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Mechanisms of Integrin Activation in Regulatory T cells

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

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by

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2019

The Thesis of Hsin Wang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

2019

DEDICATION

I dedicate this thesis to my family for their infinite love and supports.

TABLE OF CONTENTS

LIST OF FIGURES

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ABSTRACT OF THE THESIS

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Integrins have been known for its role in facilitating cell migration. Recent studies have revealed that integrin activation also plays important roles in maintaining T cell homeostasis and peripheral tolerance, but the mechanism of integrin activation in regulatory T cells, a subset of T cells that maintains peripheral tolerance, remains obscure. Here, by using various Treg-specific

gene knocked out mice, we found talin and Rap1 are critical for integrin activation in regulatory T cells. The connection between Rap1 and talin is mediated by an adaptor, RIAM. Surprisingly, RIAM knockout did not affect integrin activation in Tregs. Using mutant mice, we found another adaptor, Lamellipodin, can provide some of the connection between Rap1 and talin. In addition, we found that in lymphocytes, direct binding of Rap1 to two sites in talin makes another contribution to integrin activation in Tregs. Taken together, distinct pathways are involved in Tregs and enable their ability to maintain immune homeostasis through their suppression function.

INTRODUCTION

Our immune system protects us from pathogen invasion by having innate and adaptive immunity (Medzhitov & Janeway, 1997). The innate immunity provides nonspecific protections through skin barriers and myeloid (blood) cells, while adaptive immunity utilizes more specific and stronger protections via versatile lymphocytes (Parham, 2015). Among the lymphocytes in the adaptive immune system, T cell is the most well-studied cell type (Sakaguchi et al., 2008). T cells can be divided into two subgroups: cytotoxic T cells (CD8 T cells) and helper T cells (CD4 T cells). CD8 T cells scan through host cells and eliminate the infected ones. CD4 T cells are commonly divided into regulatory T cells (Tregs) and conventional T cells (Tconvs). Tregs are the primary mediators of peripheral tolerance that play a critical role in preventing autoimmune diseases and chronic inflammatory. Tconvs control adaptive immunity by activating other effector immune cells in a pathogen-specific manner. CD4 T cells not only facilitate the activation of both myeloid cells and B cells but also secrete cytokines to recruit corresponded white blood cells to the inflammation sites (Trifari et al., 2009).

To generate T cell receptors that recognize millions of pathogens, our immune system goes through a series of procedures that produce almost unlimited variations of receptors to recognize millions of potential threats (Parham, 2015). Some of the newly made T cell receptors would inevitably recognize self-antigen as pathogen-derived antigens. Fortunately, the immune system has two mechanisms to prevent self-targeting T cells from attacking the hosts.

The first mechanism is called “recessive mechanism”: once the receptors of a precursor T cell are synthesized in the thymus, the receptors go through self-antigens affinity checking, and the cells with high affinity against self-antigen will be eliminated (Hogquist et al., 1994). As a result, those self-targeting T cells won’t be released into the body (Sakaguchi et al., 2008).

The second mechanism is called the “dominant mechanism”. Unlike the recessive mechanism, this process is executed by Tregs. (Sakaguchi et al., 2006). The major suppression mechanisms of Tregs include secreting inhibitory cytokines, disrupting regular metabolism, and modulating dendritic cell maturation or function (Corthay, 2009). Kim and colleagues (2007) found out that Tregs are important to maintain self-tolerance. The mice showed serious autoimmune diseases and died within 10 days after Tregs removal. Besides, spleens and lymph nodes of the Tregs deleted mice were about 4-fold larger than those of the healthy ones. Lymphoid organ enlargement was caused by self-targeting T cells attack. Therefore, Tregs maintain self-tolerance by suppressing self-targeting T cells in the host (Dominguez-Villar & Hafler, 2018).

Although the adaptive immune system is a powerful tool, the system could turn against the host when it lost homeostasis. For example, inflammatory bowel disease (IBD), one of the most common autoimmune diseases, is characterized by chronic inflammation in the gastrointestinal tract (“Autoimmune Diseases | NIH: National Institute of Allergy and Infectious Diseases,” n.d.). The inflammation sites could be either in large intestine and rectum (Ulcerative colitis) or lining of any part of the intestine (Crohn's disease). Although the exact cause of IBD is not clear, it is known that the disease is the result of immune system disorder (Khor et al., 2011). The constant large intestine inflammation is caused by abnormal leukocytes recruitment from blood vessels to the intestine’s inflammation sites with decreased Tregs recruitment (Maul et al., 2005). Besides, aberrant infiltration of mononuclear phagocytes, neutrophils, and inflammatory leukocytes was observed in the colonic lamina propria of IBD patients (Smith, Ochsenbauer-Jambor, & Smythies, 2005). This rapid leukocytes recruitment from the circulation during IBD provides a potential target for pharmaceutical inhibition of the gastrointestinal inflammation

(Cominelli, 2013). Thus, since IBD mainly involves in leukocyte migration, integrin could be a potential therapeutic target for IBD.

Integrins are a family of cell transmembrane receptors that regulate leukocyte migration, adhesion, and cell-extracellular matrix interaction (Baade et al., 2019). Besides, integrins play important roles in embryonic and disease development. (Shattil, Kim, & Ginsberg, 2010). Integrins are heterodimers that consist of α and β chains; each chain has several subtypes (24 α chains and 9 β chains in mammals) that correspond to different ligands. The receptors are usually in low-affinity state (resting) that keeps cells immobilized. Upon agonists stimulation, integrins switch to high-affinity state (activate) and mediate cell migration and cell-cell adhesion (Lagarrigue, Kim, & Ginsberg, 2019). Integrins play an important role in Treg-mediated immune regulation. Colitis, for example, cannot be prevented by CD103(α chain of αEβ7 integrin) deficient Tregs because the cells lose their migration ability (Chen & Bromberg, 2006). Notably, integrins are especially critical in T cell function. Integrin α4β1 facilitates T cell migration to inflammatory sites, while integrin αLβ2 both facilitates T cell migration to inflammatory sites and directs T cells homing to lymph node (Klann et al., 2018). Therefore, integrin could be a potential therapeutic target against autoimmune diseases by having the ability to retaining self-targeting T cells in the epithelium.

Integrin activation is regulated by intracellular signal cascades that initiated by signals from several receptors (Abram & Lowell, 2009). In leukocytes, the receptors include inflammatory-activated G-protein coupled receptors, antigen-activated B cell receptor (BCR), or T cell receptor (TCR) (Abram & Lowell, 2009). As a result, A ‘canonical’ pathway to integrin activation has emerged, mostly involving the generation of Ca^{2+} and diacylglycerol (DAG), together activating Protein Kinase C (PKC) and the GTPase Rap1. Rap1 subsequently binds the

effector protein Rap1-GTP-Interacting-Adaptor Molecule (RIAM, also known as *Apbb1ip*), which recruits the cytoplasmic protein talin-1 to the plasma membrane.

The most important molecule in the integrin activation pathway is talin. Talin is an intracellular integrin-binding protein, also known as the last key of integrin activation via 'inside-out' signaling (Shattil et al., 2010). Talin is comprised of the C-terminal domain and N-terminal domain that consists F0, F1, F2, and F3 subdomain (Shattil, Kim, & Ginsberg, 2010). Structural studies have confirmed that binding of talin N-terminal F3 domain to integrin β cytoplasmic domain activates integrins (Anthis et al., 2009), and that Rap1 can bind both F0 and F1 domain directly (Gingras et al., 2019). Previous studies showed that both deletion and point mutation of talin in mouse blood cells result in failing of platelet aggregation and thrombosis (Lagarrigue et al., 2019). Besides, talin plays a critical role in Treg-mediated maintenance of immune homeostasis. Treg-specific deletion of Talin leads to spontaneous lymphocyte activation due to numerical and functional deficiencies of Tregs in the periphery. Moreover, previous studies showed talin-mediated integrin is essential in facilitating Tregs contact-mediated suppression and controlling peripheral tolerance by maintaining Treg function, suggesting an important role of talin in maintaining Treg cell-mediated immune homeostasis (Klann et al., 2018)

A small GTPase in the Ras family, Rap1, is one of the well-studied molecules directly involved in the integrin activation pathway. (Bos, 1997). The connection between Rap1 and integrin was first discovered in Rap1-deficient T and B cells. Compared to wild-type cells, the Rap1-deficient cells have decreased adhesion capacity against ICAM-1 (Duchniewicz et al., 2006). Rap1 is a G-protein that continuously circulates between inactive (GDP bound) and active (GTP-bound) forms. Guanine nucleotide exchange factor (GEFs) activates Rap1 by converting

its GDP to GTP. Several GEFs can activate Rap1 depend on different cell types. For instance, Ca^{2+} and DAG-regulated GEF1 (CalDAG-GEF1) regulate Rap1 activation in platelets (Lozano et al., 2016), while RapGEF1, RapGEF3, and RapGEF6 activate Rap1 in leukocytes and promote integrin activation(Abram & Lowell, 2009). Activated Rap1 then goes through conformational change, allowing both recruitment and binding to its effectors.

