protein made up of a 45-kDa N-terminal head domain and a 190kDa C-terminal rod domain (Rees, et al., 1990). The N-terminal head domain is responsible for integrin activation through its integrin binding site while the C-terminal domain is composed of the actin-binding site. Talin is a potent activator of integrins including VLA-4, $\alpha 4\beta 7$, and LFA-1, which play roles in lymphocyte migration, immune synapse formation, and antigen-dependent activation (Evans, et al., 2009; Kandula, et al., 2004). Following TCR engagement, Talin has been shown to be important in regulating both the affinity of LFA-1 on the T cell for its ligand ICAM-1 and its polarization towards the site of contact (Simonson, et al., 2006).

Outside of its role in activating integrins and undergoing polarization at the immune synapse, the function of Talin in T cells is not fully understood. Previous work

by our lab has shown LFA-1 to be disparately inherited by a dividing CD8+ T cell responding to a microbial infection *in vivo* (Chang, et al., 2007). In CD8+ T cells, the asymmetric segregation of proteins during the first division of a naïve cell in response to a pathogen has been suggested to give rise to daughters differentially fated toward the effector and memory lineages (Chang, et al., 2007). In order to provide for both acute and long-term defense, naïve T cells need to be able to differentiate into effector and memory T cell lineages, respectively. In addition, CD4+ T cells have the ability to differentiate into specialized subsets including the Th1, Th2, Th17 and Treg subsets that respond to specific types of pathogens (Zhu, et al., 2011). Because Talin polarizes during immune synapse formation and regulates LFA-1 polarization as well, we hypothesized that Talin might regulate T cell differentiation and immunity to microbial pathogens.

Since deletion of Talin results in embryonic lethality, we bred floxed Talin-deficient mice to CD4-Cre mice. Unexpectedly, we found that these mice exhibited splenomegaly due to an increase in CD4+ T cells with a previously activated CD44hi phenotype, suggested that immunological tolerance in these mice was compromised.

Immunological tolerance, or the ability of the immune system to react to foreign antigens while ignoring self-antigens, is critical in the prevention of autoimmune disorders. Autoimmunity occurs when the cells of the immune system fail to distinguish between self versus non-self and react against the body's own cells and tissues (Nurieva, et al., 2010). The failure of immune cells to distinguish between foreign and innocuous antigens can lead to diseases including Type 1 diabetes, lupus, Graves' disease and rheumatoid arthritis. Mechanisms underlying T cell tolerance include the requirement

for costimulation for T cell activation during antigen presentation (Mueller, et al., 1989), T-regulatory cells that suppress immune responses (Rudensky, 2011), and inhibitory signals to induce T cell anergy (Alegre, et al., 2001).

One critical player that has been identified in the maintenance of immunological tolerance is CTLA-4. CTLA-4 is an inhibitory receptor that is homologous to CD28 and binds to the same ligands (CD80 and CD86) with higher affinity. CTLA-4 is not expressed by naïve T cells and is expressed on the surface only after their activation. Whereas the role of CD28 is to promote T cell activation, ligation of CTLA-4 induces cell cycle arrest and termination of activation, making it a critical regulator of peripheral tolerance (Alegre, et al., 2001). CTLA-4 deficient mice exhibit a severe lymphoproliferative disorder driven by accumulation of activated CD4+ T cells in both lymphoid and non-lymphoid organs which results in death by 3-5 weeks of age (Chambers, et al., 1997; Tivol, et al., 1995). In a naïve T cell, CTLA-4 is found intracellularly in lysosomal compartments and becomes secreted to the surface upon TCR stimulation where it exerts its immunosuppressive effects (Iida, et al., 2000). TCR signal strength has been shown to regulate the cell surface expression of CTLA-4 (Egen, et al., 2002), but beyond this the signals required for CTLA-4 on the surface of the T cell after activation are unknown.

Our study suggests a role for Talin in regulating immune tolerance. We found that the majority of the T cells in CD4-cre positive $Talin^{fl/fl}$ mice displayed a previously activated phenotype. CD4+CD44hi T cells are functional and capable of secreting IFNγ and IL-17, while CD8+CD44hi T cells fail to produce IFNγ and TNFα. The activated CD44hi phenotype seems to result from an inability of Talin-deficient CD44hi T cells to

express CTLA-4. These findings suggest that Talin plays a critical role in regulating peripheral tolerance through effects on CTLA-4.

Materials and Methods

Mice

Mice were maintained in a specific pathogen-free vivariam at UCSD. Talin$^{fl/fl}$ mice were bred to CD4-cre mice. Offspring from these breeding pairs that were CD4-Cre positive Talin$^{fl/fl}$ were confirmed for Talin deletion in T cells through PCR. CD4-cre negative littermates were used as wild-type controls.

Antibodies and Flow Cytometry

For surface staining, cells were stained at 4 degrees in a buffered saline solution. For intracellular staining, cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes and then stained with antibodies in permeabilization buffer made up of 1% FBS, 0.2% saponin, and 0.1% NaN_3 in PBS. For analysis of transcription factors, cells were fixed with the Fixation and Permeabilization Buffer kit (eBioscience). The following antibodies were purchased from eBioscience: anti-CD4-FITC, CD8-FITC, CD44-FITC, CD4-PerCPCy5.5, CD8-PerCPCy5.5, IFNγ-PerCPCy5.5, CD4-PE, CD8-PE, CD62L-PE, IFNγ-PE, CTLA-4-PE, IL-17-PE, IL-4-PE, B220-PE, CD4-APC, CD8-APC, CD44-APC, FOXP3-APC, IFNγ-APC, IL-17-APC, IL-4-APC, and TNFα-APC. The samples were analyzed on a BD Accuri C6 cytometer and analyzed with Flowjo software (Treestar).

Adoptive transfer of cells to assess trafficking

Whole splenocytes were harvested from CD4-Cre positive- and negative- Talin$^{fl/fl}$ mice and then differentially labeled with a 1:1000 and a 1:20,000 dilution of Cell Proliferation Dye, respectively. The cells were then combined at a 1:1 ratio and injected into wild-type recipients. The following day, the blood, spleen, and lymph nodes were harvested, stained with either anti-CD4 or anti-CD8, and then analyzed by flow cytometry.

In vitro proliferation

Splenocytes were stained with CD4-FITC, CD8-PE and CD44-APC, and naïve T cells (CD4+CD44 lo and CD8+CD44lo) were sort-purified using a BD FACS ARIA flow cytometer. Sort-purified cells were combined with irradiated, T-depleted APCs and labeled with Cell Proliferation Dye. After labeling, cells were cultured with IL-2, anti-CD3, and anti-CD28 for four days and then analyzed by FACS.

