UNIVERSITY OF CALIFORNIA SAN DIEGO

Rap1 Binding to Talin1 Promotes Integrin Activation in T Lymphocytes

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

in

Biology

by

Boyang Tan

Committee in Charge:

Professor Mark Ginsberg, Chair
Professor Nao Hao, Co-Chair
Professor Li-fan Lu

2020

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

Co-Chair

Chair

University of California San Diego

2020

DEDICATION

I dedicate this thesis to the members of the Ginsberg Lab and my family for their infinite love and supports.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENT

First, I would like to thank Dr. Ginsberg for serving as my committee chair and providing me all the facilities during my master program. I would also like to thank Dr. Hao and Dr. Lu for serving as my committee members.

I cannot express enough thanks to my mentor Dr. Sun for her continued support, patient assistance, and invaluable guidance throughout my master program. My completion of the master program could not have been accomplished without the support of Dr. Sun.

Finally, I'd like to thank my lab members, Hsin Wang, Qinyi Du, Bryant Nguyen, and Weiwei Qi. It was great and enjoyable to work with you.

This thesis is part being prepared for submission for publication of the material. Sun, Hao; Lagarrigue, Frederic; Gingras, Alexandre; Lopez-Ramirez, Miguel; Tan, Boyang; Ginsberg, Mark H. The thesis author will be an author of the paper.

ABSTRACT OF THE THESIS

Rap1 Binding to Talin1 Promotes Integrin Activation in T Lymphocytes

by

Boyang Tan

Master of Science in Biology

University of California San Diego, 2020

Professor Mark Ginsberg, Chair
Professor Nan Hao, Co-Chair

Integrins are essential for all the T cell functions. Integrins can enable T cells to migrate in and out of lymph nodes and other tissues and mediate cell-to-cell interactions. Integrin activation also plays a critical role in maintaining regulatory T (Treg) cell function. Rap1 is a small GTPase playing an essential role in recruitment and tethering of talin1 to the integrin cytoplasmic domain, which results in integrin activation. Rap1 can bind to talin1 directly. Previous work showed that the Rap1–talin1 F0 domain interaction has a minimum effect on platelet activation. Our lab recently identified an additional Rap1 binding site in the talin1 F1 domain, which is critical for integrin activation in platelets. However, the relative roles of these

two binding sites for integrin activation on lymphocytes are still obscure. Here, by testing talin1 mice bearing point mutations, which block Rap1 binding without affecting talin1 expression, in talin1 F0 and F1 domains (R35E, R118E), we found that the binding between talin1 and Rap1 plays a key role in T cell adhesion and migration. The loss of direct interaction between talin1and Rap1 also leads to defects in the suppressive function of Treg cells specifically. Taken together, talin1 is a direct effector of Rap1 GTPase that mediates integrin activation in both $CD4^+$ T cells and Treg cells.

INTRODUCTION

The blood cells circulating in our body protect us from pathogen invasion and maintain homeostasis. Both innate and adaptive immunity relies on the activities of the leukocytes, where innate immunity mainly consists of granulocytes and macrophages and adaptive immune response largely involves the activity of lymphocytes (Janeway, 2001). T lymphocyte is the most well-studied cell type among the lymphocytes of the adaptive immune system (Sakaguchi et al., 2008). T lymphocytes continuously recirculate from the blood vessel through the secondary lymphoid organs (SLOs) of the body, where they encounter and recognize specific antigens presented by the antigen-presenting cells (APCs), such as dendritic cell, and differentiate into antigen-specific effector cells (Janeway, 2001). The effector T cells can subsequently migrate into peripheral non-lymphoid sites to perform their protective functions, including the direct killing of virus-infected cells by $CD8^+$ cytotoxic T cells and activation of macrophages and B cells by $CD4^+$ T cells (Alberts, 2002). These processes are critical for maintaining adaptive immunity against invading pathogens.

Integrins are essential for all T cell functions, from thymocyte differentiation to the formation of the immunological synapse and finally migrating to target tissues (Bertoni, 2018). Successful adaptive immune response relies on the regulated adhesion of lymphocytes to vascular endothelium and APCs (Mor, 2007). Integrins are a major class of adhesion receptors that regulate such cell-to-cell and cell-to-extracellular matrix interaction. They are heterodimeric transmembrane proteins consisting of large α chain that pairs non-covalently with a smaller β subunits (Kim, 2011). There are 24 different integrins, comprised of 18 α subunits and 8 β subunits, existing in different tissues and exhibiting different binding affinities to particular ligands in mammals (Humphries et al. 2006). Among these integrins, three of them are the most important on lymphocytes, which are $\beta2$, $\beta7$ and $\beta1$ integrins. All T lymphocytes express the $\alpha L\beta2$ integrin

known as lymphocyte function-associated antigen-1 (LFA-1), which binds to intercellular adhesion molecules (ICAMs) expressed on endothelium and APCs. Lymphocyte Peyer's patch adhesion molecule (LPAM-1), consisted of α4 and β7 integrins, is expressed on a subset of naive T cells and responsible for T lymphocyte homing into gut-associated lymphoid tissues by binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Very late activation antigen-4 (VLA-4) is integrin α4β1 that binds to vascular cell adhesion molecule-1 (VCAM-1) on activated endothelium, which is critical for recruiting T cells into sites of infection (Janeway, 2001). Besides mediating T lymphocyte migration and the interactions of T lymphocytes with APCs, integrins also play essential roles in facilitating Treg cell contact-mediated suppression of self-reactive effector T cells, which is crucial for maintaining homeostasis and preventing autoimmune diseases (Klann, 2018; Vignali, 2008). Given the importance of integrins during homeostasis and infection, the regulatory mechanisms of integrin activation have been studied extensively for the past two decades.