RIAM is another critical intracellular protein associated with integrin activation. RIAM recruits talin to the cytoplasm membrane and facilitates the binding of talin and integrin β chain (Lagarrigue et al., 2016). Gene deletion of RIAM results in β2 integrin inactivation, which disables β2-mediated cell migration and adhesion (Klapproth et al., 2015). Lagarrigue and colleagues (2017) demonstrated that loss of RIAM in T cells prevents antigen-dependent autoimmunity by disrupting cell-cell conjugation between effector T cells and dendritic cells. Furthermore, recent data from our lab demonstrat that RIAM-deficient Tconvs prevent adoptive T cell-transfer induced colitis by inhibiting integrin-activation mediated leukocyte migration. Interestingly, RIAM-deficient Tregs can also prevent colitis by exhibiting normal suppression and migration function (Supplement 1, unpublished). These results suggest that RIAM can be a potential therapeutic target against autoimmune disease and alternative integrin activation mechanisms might exist in Tregs.

Most of the integrin studies have been focused on the importance of integrin activation in T cells. However, little is known about the role of integrin activation in Tregs and the details of the activation mechanism. Here, we utilized different Treg-specific gene-deficient mice to assess the role of integrin activation in Tregs and investigated the signaling pathways. We found that integrin activation also plays an important role in Tregs migration and suppressive function. Two distinct signaling pathways are involved in integrin activation in Tregs. One is mediated by Rap1/

Lamellipodin (Lpd)/ RIAM/ Talin pathway; the other is mediated by Rap1 and talin directly binding. This study demonstrates the important role of integrin activation on Tregs function and provides new avenues for therapeutic intervention in the treatment of T cell autoimmunity disorders.

RESULTS

Talin -dependent integrin activation is important for Tregs function.

Talin plays a critical role in Treg cell-mediated immune homeostasis (Klann, Remedios, et al., 2018). Treg cell-specific deletion of talin results in spontaneous lymphocyte activation due to the numerical and functional deficiencies of Tregs in the periphery. To examine whether talin-dependent integrin activation is required for Tregs function, we exploited the talin(L325R) mutant that inhibits integrin activation via binding to integrins (Klann, Kim, et al., 2018). The $Tln^{L325R/fl}Foxp3^{cre}$ mice appeared to be significantly smaller and weaker than the $Tln^{wt/fl}Foxp3^{cre}$ control mice (Fig. 1A). Besides, we observed that the mutated mice had significant enlargement in the size of secondary lymphoid organs and a significant size reduction in the thymus (Fig. 1B). Furthermore, the number of leukocytes in the peripheral blood of mutated mice was greatly boosted (Fig. 1C). Overall, these results suggest leukocytosis in the $Tln^{L325R/fl}Foxp3^{cre}$ mice.

Since talin is the key to integrin-mediate adhesion, we investigated integrin activation on the *talin* (L325R) mutant Tregs. Comparing to the control, *talin*(L325R) mutant Tregs showed a dramatical reduction in binding to the integrin ligands after Phorbol 12-myristate 13-acetate (PMA) stimulation (Fig.1D), indicating a disrupted integrin activation on Tregs by the talin(L325R) mutation. We next examined the roles of *talin*(L325R) on Tregs migration by using a competitive homing assay. As we predicted, *talin*(L325R) mutant Tregs homing to the peripheral lymph node, mesenteric lymph node, and Peyer's patch were dramatically reduced (Fig. 1E). However, compared to WT Tregs, the homing of mutated Tregs to spleen showed no notable change (Fig. 1E). We next assessed whether talin-dependent integrin activation was required for Tregs suppressive capacity. Using an in vitro suppression assay, we observed that *talin*(L325R) mutant Tregs failed to suppress the Tconv cells (Fig. 1F). In conclusion,

talin(L325R) mutant impaired both the suppressive capacity and migration to secondary lymph nodes of Tregs, suggesting talin-dependent integrin activation is critical to Tregs hemostasis and function.

Rap1 plays an important role in maintaining homeostasis and functions of Tregs.

Previous works have shown that agonist stimulation induces the generation of the second messengers Ca^{2+} and diacylglycerol (DAG), together activating the GTPase Rap1 on the membrane to recruit an effector called RIAM that in turn recruits talin (Shattil, Kim, & Ginsberg, 2010). To explore the role of Rap1 in Tregs, we used $Rap1A^{fl/fl}Rap1B^{fl/fl}Foxp3^{Cre}$ mice, which had Rap1 deletion specifically in Tregs. Compared to the littermates of $Rap1A^{wt/wt}Rap1B^{wt/wt}Foxp3^{Cre}$ mice, the size of the $Rap1A^{fl/fl}Rap1B^{fl/fl}Foxp3^{cre}$ mice was significantly smaller (Fig. 2A). The thymus of the $Rap1A^{fl/fl}Rap1B^{fl/fl}Foxp3^{cre}$ mice was decreased in size, while having enlarged lymph nodes and spleen (Fig. 2B), suggesting inflammation in the $Rap1A^{fl/fl}Rap1B^{fl/fl}Foxp3^{cre}$ mice. Therefore, we examined the number of leukocytes of peripheral blood and found a significantly increasing number of leukocytes with a decreased concentration of hemoglobin (Fig. 2C). Next, we inspected whether Rap1-deficient in Treg would affect its migration ability. Examination of integrin expression on Treg showed that Tregs from $Rap1A^{fl/fl}Rap1B^{fl/fl}\ Foxp3^{Cre}$ mice expressed a similar amount of integrins as the WT controls (Fig. 2D). Analysis of integrin-ligand binding confirmed that PMA could not activate integrin on Rap1-deficient Tregs, indicating that Rap1 is required for integrin activation on Tregs (Fig. 2E). Finally, the homing assay showed that compared to wildtype controls cells, the presence of Rap1-deficient Tregs was much less in the popliteal lymph node, mesenteric lymph node, and Peyer's patch but increased in the spleen (Fig. 2F). This result implies that Tregs could immobilize and accumulate in the spleen.

Previous studies showed that Treg-specific deletion of talin mice resulted in spontaneous lymphocyte activation. Based on this result, we hypothesized that Treg-specific Rap1-deficient mice would also lead to spontaneous lymphocyte activation. First, the suppression assay showed that Rap1-deficient Tregs were not able to suppress Tconv cells (Fig. 2G). Furthermore, examination of the naive ($CD44^{lo}CD62L^{hi}$) and activated ($CD44^{hi}CD62L^{lo}$) $CD4^{+}$ T cell compartments suggested that Rap1-deficient Tregs affected T lymphocyte activation in $Rap1A^{fl/fl}Rap1B^{fl/fl}$ $Foxp3^{Cre}$ mice (Fig. 2H). In conclusion, these data imply that Rap1 plays an important role in maintaining homeostasis and functions of Tregs.