Ex vivo restimulation

After harvest, splenocytes were immediately stimulated with PMA (50 ng/mL) and ionomycin (0.5 ug/mL) for 4 hours in the presence of brefeldin A. After stimulation, cells were harvested, fixed in PFA, stained with antibodies and then analyzed by FACS.

In vitro differentiation

Splenocytes were stained with CD4-FITC and CD44-APC, and naïve CD4+ T cells (CD44 lo) were sort-purified using a BD FACS ARIA flow cytometer. Cells were

then cultured in the presence of soluble anti-CD3 (2.5 ug/mL) and anti-CD28 (1 ug/mL) with irradiated T-depleted splenocytes (5:1 splenocyte to T cell ratio). The following conditions were used: Th1-- IL-12 (10 ng/mL) and IL-2 (10ng/mL); Th17-- TGFβ (5 ng/mL), IL-6 (10 ng/mL), anti-IL-4 (10 ug/mL), anti-IFNγ (10 ug/mL), and IL-2 (2.5 ng/mL); induced Treg (iTreg)-- TGFβ (5 ng/mL), anti-IL-4 (10 ug/mL), and anti-IFNγ (10 ug/mL); Th2-- IL-4 (10 ng/mL), IL-2 (10ng/mL), anti-IFNγ (10 ug/mL), and anti-IL-12 (10 ug/mL). Cells cultured in Th2-promoting conditions were washed at 4 days, rested for 2 days in fresh media without anti-CD3 and anti-CD28, then restimulated with 4 hours with PMA/ionomycin. Cells cultured in Th1, Th17, and Treg conditions were restimulated with PMA/ionomycin and BFA for 4 hours before harvest on day 4 of culture.

For CD8 differentiation, splenocytes were stained with CD8-PE and CD44-APC and naïve CD4+ T cells (CD44lo) were sort-purified using a BD FACS ARIA flow cytometer. Cells were then cultured in the presence of soluble anti-CD3 (2.5ug/mL), anti-CD8 (1ug/mL), and IL-2 with irradiated T-depleted splenocytes (5:1 splenocyte to T cell ratio) for four days. After culture, cells were restimulated for 4 hours with PMA/ionomycin to look for IFNγ and TNFα production.

Results

Talin-deficient mice have increased numbers of splenic CD4+ T cells

We observed that CD4-cre positive Talin$^{fl/fl}$ (Talin-deficient) mice weighed less than Cre-negative (wild-type) littermates at 8 weeks of age, and this difference was more pronounced at 4 months of age (Figure 1A). In addition, Talin-deficient mice exhibited splenomegaly, with larger spleen sizes as well as higher cell counts (Figures 1B and C). The thymi of Talin-deficient mice were comparable in size to the wild-type (data not shown), with similar percentages of double-negative CD4-CD8-, double-positive CD4+CD8+, and single-positive CD4+CD8- and CD4-CD8+ thymocytes (Figure 1D), suggesting that thymic development was normal. However, there was an increased number of CD4+ T cells in the spleens of Talin-deficient mice compared to wild-type mice (Figures 1E and F), both in terms of percentage and in absolute number (3-fold higher). This was accompanied by a 2-fold decrease in the number of CD8+ T cells (Figures 1E and F), while absolute numbers of B cells were comparable in wild-type and Talin-deficient mice (Figure 1F). In the lymph nodes, by contrast, we observed a 5-fold decrease in the number of Talin-deficient CD4+ T cells and a 11-fold decrease in the number of Talin-deficient CD8+ T cells (Figure 1G).

Talin-deficient T cells have a defect in trafficking to lymph node

Because Talin is a potent activator of integrins such as LFA-1, $\alpha 4\beta 7$, and VLA-4 which are required for trafficking to the lymph nodes (Berlin-Rufenach, et al., 1999), we hypothesized that Talin deficiency might prevent the entry of T cells into

lymph nodes. To test this hypothesis, Talin-deficient splenocytes and wild-type splenocytes were differentially labeled with a fluorescent dye, Cell Proliferation Dye (CPD), mixed in equal numbers, and adoptively transferred into wild-type recipients. Sixteen hours later, cells from the blood, spleen, and lymph nodes were stained with anti-CD4 and anti-CD8 and analyzed using flow cytometry. We observed that the ratio of wild-type and Talin-deficient T cells remained equal in the blood and spleen (Figure 2). In the lymph nodes, by contrast, we observed higher numbers of wild-type T cells relative to Talin-deficient T cells, with 7-fold more wild-type CD4+ T cells (Figure 2A) and 5-fold more wild-type CD8+ T cells (Figure 2B) than Talin-deficient cells. These results suggest a critical role for Talin in regulating T lymphocyte trafficking to lymph nodes.

T cells in the periphery of Talin-deficient mice are CD44hi

We next examined the activation status of Talin-deficient T cells. Expression of the markers CD44 and CD62L can been used to identify distinct subsets of T cells: naïve (CD44lo, CD62L hi), effector memory (CD44 hi, CD62L lo), and central memory (CD44 hi, CD62L hi). We observed that the vast majority of CD4+ T cells from Talin-deficient mice were CD44hi (Figure 3A), in contrast to the majority of wild-type CD4+ T cells, which were CD44lo. Among CD8+ T cells, we observed a similar increase in the proportion of CD44hi cells from Talin-deficient mice, but to a lesser degree than among CD4+ T cells (Figure 3B). These results suggest that the majority of circulating CD4+ and CD8+ T cells in Talin-deficient mice were previously activated. To determine whether activation of T cells occurred in the thymus or in the periphery, we

examined CD44 and CD62L expression in single positive (SP) CD4+ and SP CD8+ thymocytes. The vast majority of wild-type and Talin-deficient SP CD4+ and SP CD8+ T cells were CD44lo (Figure 3C), suggesting that activation of T cells in Talin-deficient mice occurs in the periphery.

CTLA-4 expression is defective in Talin-deficient CD44hi cells but not in nTregs

The high numbers of activated CD44hi T cells we observed in Talin-deficient mice was reminiscent of the phenotype of CTLA-4-deficient mice. CTLA-4 has an important role in setting the threshold for T cell activation and maintenance of peripheral tolerance (Alegre, et al., 2001). Mice lacking CTLA-4 develop a severe lymphoproliferative disorder due to T cell activation and infiltration into lymphoid and non-lymphoid tissues (Tivol, et al., 1995). Because CTLA-4 is expressed on the surface of a T cell following activation (Iida, et al., 2000), we examined CTLA-4 expression in splenic CD44hi T cells. While wild-type CD4+ and CD8+ CD44hi T cells expressed CTLA-4, Talin-deficient CD44hi T cells did not (Figure 4A). Because CTLA-4 is also constitutively expressed on Tregs and is necessary for Treg function (Takahashi, et al., 2000; Wing, et al., 2011), we examined CTLA-4 on Talin-deficient Tregs. Wild-type and Talin-deficient Tregs expressed similar levels of CTLA-4 (Figure 4B). Although CTLA-4 expression on Talin-deficient Tregs is normal, the inability of activated naïve T cells to express CTLA-4 may result in their activation in Talin-deficient mice.