RIAM is dispensable for Tregs functions

RIAM, an effector of the RAP1 GTPases, interacts with talin to promote its recruitment to integrins, thereby mediating integrin activation (Klapproth et al., 2015). To characterize the role of RIAM on Tregs function, we crossed floxed RIAM mice with $Foxp3^{Cre}$ mice to specifically delete RIAM in Tregs ($Apbb1ip^{fl/fl}Foxp3^{Cre}$). RIAM expression was completely silenced in the modified Tregs while integrin expression level was similar to WT mice (Fig. 3A). Besides, $Apbb1ip^{fl/fl}Foxp3^{Cre\text{-}YFP}$ mice were born at expected frequencies and developed normally with no overt signs of pathology. We next investigated whether RIAM is also important for integrin activation in Tregs. Both the WT and RIAM-deficient Tregs had a similar level of binding affinity against soluble ICAM-1/VCAM-1/MAdCAM-1 upon PMA stimulation (Fig. 3B), suggesting other physiological stimuli could not affect RIAM-deficient Tregs binding to integrin ligands.

We next assessed whether the expression of RIAM was required for Treg cell function. By using an in vitro suppression assay, we observed that RIAM-deficient Tregs were functionally normal (Fig. 3C). Besides, both WT and RIAM-deficient Tregs homed equally well

to MLN, PP, PLN and SP (Fig. 3D). We also tested if RIAM is also important for Treg function. Examination of the naive ($CD44^{lo}CD62L^{hi}$) and activated ($CD44^{hi}CD62L^{lo}$) $CD4^+$ T cell compartment suggested that RIAM-deficient Tregs do not affect $CD4^+$ T lymphocyte activation in *Apbb1ip*$^{fl/fl}$*Foxp3*$^{Cre\text{-}YFP}$ mice (Fig. 3E). In summary, these data indicate that RIAM is dispensable for Tregs function and integrin activation on Tregs, suggesting the existence of an alternative protein that connects Rap1 and talin.

Lamellipodin (Lpd), together with RIAM, plays an important role on Tregs function.

RIAM is dispensable on Tregs homeostasis and functions. Therefore, there must be an alternative integrin activation pathway other than the Rap1-RIAM-talin axis. Lamellipodin (Lpd) is a RIAM paralogue that is also present in leukocytes and plays an important role in cell migration (Lagarrigue, Kim, & Ginsberg, 2019). We found out that Lpd expressed more in Tregs than in Tconv cells (Fig. 4A). Therefore, we next accessed whether Lpd plays an important role in Tregs function. To explore the function of Lpd and RIAM, we crossed floxed Lpd mice or/and RIAM mice with *Foxp3*^{Cre} mice to specifically delete Lpd or/and RIAM in Tregs (*Raph1*$^{fl/fl}$*Foxp3*^{Cre} and *Raph1*$^{fl/fl}$*Apbb1ip*$^{fl/fl}$*Foxp3*^{Cre}). The morphology of the lymphoid organs from the *Raph1*$^{fl/fl}$*Apbb1ip*$^{wt/wt}$*Foxp3*^{Cre} mice had no significant difference comparing to the organs from WT mice, except for a larger spleen observed in the *Raph1*$^{fl/fl}$*Apbb1ip*$^{fl/fl}$*Foxp3*^{Cre} mice (Fig. 4B). We next checked the peripheral blood of WT, *Raph1*$^{fl/fl}$*Foxp3*^{Cre}, and *Raph1*$^{fl/fl}$*Apbb1ip*$^{fl/fl}$*Foxp3*^{Cre} mice. *Raph1*$^{fl/fl}$*Apbb1ip*$^{fl/fl}$*Foxp3*^{Cre} mice exhibited a significantly increased number of leukocytes and lower hemoglobin concentration (Fig. 4C), suggesting the mice had more inflammation.

Then we investigated the role of Lpd on integrin activation on Tregs. We first checked on the integrin expression of Lpd/RIAM-deficient Tregs. No significant difference was found (Fig.

4D). We then tested integrin activation by measuring cells binding to integrin ligands ICAM-1, VCAM-1, and MAdCAM-1. The integrin activation of *Lpd*$^{fl/fl}$*Foxp*3^{cre} Tregs showed about a one-third reduction on their binding to ICAM-1 and MAdCAM-1, indicating that Lpd is required for integrin activation on Tregs. However, Lpd/RIAM-deficient Tregs showed dramatically decrease in binding to integrin ligands (Fig. 4E). Lastly, we evaluated whether Lpd is also involved in Treg suppression function. We observed that the suppressive capacity of Lpd-deficient Tregs declined compared with WT control. In addition, the suppressive function of Lpd/RIAM double deficient Tregs was greatly impaired (Fig. 4F). Taken together, these results suggest that both Lpd and RIAM are required for Treg homeostasis and function, and Lpd can compensate for RIAM function.

Rap1 can partially bind to the talin F0F1 domain directly and activate integrin

A recent study reported that Rap1 can bind talin directly through F0 and F1 domains (F0F1) in both Chinese hamster ovary cells and platelets. A structure-guided point mutant (R118E in the F1 domain and R35E in the F0 domain) could block Rap1 binding and abolish the capacity of Rap1 to potentiate talin-induced integrin activation (Lagarrigue et al., 2018). To understand the role of the Rap1-talin pathway on cell function and integrin activation on Tregs, we crossed floxed talin mice, R118E in F1 domain and R35E in F0 domain double mutant mice, with *Foxp3*^{Cre} mice to specifically mutate the R118E in F1 domain and R35E in F0 domain in Tregs (*Tln*$^{F0F1/fl}$*Foxp*^{Cre}). No significant difference in both the morphology or phenotype were observed between *Tln*$^{F0F1/fl}$*Foxp*^{Cre} mice and the control *Tln*$^{wt/fl}$*Foxp*^{Cre} mice (Fig. 5A-B). However, the peripheral blood from the *Tln*$^{F0F1/fl}$*Foxp*^{Cre} mice showed an abnormal number of leukocytes increase compared to the peripheral blood from the *Tln*$^{wt/fl}$*Foxp*^{Cre} control mice (Fig. 5 C), illustrating that homeostasis of the immune system of the *Tln*$^{F0F1/fl}$*Foxp*^{Cre} mice was

disturbed. Consistent with our hypothesis that the F0F1 domain contributes to integrin activation, Treg cell from *Tln*$^{F0F1/fl}$*Foxp*^{Cre} mice exhibited a reduction of integrin activation in binding to both ICAM-1 and MadCAM-1 in the soluble binding assay (Fig. 5D). Lastly, talin F0F1-deficient Tregs had slightly reduced suppression capacity against Tcov cells in vitro (Fig. 5E). Overall, the talin F0F1 domain plays a role in integrin activation in Tregs.

FIGURES

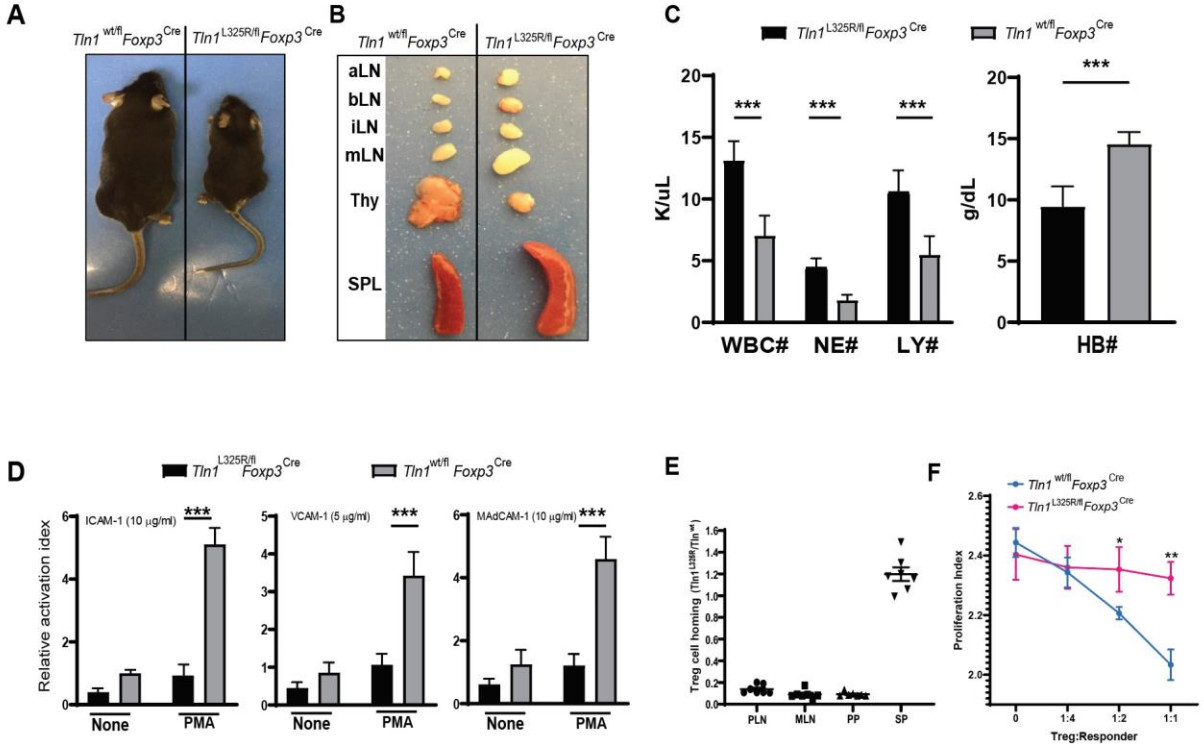
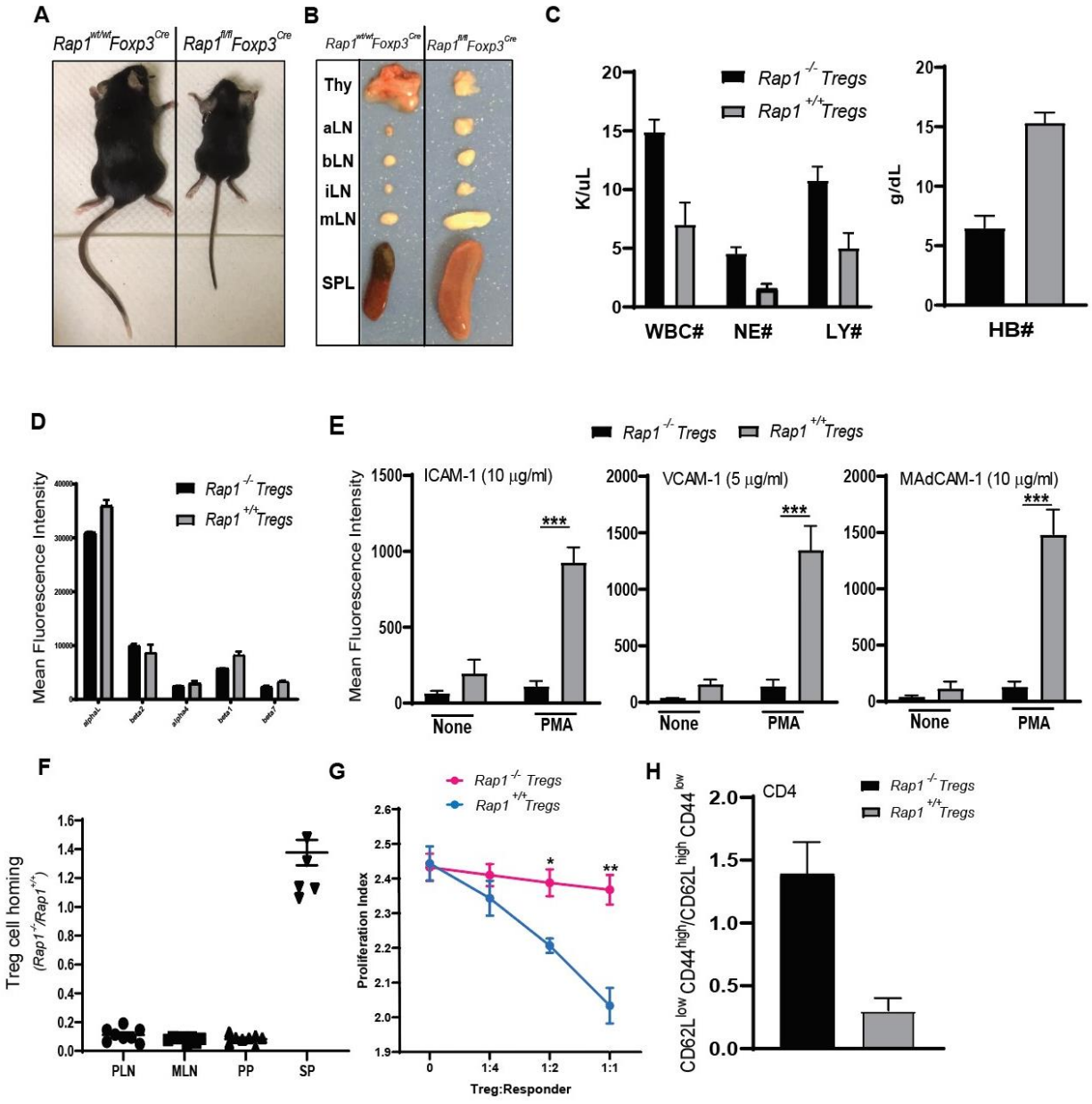


Figure 1. Talin controls integrin activation in Tregs. (A) Morphology of *Tln1*$^{wt/fl}$*Foxp3*^{Cre} or *Tln1* $^{L325R/fl}$ *Foxp3*^{Cre} mouse. (B) Second lymphoid organs (Thy(Thymi), lymph nodes [aLN(arotic), bLN(brachial), iLN(inguinal), mLN(mesenteric)] and SPL(Spleen) of *Tln1*$^{wt/fl}$*Foxp3*^{Cre} or *Tln1*$^{325/fl}$*Foxp3*^{Cre} mouse. (C) Complete blood count of *Tln1*$^{wt/fl}$ *Foxp3*^{cre} or *Tln1* $^{L325R/fl}$ *Foxp3*^{cre} mice. (D) Binding of soluble ICAM-1, VCAM-1 or MAdCAM-1 to YFP^{+} Tregs from the spleen of *Tln1*$^{wt/fl}$, *Foxp3*^{cre} (n=5) or *Tln1*$^{L325R/fl}$*Foxp3*^{cre} (n=5) mice. (E) Migration ability of Talin (L325R)- Tregs to different lymphoid tissues. YFP^{+} Tregs were isolated from *Tln1*$^{wt/fl}$ *Foxp3*^{cre} or *Tln1*$^{L325R/fl}$*Foxp3*^{cre} mice. Lymphoid organs were isolated 2hrs after injection. The ratio of *Tln1*$^{L325R/fl}$/*Tln1*$^{wt/fl}$ Tregs were shown (n=7). PLN, peripheral lymph node; MLN, mesenteric lymph node; PP, Peyer's patch; SP, spleen. (F) Treg suppression function. Tregs isolated from CD45.2 congenic *Tln1*$^{wt/fl}$*Foxp3*^{cre} or *Tln1* $^{L325R/fl}$*Foxp3*^{cre} mice were mixed with responder cells at the indicated Treg/Responder cell ratios. Responder cells are CFSE labelled CD45.1 congenic C57BL/6 CD4+CD25- naive T cells, which were activated by anti-CD3 (5 μg/ml), anti-CD28 (5 μg/ml) and IL2. CFSE populations gated on CD45.1+ cells were analyzed by flow cytometry at 72h to determine the proliferation index using FlowJo software. Data represent mean ± SEM. One-way ANOVA with Bonferroni post test. *P<0.05; **P<0.01, ***P<0.001.