Talin-deficient CD4+CD44hi T cells produce greater amounts of IL-17 and lower amounts of IFNγ than their wild-type counterparts

Previously activated (CD44hi) T cells are capable of secreting cytokines immediately upon restimulation, in contrast to naïve T cells. To determine whether CD4+CD44hi T cells from Talin-deficient mice were capable of secreting cytokines, we restimulated splenocytes from wild-type and Talin-deficient mice *ex vivo* with PMA and ionomycin. We observed that a higher percentage of CD44hi CD4+ T cells from Talin-deficient mice were capable of producing IL-17 but a lower percentage were capable of producing IFNγ (Figure 5). As a negative control, we found that naïve CD44lo CD4+ T cells from wild-type and Talin-deficient mice did not produce IL-17 or IFNγ upon restimulation. These results suggest that previously activated CD4+CD44hi T cells that we find in high numbers in Talin-deficient mice are functional and capable of secreting IFNγ and IL-17.

Talin-deficient CD8+CD44hi T cells do not produce cytokine

Because we observed higher numbers of Talin-deficient CD8+ T cells that were previously activated, we next examined the ability of these cells to produce the cytokines IFNγ and TNFα. We found that while the majority of CD8+CD44hi cells were capable of producing IFNγ and TNFα, Talin-deficient CD8+CD44 failed to do so (Figure 6). These results raised the possibility that Talin might play distinct roles in the differentiation of naïve CD4+ and CD8+ T cells.

Naïve Talin-deficient T cells proliferate normally in response to anti-CD3 and anti-CD28

To investigate the role of Talin in T cell differentiation, we first assessed the capability of the Talin-deficient T cells to proliferate *in vitro*. Because the majority of the Talin-deficient T cells are CD44hi, splenocytes were sorted into CD4+CD44lo and CD8+CD44lo populations using flow cytometry. After sorting, the cells were combined with T-depleted, irradiated wild-type splenocytes, labeled with Cell Proliferation Dye (CPD), and activated with soluble anti-CD3 and anti-CD28. Proliferation was examined by assessing dilution of CPD at 4 days after activation. We observed that Talin-deficient CD4+ and CD8+ T cells underwent proliferation comparable to that of wild-type T cells (Figure 7). These data suggest that Talin-deficient T cells are capable of undergoing proliferation in response to stimulation with anti-CD3 and anti-CD28.

Naïve Talin-deficient CD4+ T cells undergo dysregulated differentiation in vitro

A naïve CD4+ T helper cell activated by an APC bearing its cognate peptide and appropriate costimulatory signals can differentiate into distinct lineages to provide a specialized immune response depending on the type of pathogen encountered. For example, the Th1 subset is important in mediating responses to intracellular pathogens; the Th2 subset is important in responses to extracellular parasites; the Th17 subset is important for responses to extracellular bacteria. In addition, Tregs are important for regulating self-tolerance (Zhu, et al., 2008). Differentiation is driven by distinct networks of cytokine signaling and transcription factors and each T helper subset secretes a distinctive set of cytokines (Zhu, et al., 2011). Because Talin has been shown

to polarize to the immune synapse formation, we hypothesized that it could play an important role in differentiation of CD4+ T cells. Furthermore, our observation that Talin-deficient CD4+CD44hi T cells produce more IL-17 and less IFNγ than their wild-type counterparts suggested that Talin might regulate differentiation. To test this hypothesis, we sort-purified CD4+CD44lo T cells and cultured them for 4 days in conditions that promote Th1, Th2, Th17, and iTreg differentiation (Zhu, et al., 2011). Cells were either restimulated before being analyzed for intracellular cytokine production or analyzed for expression of fate-determining transcription factors.

Compared to wild-type cells, a higher percentage of Talin-deficient cells cultured in Th1 conditions differentiated into Th1 cells, as determined by IFNγ production (Figure 8A). By contrast, fewer Talin-deficient cells differentiated into Th2 or Th17 cells in the appropriate conditions (Figure 8B and C). Finally, a higher percentage of Talin-deficient cells cultured in Treg-promoting conditions differentiated into iTregs, as assessed by FoxP3 expression (Figure 8D). These results suggest a role for Talin in regulating CD4+ T cell differentiation.

Naïve Talin-deficient CD8+ T cells undergo relatively normal differentiation in vitro

We next examined the ability of CD8+ T cells to differentiate into effector cells. In order to provide effective immunity, CD8+ T cells give rise to cytotoxic T lymphocytes (CTLs) as well as long-lived memory cells. CTLs are short lived CD8+ T cells that are important in killing damaged and infected somatic cells (Rutishauser, et al., 2010). The cytotoxicity of these cells is driven by the transcription factor T-bet and are characterized by their ability to rapidly secrete IFNγ, TNFα, and granzyme (Arens, et al.,

2010). Because Talin-deficient CD8+CD44hi cells in the Talin-deficient mice appeared to have a defect in IFNγ and TNFα production, we examined the ability of naïve CD8+CD44lo cells to differentiate *in vitro*. Sorted CD8+CD44lo cells were activated *in vitro* for four days in culture and then restimulated before being analyzed for cytokine production. Activated wild-type and Talin-deficient CD8+ T cells produced similar levels of IFNγ and TNFα (Figure 9). These results suggest that naïve CD8+ T cells from Talin-deficient mice undergo normal effector differentiation *in vitro*.