Figure 2. Rap1 plays an important role in maintaining integrin activation and functions of Tregs. (A) Morphology of *Rap1a*$^{wt/wt}$ *Rap1b*$^{wt/wt}$ *Foxp3*^{cre} or *Rap1a* $^{fl/fl}$ *Rap1b*$^{fl/fl}$ *Foxp3*^{cre} mouse. (B) Second lymphoid (Thy(Thymi), lymph nodes [aLN(arotic), bLN(brachial), iLN(inguinal), mLN(mesenteric)] and SPL(Spleen) of *Rap1a*$^{wt/wt}$ *Rap1b*$^{wt/wt}$ *Foxp3*^{cre} or *Rap1a* $^{fl/fl}$ *Rap1b*$^{fl/fl}$ *Foxp3*^{cre} mouse. (C) Complete blood count of *Rap1a*$^{wt/wt}$ *Rap1b*$^{wt/wt}$ *Foxp3*^{cre} or *Rap1a* $^{fl/fl}$ *Rap1b*$^{fl/fl}$ *Foxp3*^{cre} mice. (D) Expression of integrin subunits on Tregs from *Rap1a*$^{wt/wt}$ *Rap1b*$^{wt/wt}$ *Foxp3*^{cre} or *Rap1a* $^{fl/fl}$ *Rap1b*$^{fl/fl}$ *Foxp3*^{cre} mice. (E) Binding of soluble ICAM-1, VCAM-1 or MAdCAM-1 to YFP^{+} Tregs from the spleen of *Rap1a*$^{wt/wt}$ *Rap1b*$^{wt/wt}$ *Foxp3*^{cre} or *Rap1a* $^{fl/fl}$ *Rap1b*$^{fl/fl}$ *Foxp3*^{cre} mice. (F) Migration ability of Rap1-deficient Tregs to different lymphoid tissues. YFP^{+} Tregs were isolated from *Rap1a*$^{wt/wt}$ *Rap1b*$^{wt/wt}$ *Foxp3*^{cre} or *Rap1a* $^{fl/fl}$ *Rap1b*$^{fl/fl}$ *Foxp3*^{cre} mice. Lymphoid organs were isolated 2hrs after injection. The ratio of *Rap1a* $^{fl/fl}$ *Rap1b*$^{fl/fl}$ / *Rap1a*$^{wt/wt}$ *Rap1b*$^{wt/wt}$ Tregs were shown (n=8). PLN, peripheral lymph node; MLN, mesenteric lymph node; PP, Peyer's patch; SP, spleen. (G) Treg suppression function. Tregs isolated from CD45.2 congenic *Rap1a*$^{wt/wt}$ *Rap1b*$^{wt/wt}$ *Foxp3*^{cre} or *Rap1a* $^{fl/fl}$ *Rap1b*$^{fl/fl}$ *Foxp3*^{cre} mice were mixed with responder cells at the indicated Treg/Responder cell ratios. Responder cells are CFSE labelled CD45.1 congenic C57BL/6 CD4+CD25- naive T cells, which were activated by anti-CD3 (5 μg/ml), anti-CD28 (5 μg/ml) and IL2. CFSE populations gated on CD45.1+ cells were analyzed by flow cytometry at 72h to determine the proliferation index using FlowJo software. (H) Relative expression of CD62L^{low}CD44^{high} and CD62L^{high}CD44^{low} in splenic CD4^{+} T cells from *Rap1a*$^{wt/wt}$ *Rap1b*$^{wt/wt}$ *Foxp3*^{cre} or *Rap1a* $^{fl/fl}$ *Rap1b*$^{fl/fl}$ *Foxp3*^{cre} mice. Data represent mean ± SEM. One-way ANOVA with Bonferroni post test. *P<0.05; **P<0.01, ***P<0.001.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bLN
iLN
mLN
SPL
C
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VCAM-1 (5 μg/ml)
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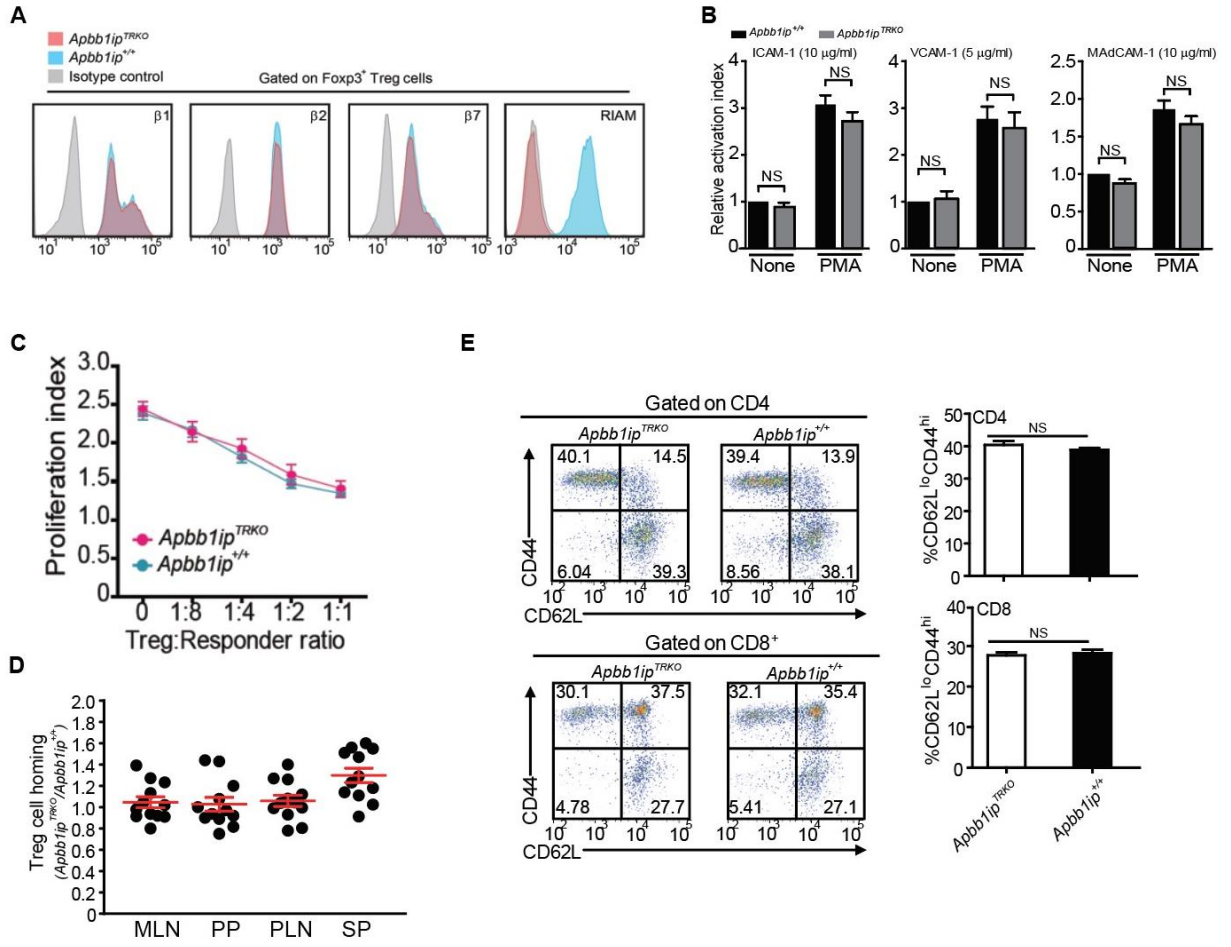

Figure 3. RIAM is dispensable in Treg function. (A)integrin β chain expression in Tregs from *Apbb1ip*$^{fl/fl}$ *Foxp3*^{cre} or *Apbb1ip*$^{wt/wt}$*Foxp3*^{cre} mice (B) Binding of soluble ICAM-1, VCAM-1 or MAdCAM-1 to YFP^{+} Tregs from the spleen of *Apbb1ip*$^{fl/fl}$ *Foxp3*^{cre} (n=5)or *Apbb1ip*$^{wt/wt}$*Foxp3*^{cre} mice(n=6). (C) Treg suppression function. Tregs isolated from CD45.2 congenic *Apbb1ip*$^{fl/fl}$ *Foxp3*^{cre} or *Apbb1ip*$^{wt/wt}$*Foxp3*^{cre} mice were mixed with responder cells at the indicated Treg/Responder cell ratios. Responder cells are CFSE labelled CD45.1 congenic C57BL/6 CD4+CD25- naive T cells, which were activated by anti-CD3 (5 μg/ml), anti-CD28 (5 μg/ml) and IL2. CFSE populations gated on CD45.1+ cells were analyzed by flow cytometry at 72h to determine the proliferation index using FlowJo software. (D) Foxp3^{+} cells ratio (*Apbb1ip*$^{fl/fl}$ *Foxp3*^{cre} / *Apbb1ip*$^{wt/wt}$*Foxp3*^{cre}) in various tissues (Mesenteric lymph node, Peyer's Patch, , popliteal lymph node, and spleen) from *Apbb1ip*$^{fl/fl}$*Foxp3*^{Cre} mice (n=12). (E) Representative expression of CD44 and CD62L in splenic CD4^{+} (upper panels) and CD8^{+} (lower panels) T cells from *Apbb1ip*$^{+/+}$*Foxp3*^{Cre} and *Apbb1ip*$^{fl/fl}$*Foxp3*^{Cre} mice (n=4). The percentage of CD62L^{low}CD44^{high} effector T cells is shown in the right. Data represent mean±SEM. Two-tailed t-test. Data represent mean ± SEM. One-way ANOVA with Bonferroni post test. *P<0.05; **P<0.01, ***P<0.001.

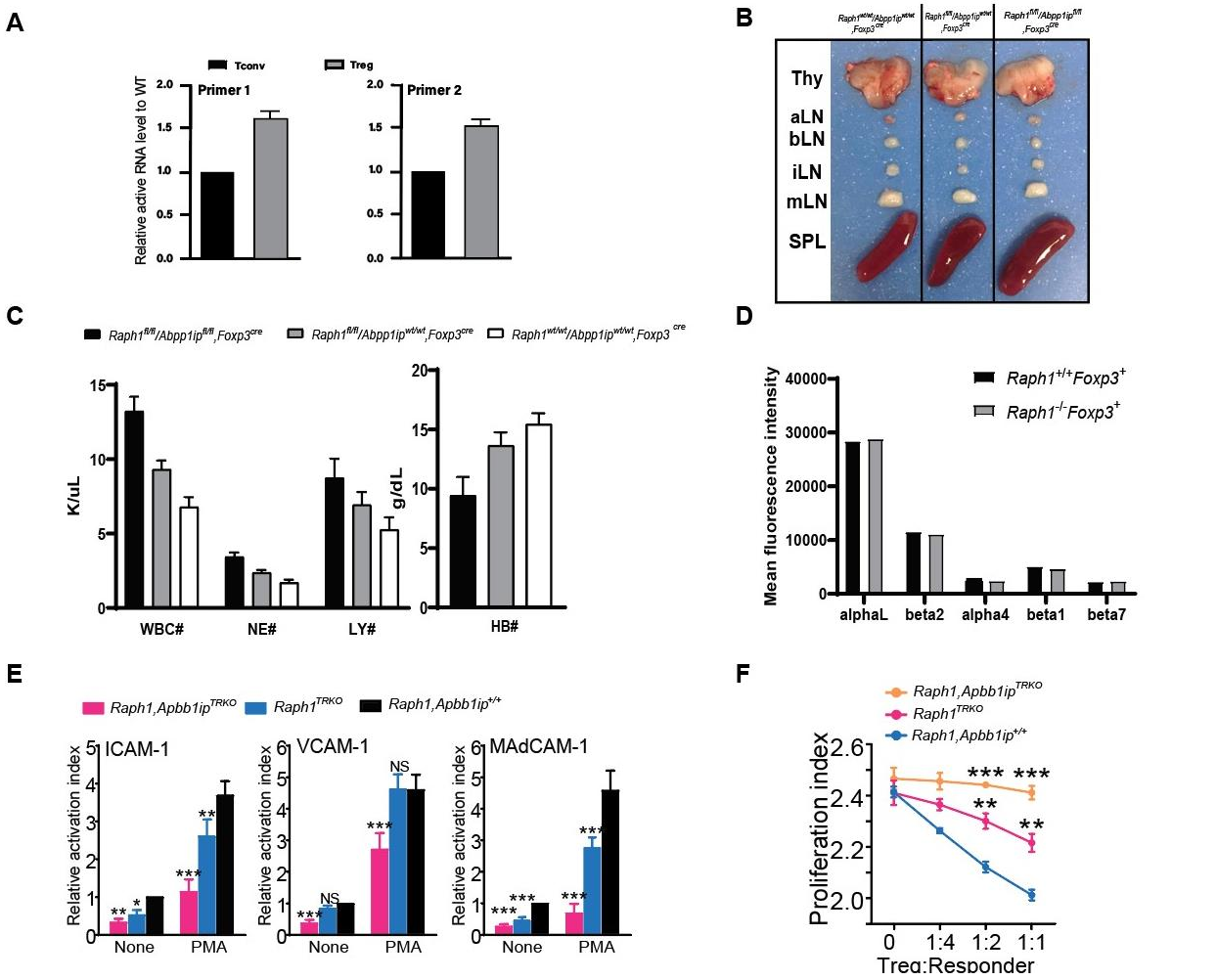

Figure 4. Lamellipodin (Lpd), together with RIAM, plays an important role on Tregs function. (A) RNA expression of Raph1 in conventional T cells and Tregs. Two pairs of primers were tested. Results were normalized to GAPDH. Data represent mean±SEM. (B) Second lymphoid organs (Thy(Thymi), lymph nodes [aLN(arotic), bLN(brachial), iLN(inguinal), mLN(mesenteric)] and SPL(Spleen) of *Rap1h*$^{wt/wt}$ *Apbb1ip*$^{wt/wt}$ *Foxp3*^{cre}, *Rap1h*$^{fl/fl}$ *Apbb1ip*$^{wt/wt}$ *Foxp3*^{cre}, or *Rap1h*$^{fl/fl}$ *Apbb1ip*$^{fl/fl}$ *Foxp3*^{cre} mouse.(C) Concentration of leukocytes in peripheral blood of *Rap1h*$^{wt/wt}$ *Apbb1ip*$^{wt/wt}$ *Foxp3*^{cre}, *Rap1h*$^{fl/fl}$ *Apbb1ip*$^{wt/wt}$ *Foxp3*^{cre}, or *Rap1h*$^{fl/fl}$ *Apbb1ip*$^{fl/fl}$ *Foxp3*^{cre} mice. (D) Surface expression of integrin αL, β2, α4, β1, and β7 in WT and *Rap1h*$^{fl/fl}$ *Apbb1ip*$^{fl/fl}$ *Foxp3*^{cre} mice. Mean fluorescence intensities (MFI) are plotted. (E) Binding of soluble ICAM-1, VCAM-1 or MAdCAM-1 to YFP^{+} Tregs from the spleen of *Rap1h*$^{wt/wt}$ *Apbb1ip*$^{wt/wt}$ *Foxp3*^{cre}, *Rap1h*$^{fl/fl}$ *Apbb1ip*$^{wt/wt}$ *Foxp3*^{cre}, or *Rap1h*$^{fl/fl}$ *Apbb1ip*$^{fl/fl}$ *Foxp3*^{cre} (n=4) mice. (F) Treg suppression function. Tregs isolated from CD45.2 congenic *Rap1h*$^{wt/wt}$ *Apbb1ip*$^{wt/wt}$ *Foxp3*^{cre}, *Rap1h*$^{fl/fl}$ *Apbb1ip*$^{wt/wt}$ *Foxp3*^{cre}, or *Rap1h*$^{fl/fl}$ *Apbb1ip*$^{fl/fl}$ *Foxp3*^{cre} mice were mixed with responder cells at the indicated Treg/Responder cell ratios. Responder cells are CFSE labelled CD45.1 congenic C57BL/6 CD4+CD25- naive T cells, which were activated by anti-CD3 (5 μg/ml), anti-CD28 (5 μg/ml) and IL2. CFSE populations gated on CD45.1+ cells were analyzed by flow cytometry at 72h to determine the proliferation index using FlowJo software. Data represent mean ± SEM. One-way ANOVA with Bonferroni post test. *P<0.05; **P<0.01, ***P<0.001.