Figures

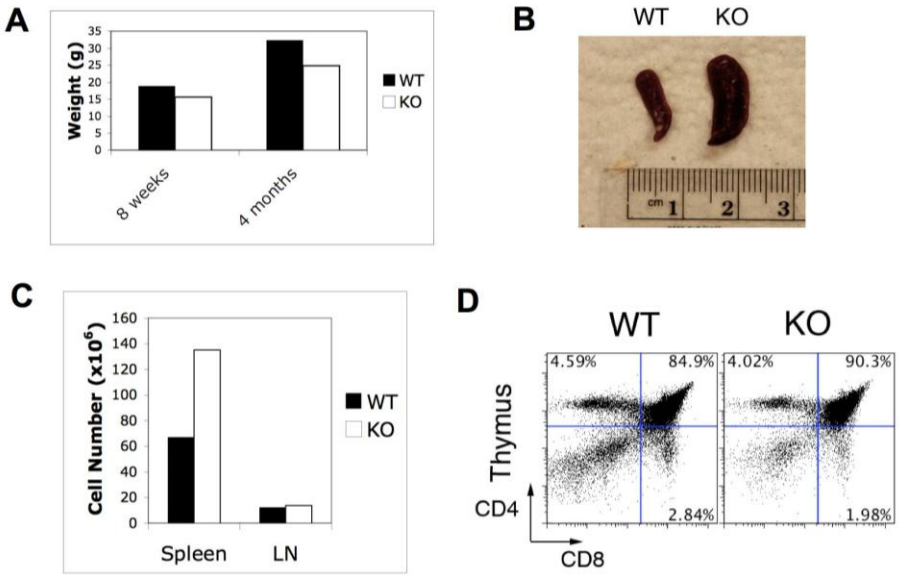

Figure 1: Increased numbers of CD4+ T cells in Talin-deficient mice.
(A) CD4-Cre negative ("WT", black bars) and CD4-Cre positive Talin$^{fl/fl}$ ("KO", white bars) mice were weighed at 8 weeks and 4 months.
(B) Spleens from CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were photographed at 4 months of age.
(C) Cell numbers from spleens and lymph nodes (LN) of CD4-Cre negative (black bars) and CD4-Cre positive Talin$^{fl/fl}$ (white bars) mice were quantitated.
(D) Thymocytes from CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were stained for CD4 and CD8 and analyzed by FACS.

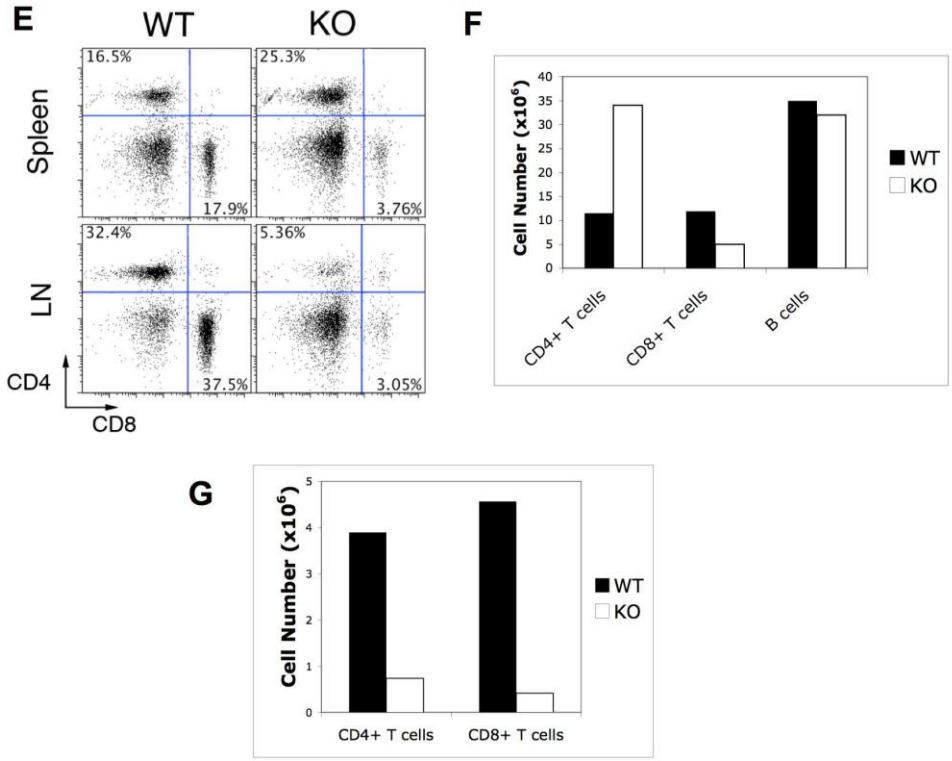

Figure 1 continued

(E) Splenocytes (top panels) and lymph node cells (bottom panel) from CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were stained for CD4 and CD8 and analyzed by FACS.

(F) Absolute numbers of CD4+ T cells, CD8+ T cells, and B cells in spleens of CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were quantitated using flow cytometry.

(G) Absolute numbers of CD4+ T cells and CD8+ T cells in lymph nodes of CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were quantitated using flow cytometry.

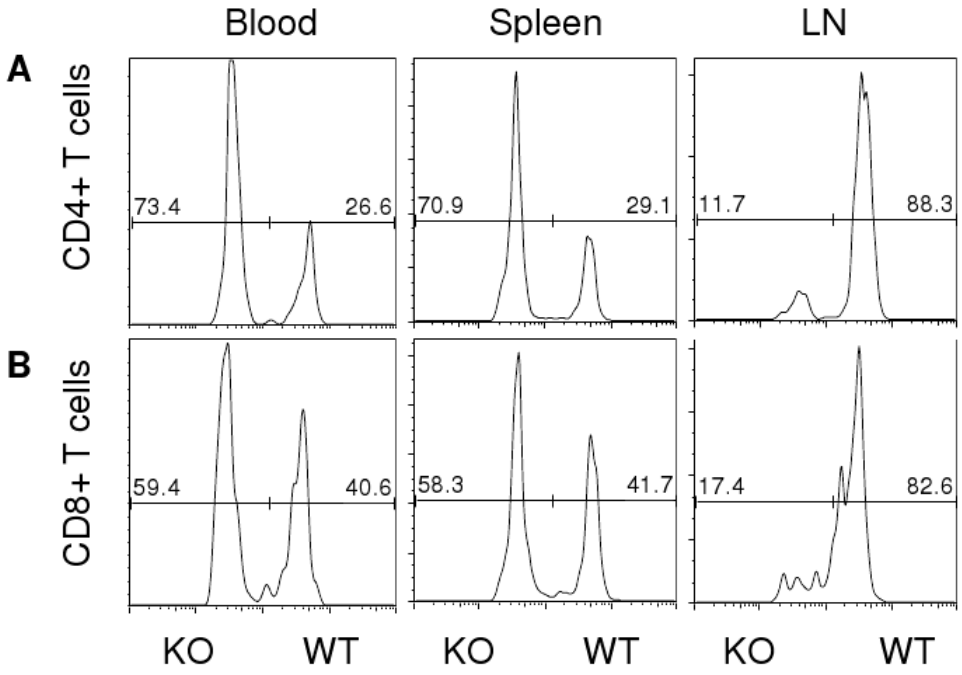

Figure 2: Talin-deficient T cells fail to traffic to lymph nodes.
CD4-Cre positive *Talin*$^{fl/fl}$ splenocytes were labeled with a low concentration of Cell Proliferation Dye (CPD) and CD4-Cre negative splenocytes were labeled with a high concentration of CPD. Cells were then mixed at a 1:1 ratio and adoptively transferred into wild-type recipients. Sixteen hours later, blood, splenocytes and lymph node cells were stained with CD4 (A) and CD8 (B) and analyzed by FACS.