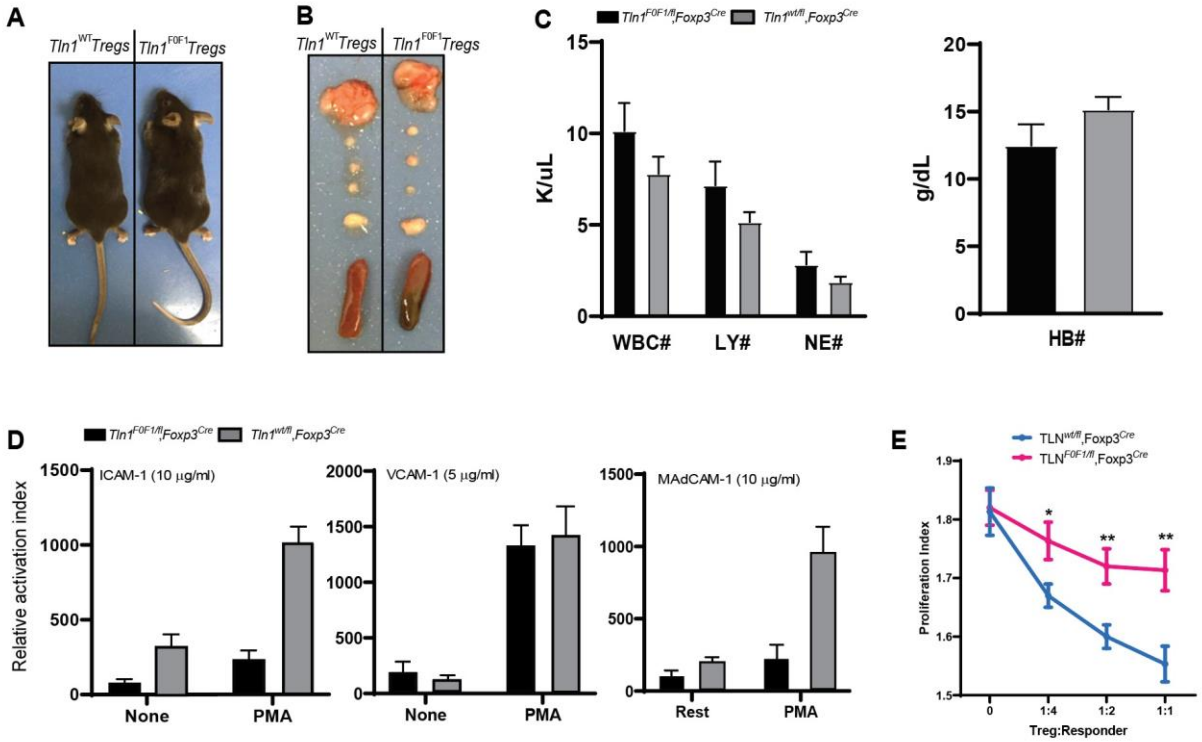

Figure 5. Talin-Rap1 direct binding also palys a role in integrin activation in Tregs. (A) Morphology of *Tln*$^{wt/fl}$ *Foxp3*^{cre} or *Tln* $^{F0F1/fl}$*Foxp3*^{cre} mouse. (B) Second lymphoid organs (Thy(Thymi), lymph nodes [aLN(arotic), bLN(brachial), iLN(inguinal), mLN(mesenteric)] and SPL(Spleen) of *Tln*$^{wt/fl}$ *Foxp3*^{cre} or *Tln* $^{F0F1/fl}$ *Foxp3*^{cre} mouse. (C) Number or concentration of leukocytes in peripheral blood of *Tln*$^{wt/fl}$ *Foxp3*^{cre} or *Tln* $^{F0F1/fl}$ *Foxp3*^{cre} mice. (D) Binding of soluble ICAM-1, VCAM-1 or MAdCAM-1 to YFP^{+} Tregs from the spleen of *Tln*$^{wt/fl}$ *Foxp3*^{cre} (n=3) or *Tln*$^{wt/fl}$ *Foxp3*^{cre} (n=4) mice. (E) Treg suppression function. Tregs isolated from CD45.2 congenic *Tln*$^{wt/fl}$ *Foxp3*^{cre} or *Tln* $^{F0F1/fl}$ *Foxp3*^{cre} mice were mixed with responder cells at the indicated Treg/Responder cell ratios. Responder cells are CFSE labelled CD45.1 congenic C57BL/6 CD4+CD25- naive T cells, which were activated by anti-CD3 (5 μg/ml), anti-CD28 (5 μg/ml) and IL2. CFSE populations gated on CD45.1+ cells were analyzed by flow cytometry at 72h to determine the proliferation index using FlowJo software. Data represent mean ± SEM. One-way ANOVA with Bonferroni post test. *P<0.05; **P<0.01, ***P<0.001.

Discussion

Integrin activation has been shown critical for maintaining peripheral tolerance. Disrupting integrin activation by talin ablation in Tregs results in spontaneous T cell activation and inflammation in mice organs. However, the integrin activation mechanism on Tregs is still unknown. Here, our results demonstrate that talin and Rap1 are essential for integrin activation in Tregs as well as RIAM and Lpd. In addition, Rap1 direct binding to talin1 is important for integrin activation in Tregs. Moreover, the results suggest that Tregs have distinct integrin activation mechanisms from Tconv cells by having compensative proteins and other pathways that bypass the intermediate proteins.

Based on the observation from previous studies that integrin activation is critical for Treg-mediated peripheral tolerance, we hypothesized that Tregs might also share the same integrin pathway as $CD4^+$ T cell. Our study, however, instead of deleting talin, we utilized L325R mutation in talin that inhibited integrin activation without alternating the binding between talin and the β chain of the integrin. We observed that talin-mutated mice developed serious systemic autoimmunity and abnormal increased leukocytes number in peripheral blood. Additionally, the mutated Tregs also failed to suppress the conventional T cells in vitro, indicating that the Tregs were dysfunctional. These results support the previous conclusion that integrin activation governs Treg-mediated homeostasis and peripheral tolerance.

However, although deletion of Treg-specific Rap1 or talin exhibited similar autoimmunity symptoms, loss of RIAM in Tregs only showed a very limited impact on integrin activation in Tregs. Previously, our lab found out that Treg- specific RIAM deletion prevents colitis mouse model, and RIAM-deficient Treg cell injection could rescue mice from colitis (Supplement 1, unpublished). The results suggest a different role of RIAM in Tregs from $CD4^+$

T cells. In the current study, by using *apbb1ip*$^{fl/fl}$*Foxp3*^{Cre} mice, we confirmed that RIAM is dispensable in maintaining Treg migration and function. No significant change was observed in the mice phenotype, integrin activation states, or suppressive capacity level. Taken together, the results suggest that RIAM does not serve an important role as it does in the conventional T cells, and there is a distinct mechanism that exists in integrin activation of Tregs.

As mentioned before, RIAM's paralogue protein, Lamellipodin (Lpd, *Raph1*), is able to bind talin and form a complex. We also found the RNA expression level of Lpd is higher in Tregs than conventional T cells. Thus, we utilized *Raph1*$^{fl/fl}$*Foxp3*^{Cre} mice and explored the role of Lpd in integrin activation of Tregs. Interestingly, similar to the mice with RIAM-deficient Tregs, Treg-specific Lpd deleted mice were normal from appearance. However, Lpd-deficient Tregs showed a notable decrease in integrin activation level and a minor but significant decreased in suppressive capacity, suggesting that Lpd plays a more important role in integrin activation in Tregs than RIAM does. Surprisingly, when we crossed *Raph1*$^{fl/fl}$*Foxp3*^{Cre} mice with *Abpp1ip*$^{fl/fl}$ *mice,* the mice with both Lpd and RIAM deficient in Treg exhibited a dramatic defect on Treg cell function. The Lpd/RIAM double-deficient mice also had inflammation in the lymphoid organs and a significant decrease in the integrin activation level. These results suggest that Lpd plays a similar but more important role compared to RIAM in Tregs by compensating RIAM and forms an alternative integrin activation pathway with talin.

Recent studies showed that Rap1 can bind to the talin directly (Gingras et al., 2019). We hypothesized that the Rap1-talin direct binding could also facilitate integrin activation in Tregs. We used *Tln*$^{F0F1/fl}$*Foxp3*^{Cre} mice that had the talin R35E/R118E double mutations in all Tregs. We observed that although the appearance of the Tregs-specific R35E/R118E double mutant mice showed no significant differences compared to WT mice, the integrin binding to ICAM-1

and MAdCAM-1ligands exhibited a significant decrease. In addition, R35E/R118E double mutant Tregs' suppressive capacity also reduced significantly. These data indicate that the Rap1-talin directly binding through R35 in the F0 domain and R118 in the F1 domain facilitate part of integrin activation.