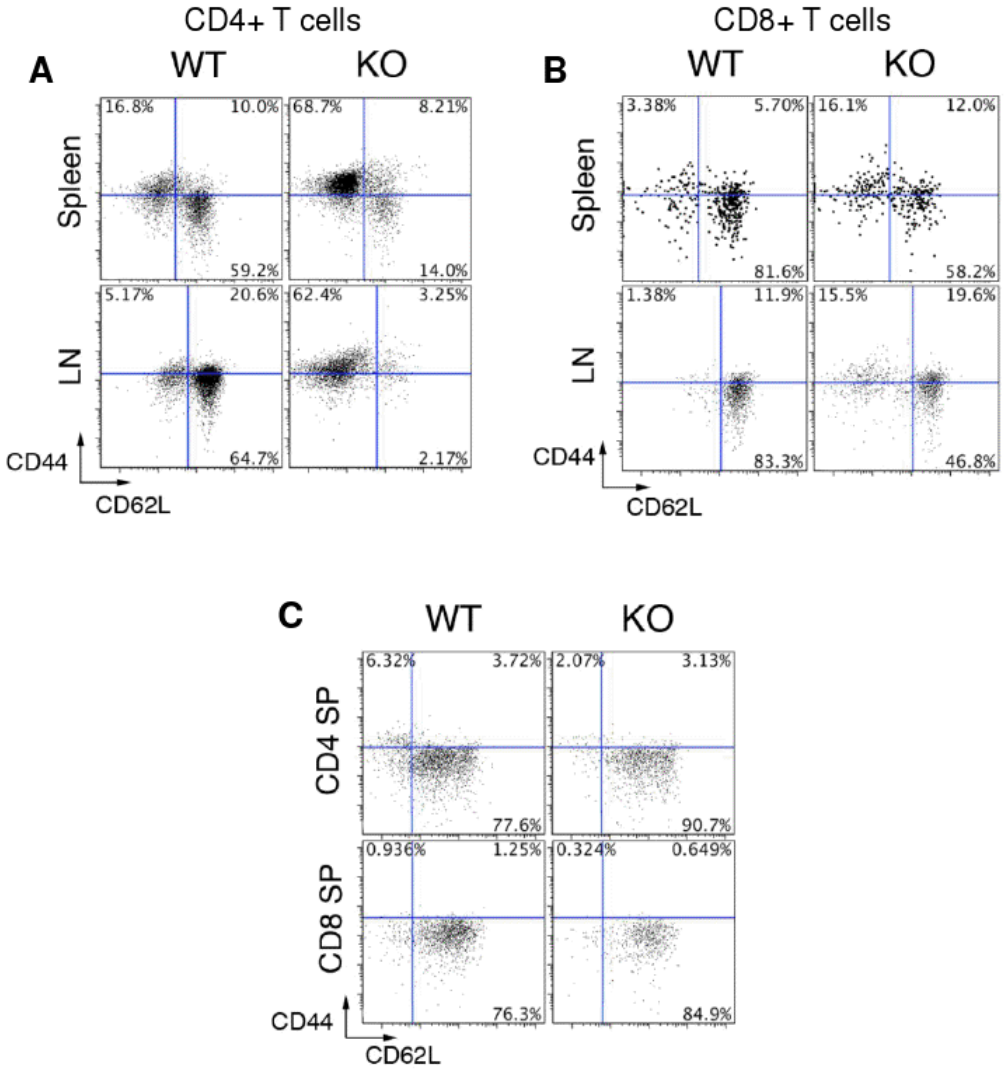

Figure 3: T cells in the periphery of Talin-deficient mice are CD44hi.
(A and B) Splenocytes (top row) and lymph node cells (bottom row) from CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were analyzed by FACS. Cells were gated on CD4 (A) or CD8 (B) and levels of CD44 and CD62L were analyzed.
(C) Thymocytes from CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were analyzed by FACS. Cells were gated on CD4 (top panel) and CD8 (bottom panel) single positive T cells and levels of CD44 and CD62L were analyzed.

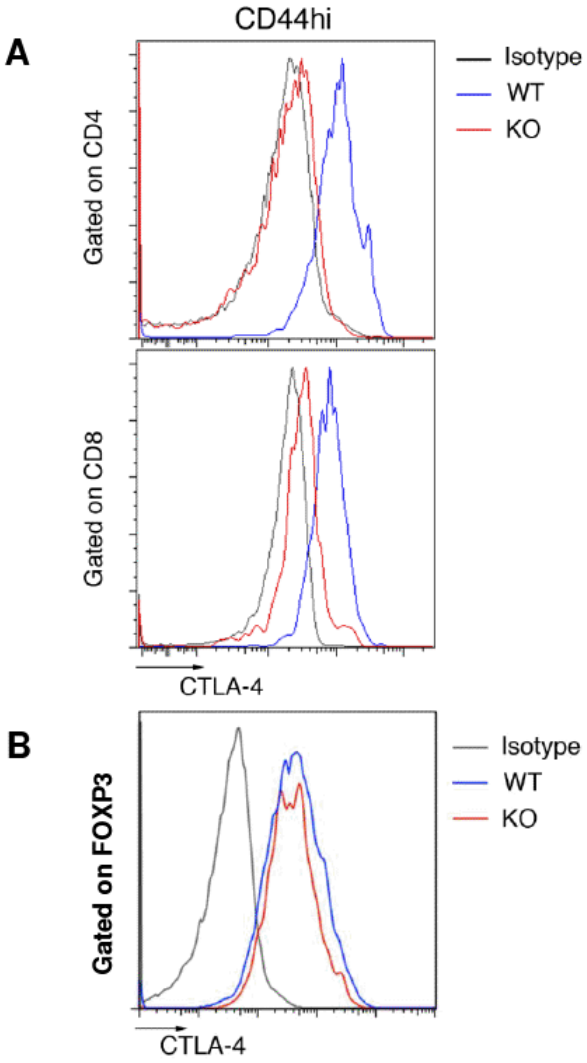

Figure 4: CTLA-4 expression is defective in Talin-deficient CD44hi cells but not in Tregs.
(A) Splenocytes were stained for CTLA-4 on CD4+CD44hi (upper), or CD8+CD44hi (lower) from CD4-Cre negative (blue) and CD4-Cre positive Talin$^{fl/fl}$ (red) mice and analyzed through comparison with an isotype control (grey).
(B) Thymocytes from CD4-Cre negative (blue) and CD4-Cre positive Talin$^{fl/fl}$ (red) mice were surface stained for CD4 and CTLA-4, and then fixed and permeabilized to stain for intracellular FOXP3. The cells were gated on the CD4+FOXP3+ population and then analyzed for CTLA-4 surface expression through comparison with an isotype control (grey).

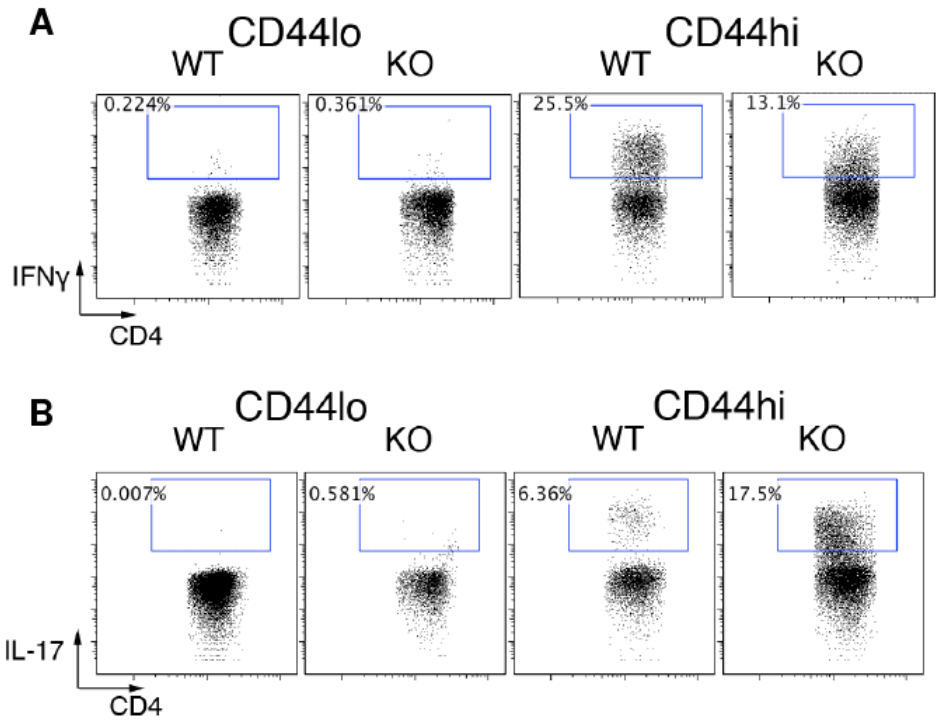

Figure 5: Talin-deficient CD4+CD44hi T cells produce greater amounts of IL-17 and lower amounts of IFNγ than their wild-type counterparts.
Splenocytes from CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were isolated and stimulated ex vivo for 4 hours, stained, and analyzed by FACS. CD4+ T cells were gated on CD44; CD44lo (left) and CD44hi (right) were analyzed for intracellular IFNγ (top) and IL-17 (bottom) production.

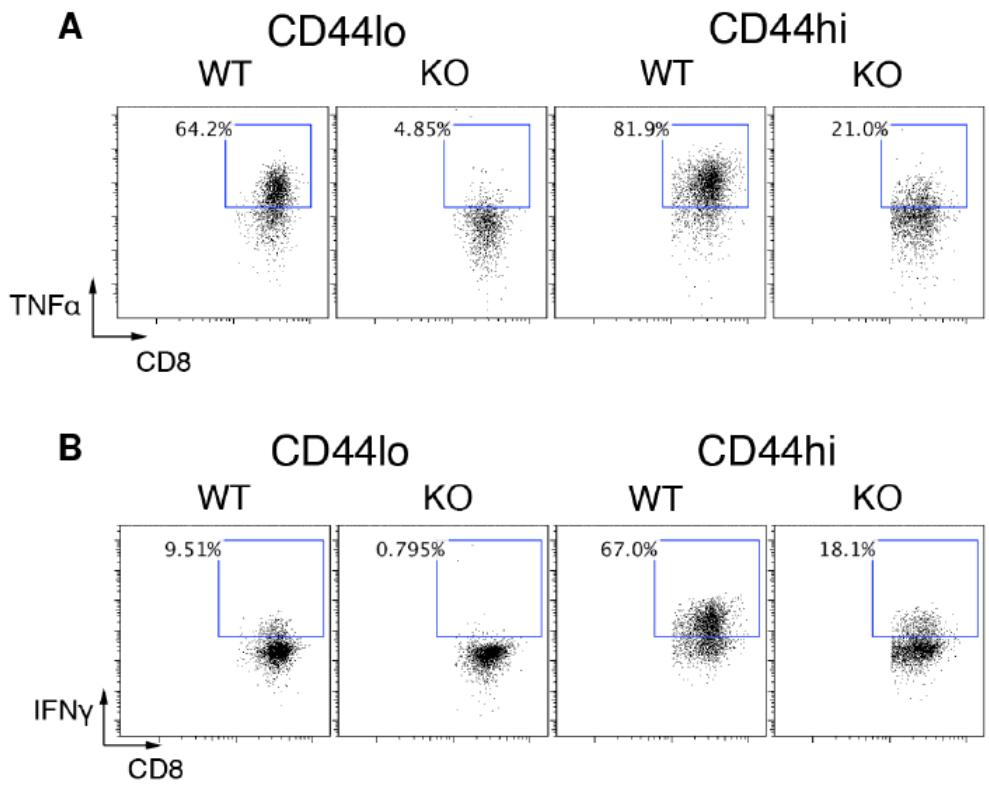

Figure 6: Talin-deficient CD8+CD44hi T cells do not produce TNFα or IFNγ. Splenocytes from CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were isolated and stimulated ex vivo for 4 hours, stained, and analyzed by FACS. CD8+ T cells were gated on CD44; CD44lo (left) and CD44hi (right) were analyzed for intracellular TNFα (top) and IFNγ (bottom) production.

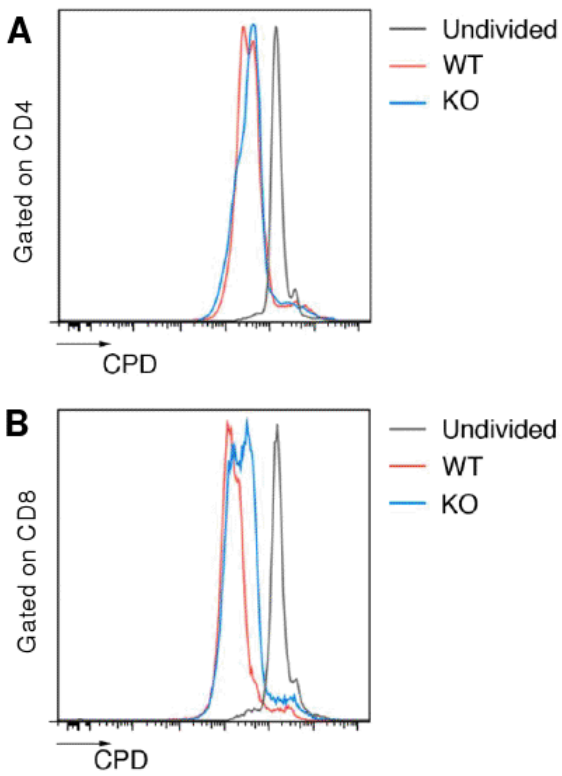

Figure 7: Talin-deficient T cells proliferate normally *in vitro*.