Many different cell types express integrin on their surfaces and have different integrin activation mechanisms. Platelet, for example, has much higher talin and Rap1 expression and low expression on RIAM. As a result, RIAM is also dispensable in platelet, and only the point mutations on the F0F1 domain can inhibit integrin activation on the platelet. Our findings demonstrate that integrin activation plays a critical role in maintaining Treg homeostasis and suppression function. Meanwhile, this mechanism of integrin activation is unique to Tregs and different from other cell types. New studies are needed for exploring the importance and mechanisms of integrin activation in other cell types. Our future study will continue discovering the roles of integrin activation in the other autoimmunity related lymphocytes. There are potentials that our future research can reveal other candidates as therapeutic targets just as RIAM.

Materials and Methods

Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego, and were conducted in accordance with federal regulations as well as institutional guidelines and regulations on animal studies. All mice were housed in specific pathogen-free conditions. C57BL/6 (CD45.1), C57BL/6 (CD45.2), *Rag1*$^{KO/KO}$ mice were from The Jackson Laboratory. *Abpp1ip*$^{KO/KO}$, *Abpp1ip*$^{fl/fl}$, *Tln1*$^{fl/fl}$, *Tln1*$^{L325R/fl}$, *CD4*^{Cre}, *Foxp3*$^{Cre\text{-}YFP}$ and *Foxp3*^{GFP} mice have been described previously. F0^{R35}F1 ^{R} *Rap1A*$^{fl/fl}$*Rap1B*$^{fl/fl}$ mice were obtained from Dr. Mark Philips.

Mononuclear cells were isolated from mesenteric lymph node (MLN), Peyer's patch (PP), peripheral lymph node (PLN), spleen (SP) and colonic lamina propria as previously described. Cell counting with immunofluorescence cytometry was performed using an Accuri C6 Plus and FACSCalibur (BD Biosciences).

Antibodies and reagents

The following Antibodies were from BioLegend: CD3 (17A2), CD4 (GK1.5), CD8 (53-6.7), CD44 (1M7), CD62L (MEL-14), β7 (FIB504), Foxp3 (MF-14), anti-CD3 (2C11), anti-CD28 (37.51) and TGF-β1 (TW7-16B4). Secondary AlexaFluor-labelled antibodies were from Jackson ImmunoResearch. Foxp3 transcription factor fixation/permeabilization kit was purchased from eBioscience. CFSE and eFluor 670 were purchased from Invitrogen and Biolegend respectively. 12-O-Tetradecanoylphorbol-13-acetate (PMA) and piroxicam were from Sigma. Ionomycin, brefeldin A and monensin were from BioLegend. MojoSort™ mouse CD3 T cell isolation kit and mouse CD4 T cell isolation kit were from BioLegend. Liberase TL (Research Grade) and DNAse I were from Roche. Recombinant mouse ICAM-1-Fc and VCAM-1-Fc were from R&D

Systems. Recombinant mouse MAdCAM-1-Fc was purified by ProteinA beads as previously described[52]

Soluble ligand binding assays

Cells isolated from mouse tissues were washed and resuspended in HBSS containing 0.1% BSA and 1 mM Ca^{2+}/Mg^{2+} and stained with conjugated antibody for 30 min at 4 °C. Then cells were washed twice before flow cytometry analysis using a Accuri C6 Plus or FACSCalibur (BD Biosciences). Data were analyzed using FlowJo software. For soluble ligand binding assay, 5×10^6 cells were washed and resuspended in HBSS containing 0.1% BSA and 1 mM Ca^{2+}/Mg^{2+}, prior to incubation with integrin ligands for 30 min at room temperature in presence with or without 100 nM PMA. Cells were then incubated with AlexFluor647-conjugated anti-human IgG (1:200) for 30 min at 4 °C. For intracellular detection of cytokines, splenocytes were stimulated ex vivo with PMA and ionomycin in the presence of brefeldin A and monensin for 6 h 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 IL10, TGF-β1 and Foxp3 staining.

Treg cell suppression assays

$CD4^+CD25^-$ T cells (Responder cells) were isolated from spleens of C57BL/6 (CD45.1) WT mice by magnetic separation using the $CD4^+$ T cell negative isolation kit (Biolegend); a biotin-conjugated anti-CD25 (PC61; BioLegend) Ab was included to deplete Tregs. YFP^+ Tregs were sorted with a FACSAria 2 (BD Biosciences). Responder cells were labelled with CFSE and cocultured with Tregs (8:1, 4:1, 2:1 and 1:1 ratios) in the presence of 5 μg/ml immobilized anti-

CD3 (2C11) and anti-CD28 (37.51) and *IL2* for 4 days at 37°C. Proliferation index was calculated by FlowJo v10.

Cell homing experiment

For competitive homing assay of RIAM-deficient Tregs, YFP^+ Tregs were sorted with a FACSAria 2 (BD Biosciences) from $Abpp1ip^{WT/WT}Foxp3^{Cre\text{-}YFP}$ or $Abpp1ip^{fl/fl}Foxp3^{Cre\text{-}YFP}$ mice and labeled with 1μM and 10 μM of eFluor670, respectively. For competitive homing assay of talin-deficient Tregs, YFP^+ Tregs were sorted with a FACSAria 2 (BD Biosciences) from $Tln^{WT/WT}Foxp3^{Cre\text{-}YFP}$ or $Tln^{L325R/fl}Foxp3^{Cre\text{-}YFP}$ mice and labeled with 1μM and 10 μM of eFluor670, respectively. . For competitive homing assay of Rap1-deficient Tregs, YFP^+ Tregs were sorted with a FACSAria 2 (BD Biosciences) from $Rap1A^{WT/WT}/Rap1B^{WT/WT}$, $Foxp3Cre^{\text{-}YFP}$ or $Rap1A^{fl/fl}/\ Rap1B^{fl/fl}$,$Foxp3Cre^{\text{-}YFP}$ mice and labeled with 1μM and 10 μM of eFluor670, respectively Equal numbers (1×10^7) of differentially labelled Tregs were mixed and then intravenously injected into C57BL/6 recipient mice. Lymphoid organs were harvested 3hrs after injection and isolated cells were analyzed by flow cytometry. The ratio of $Abpp1ip^{KO}$ Tregs ($eFluor670^{high}$) to $Abpp1ip^{WT}$ Tregs ($eFluor670^{low}$) from different lymphoid organs are shown. MLN, mesenteric lymph node; PP, Peyer's patch; PLN, peripheral lymph node; SP, spleen.

Statistical Analysis

Statistical analysis was performed using PRISM software (version 6.00, GraphPad Software), and all datasets were checked for Gaussian normality distribution. Data analysis was performed using one-way ANOVA or two-way ANOVA followed by Bonferroni post test as indicated in the figure legends. The resulting *P* values are indicated as follows: NS: not significant, $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$. Plotted data are the mean ±SEM of at least three independent experiments.

Real-time quantitative PCR analyses

Total RNAs were isolated from colon using tissue homogenizer (JXFSTPRP-24, ThunderSci) and TRIzol reagent according to the manufacturer's protocol (Thermo Fisher Scientific). For gene expression analysis, single-stranded cDNA was produced from 10 ng total RNA of colon using SuperScript III First-Strand synthesis and oligo-dT primers according to the manufacturer's protocol (Thermo Fisher Scientific). Kapa SybrFast qPCR kit (Kapa Biosystems) and thermal cycler (CFX96 Real-Time System; Bio-Rad) were used to determine the relative levels of the genes analyzed (primer sequences are shown in Table 1) according to the manufacturer's protocol. Actin mRNA levels were used as internal control, and the $2^{-\Delta\Delta CT}$ method was used for analysis of the data. Each control value (WT mice or $Rag1^{KO/KO}$ mice injected with PBS) was normalized to 1.

Statistical Analysis

Statistical analysis was performed in PRISM software (version 6.00, GraphPad Software), and all datasets were checked for Gaussian normality distribution. Data analysis was performed using one-way ANOVA or two-way ANOVA with Bonferroni post test which are indicated in figure legends. The resulting P values are indicated as follows: NS: not significant, $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$. Data represent the mean ±SEM of at least three independent experiments.

This thesis is part being prepared for submission for publication of the material. Sun, Hao; Wang, Hsin; Lopez-Ramirez, Miguel; Ginsberg, Mark. The thesis author will be the primary author of this paper

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