CD4+CD44lo (A) and CD8+CD44lo (B) T cells from CD4-Cre negative (red) and CD4-Cre positive Talin$^{fl/fl}$ (blue) mice were sorted by FACS, combined with T-depleted irradiated splenocytes, labeled with Cell Proliferation Dye (CPD) and activated with IL-2, anti-CD3, and anti-CD28 for four days. Proliferation of was analyzed using FACS by assessing CPD dilution from undivided irradiated splenocytes (grey).

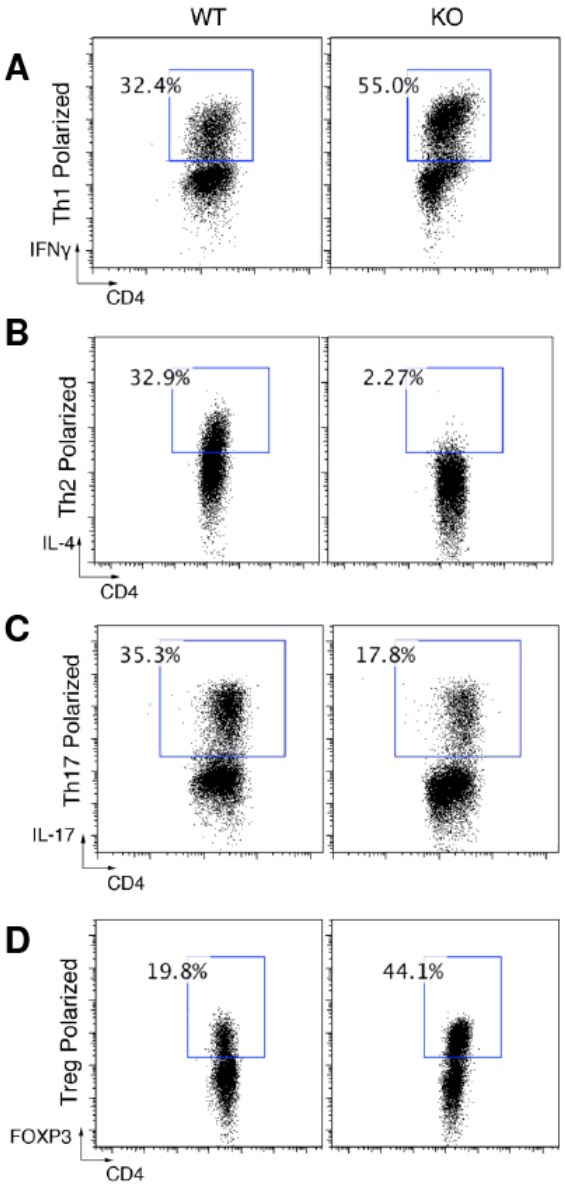

Figure 8: Talin-deficient CD4+ T cells undergo abnormal differentiation *in vitro*. Sort-purified CD4+CD44lo cells from CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were activated with anti-CD3 and anti-CD28 for four days in Th1 (A), Th2 (B), Th17 (C), and Treg (D) polarizing conditions. Cells were then restimulated with PMA and ionomycin and analyzed by FACS. Cells that were cultured in Th1 conditions were stained for IFNγ (A), those in Th2 conditions were stained for IL-4 (B), those in Th17 conditions were stained for IL-17 (C), and those in Treg conditions were stained for FOXP3 (D).

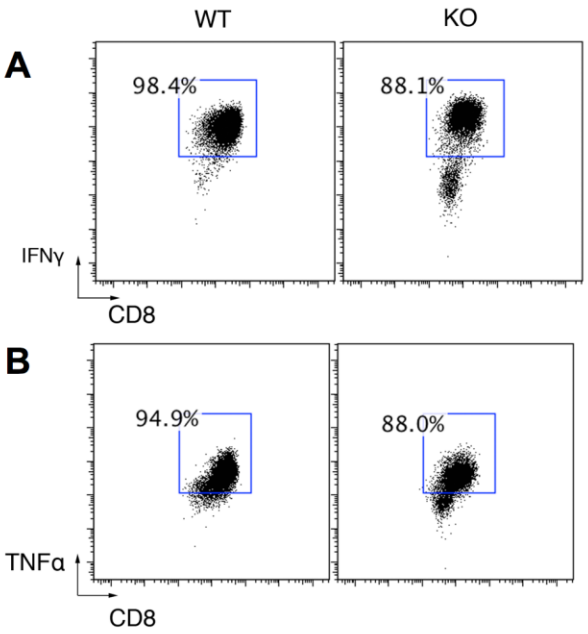

Figure 9: Talin-deficient CD8+ cells undergo normal differentiation *in vitro*. Sort-purified CD8+CD44lo cells from CD4-Cre negative and CD4-Cre positive Talin$^{fl/fl}$ mice were activated with anti-CD3 and anti-CD28 and then washed on day 2 and cultured for two more days. Cells were then restimulated with PMA and ionomycin and analyzed by FACS for the production of IFNγ (A) and TNFα*(B).*

Discussion

Since Talin is an important regulator of the integrins involved in immune synapse formation, we initially hypothesized that loss of Talin would inhibit T cell activation. Instead, we found that T cells in Talin-deficient mice display a previously activated phenotype. Single-positive CD4+ and CD8+ thymocytes are not CD44hi, suggesting that these CD44hi cells undergo activation in the periphery. This phenotype is more pronounced in CD4+ T cells, although CD8+ T cells are also affected. In addition, Talin-deficient mice exhibit splenomegaly and seem to lose weight with age. Despite our findings, Talin-deficient mice up to 4 months of age appear healthy and do not seem to be affected by overt autoimmunity, which may be explained by our observation that Talin is required for lymphocyte trafficking.

Our study suggests that Talin is necessary for T cell entrance into lymph nodes. Defects in migration have also been seen in Talin-deficient B cells (Manevich-Mendelson, et al., 2010) and LFA-1 deficient T cells (Kandula, et al., 2004). Because of their roles in lymphocyte migration, integrins play an important role in the pathogenesis of autoimmune disease. The deletion of LFA-1 and its ligand ICAM-1 have been shown to protect mice against experimentally induced colitis through an inability of the T cells to traffic to the mesenteric lymph nodes and intestine (Bendjelloul, et al., 2000; Pavlick, et al., 2006). If Talin is required for lymphocyte entry into lymph nodes, it may also be required for entry into non-lymphoid tissues. In future experiments, we intend to examine tissues including the small intestine and colon for T cells and expect that T cells will fail to traffic to tissue in Talin-deficient

mice. Although T cells in the Talin-deficient mice aberrantly undergo activation, they may not be able to infiltrate into the tissues where they would otherwise cause damage.

While the Talin-deficient mice do not appear to be affected by overt autoimmunity, the high numbers of activated T cells suggest that immunological tolerance has been compromised. Immunological tolerance allows the immune system to distinguish between foreign and self-antigens present in the body. To determine if Talin-deficient T cells are reacting against self-antigens, Talin-deficient mice are currently being bred to OT-II and OT-1 T-cell receptor (TCR) transgenic mice. OT-II and OT-I TCR transgenic mice have CD4+ and CD8+ T cells that recognize a single, specific epitope of ovalbumin, respectively. Thus, T cells in these mice should not react to endogenous self-antigens. It has been shown that when CTLA-4 deficient mice are bred to various TCR transgenic mice, they do not develop the lymphoproliferative disorder (Waterhouse, et al., 1997), demonstrating that a polyclonal T-cell repertoire to endogenous antigens is needed to drive autoimmunity. Therefore, we hypothesize that breeding Talin-deficient mice to OT-I or OT-II TCR transgenic mice will rescue the CD44hi phenotype, even if the naïve cells lack the ability to express surface CTLA-4.

CTLA-4 is critical for setting the threshold for T cell responses during early and late stages of T cell activation (McCoy, et al., 1999). In a naïve cell, CTLA-4 is located intracellularly in lysosomes and becomes secreted to the surface upon TCR stimulation (Iida, et al., 2000). TCR signal strength has also been shown the affect the cell surface expression of CTLA-4 (Egen, et al., 2002). Beyond TCR stimulation, the signals needed for CTLA-4 expression on the cell surface are unknown. In our study, the Talin-deficient CD44hi T cells did not express CTLA-4 unlike their wild-type counterparts,

which suggest that Talin plays an important role in the expression of CTLA-4 following activation. This raises the question of how Talin regulates CTLA-4 expression. A recent study has shown that the transcription factor FOXO1 is a direct target of the CTLA-4 gene, and loss of FOXO1 resulted in severe autoimmunity (Kerdiles, et al., 2011). It is possible that Talin is upstream of FOXO1 activation which then induces CTLA-4 expression. Alternatively, Talin may be critical in a signaling cascade that precedes CTLA-4 expression at the cell surface following activation. These alternatives can begin to be answered by examining the intracellular expression of CTLA-4 in naïve Talin-deficient T cells.

CTLA-4 has also been implicated as an activator of LFA-1 adhesion and clustering. Studies have shown that CTLA-4 activates integrin mediated adhesion. This increases motility of T cells and limits the duration of T cell contact with APCs (Schneider, et al., 2006; Schneider, et al., 2005). This is believed to inhibit the interaction of T cells with APCs to reduce T cell activation in order to increase the TCR signaling threshold necessary for T cell activation. If both CTLA-4 and Talin cannot upregulate integrin mediated adhesion to prevent prolonged contact with APCs, this may further lower the threshold for T cell activation. Overall, our findings suggest a role for Talin in mediating CTLA-4 expression, but the underlying mechanism remains to be explored.

In addition to identifying high numbers of previously activated T cells in Talin-deficient mice, we began to explore the role of Talin in T cell differentiation. We found that Talin-deficient sort-purified naïve CD4+ T cells underwent abnormal differentiation *in vitro*. In Th1 and Treg polarizing conditions, Talin-deficient cells had enhanced

differentiation. In contrast, the Talin-deficient T cells had impaired differentiation in Th2 and Th17 polarizing conditions. However, we observed *in vivo* that a higher percentage of Talin-deficient CD4+CD44hi T cells were able to produce IL-17 and a lesser percentage of cells produced IFNγ than their wild-type counterparts. A possible explanation for the differences seen *in vivo* and *in vitro* is differences in T cell activation. In the *in vitro* culture conditions, cells were activated using artificial and very strong anti-CD3 and anti-CD28 stimuli, while T cells in vivo were likely activated by low affinity self antigens. TCR signal strength has been suggested to mediate CD4+ T cell differentiation (Nakayama, et al., 2010), and could explain the differences that we observe *in vitro* and *in vivo*. In addition, CD4+ T cells are differentiated *in vitro* using high concentrations of cytokines, which is likely to differ from the cytokine milieu experienced by naïve T cell *in vivo*.

We also explored the differentiation of naïve CD8+ T cells. While a large percentage of the CD8+ T cells in the Talin-deficient mice displayed a CD44hi phenotype, the majority of these CD44hi cells could not produce IFNγ or TNFα *ex vivo,* which suggested that Talin might play a role in the differentiation of CD8+ T cells into effector Cytotoxic T Lymphocytes (CTLs). However, when sort-purified naïve Talin-deficient CD8 cells were differentiated *in vitro,* they were able to produce similar amounts of IFNγ and TNFα compared with wild-type controls. As discussed above, it is possible that differences in TCR strength induced by anti-CD3/anti-CD28 vs. self antigens may be an explanation. Strong stimulation from anti-CD3/anti-CD28 might be able to bypass a requirement for Talin observed *in vivo*. In addition, Talin has been shown to be important in mediating adhesion during immune synapse formation

(Simonson, et al., 2006). If the Talin-deficient T cells cannot form stable immune synapses necessary for sustained TCR signaling, then they may not receive sufficient signaling to develop into effector cells.

To overcome the caveats of examining T cell differentiation using artificial anti-CD3 and anti-CD28 stimulus, we will use floxed Talin-deficient mice bred to OTI and OTII-TCR transgenic mice. Because these mice have T cells specific to OVA, they can activated *in vitro* using ovalbumin peptide, which is more physiologic than stimulation using anti-CD3 and anti-CD28. The cells from these mice can also be adoptively transferred into wild-type mice to examine the role of Talin during T cell responses to a microbial pathogen *in vivo*, such as *Listeria monocytogenes* expressing ovalbumin. This model of infection drives a Th1 response (Hsieh, et al., 1993) and it can also be used to study CD8 effector and memory responses (Berg, et al., 2005). Since Talin is found at the immune synapse during T cell activation (Monks, et al., 1998), it could have important roles in initiating T cell responses against microbial pathogens and might affect both short-term and long-term immunity. Future studies will help us understand the role of Talin in T cell differentiation and the initiation of T cell immune responses.

Overall, this study suggests an important role for Talin in maintaining peripheral tolerance due to high numbers of activated T cells in floxed Talin-deficient mice. Our findings suggest that this phenotype is due to an inability of Talin-deficient T cells to express CTLA-4 following activation. CTLA-4 expression is important for setting the threshold of immune responses, but the signals required for CTLA-4 expression remain unknown. Our study indicates that Talin may be necessary for CTLA-4 surface

expression following T cell activation. Further research will be needed to understand the mechanism of how this occurs.

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