Integrins usually maintain an inactive form when circulating in the body, and agonist stimulation leads to integrin activation via "inside-out" signaling as a result of a conformational change from a compact, bent shape to an extended conformation (Hogg, 2011). During this complex process, the intracellular proteins Rap1 and talin1 are critical regulators required as signaling elements (Han, 2006). Rap1 is a small GTPase that functions as a hub in integrin inside-out signaling. It is activated by Rap1 guanine nucleotide-exchange factors (GEFs) from the GDP-bound form to the GTP-bound form downstream of chemokine or T-cell receptor (TCR) signaling, resulting in LFA-1 and α4 integrin activation (Reedquist, 2000). Several Rap1 GEFs such as CALDAG-GEF1 (Ca^{2+} and diacylglycerol-regulated guanine nucleotide exchange factor 1), have been identified. Rap1 GEFs are activated by a diverse set of upstream stimuli, indicating that

multiple pathways can converge on the Rap1 GTPases (Abram, 2009). Talin1 is a major cytoskeletal protein consisting of an N-terminal head and a rod domain. The N-terminal talin head domain (THD) contains a FERM (band 4.1, ezrin, radixin, and moesin) domain composed of 4 subdomains: F0, F1, F2, and F3. The F3 subdomain binding to the integrin β cytoplasmic tails leads to conformational changes of integrins, which is the critical final step of integrin activation (Kim, 2011). The connection between the Rap1 and talin1 has not been completely characterized.

One model has suggested that activated Rap1 can recruit Rap1-GTP interacting adaptor molecule (RIAM), which relays Rap1 signaling to talin1 and targets talin1 to the integrin (Lagarrigue, 2016). Recent studies have shown that Rap1 could directly bind to talin1 F0 and F1 domains and therefore regulate integrin activities in platelets, which indicates a new pathway of integrin activation (Lagarrigue, 2018; Gingras, 2019). However, the effects of Rap1 directly binding to talin1 on integrin activation in lymphocytes are still unknown. Here, we utilized talin1 mice bearing point mutations that blocked Rap1 binding without affecting talin1 expression to access the regulatory function of Rap1-talin1 interaction in $CD4^+$ T cells and Treg cells. We found that loss of Rap1-talin1 interaction in $CD4^+$ T cells and Tregs decreases the activation of lymphocyte β1-, β2- and β7- integrins. Adhesion and migration of $CD4^+$ T cells are dramatically impaired when Rap1-talin1 interaction was disrupted. In Treg cells, the migration and the capacity of suppressing the proliferation of conventional T cells are also substantially impaired when Rap1-talin1 interaction was blocked. These findings indicate that talin1 is a direct effector of Rap1 GTPases that regulates integrin activation in both $CD4^+$ T cells and Treg cells.

RESULTS

Rap1 binding to talin1 F0 and F1 domains is important for integrin activation in CD4+ T cells.

The point mutations R35E in talin1 F0 domain and R118E in talin1 F1 domain do not disturb the folding of F0 and F1 and blocks binding to Rap1 (Gingras, 2018; Gingras, 2019). In order to examine the contribution of directly binding between Rap1 and talin1 to integrin activation in CD4+ T cells, we crossed talin1-floxed mice with heterozygous *Tln1*$^{R35E,R118E/wt}$ mice to get *Tln1*$^{R35E,R118E/flox}$ mice. Then, the *Tln1*$^{R35E,R118E/flox}$ mice were crossed with *CD4*^{Cre} mice to generate *Tln1*$^{R35E,R118E/flox}$ *CD4*^{Cre} mice in which the mutants (R35E) and (R118E) of talin1 were specifically expressed in CD4+ T cells. The *Tln1*$^{R35E,R118E/flox}$ *CD4*^{Cre} mice were viable and healthy, showing normal numbers of whole blood cells in comparison with *Tln1*$^{wt/flox}$ *CD4*^{Cre} littermates (Fig. 1A). We checked that the talin1(R35E, R118E) CD4+ T cells expressed the same level of Rap1 and talin1 protein as wild-type (WT) CD4+ T cells (Fig. 1B). The surface expression of αL, β2, α4, β1 and β7 integrins also showed no difference between talin1(R35E, R118E) CD4+ T cells and WT CD4+ T cells (Fig. 1C). Overall, these results suggest that *Tln1*$^{R35E,R118E/Flox}$ *CD4*^{Cre} mice are appropriate for studying the effects of directly binding between Rap1 and talin1 for integrin activities in CD4+ T cells.

Rap1 and talin1 are both essential for the activation of CD4+ T cell integrins. We assessed the importance of Rap1-talin1 directly binding for CD4+ T cells integrin activation. Addition of 12-O-Tetradecanoylphorbol-13-acetate (PMA) stimulated the binding of soluble ICAM-1, VCAM-1, and MAdCAM-1 to WT CD4+ T cells (Fig. 2A). However, talin1(R35E, R118E) CD4+ T cells showed impaired binding to all three integrin ligands, indicating that activation of integrins αLβ2, α4β1, and α4β7, respectively, was inhibited in talin1(R35E, R118E) CD4+ T cells (Fig. 2A).

Those results indicate that loss of Rap1 directly binding to talin1 substantially disrupts integrin activation in $CD4^+$ T cells.

Rap1 binding to talin1 plays an essential role in maintaining the functions of $CD4^+$ T cells.

Integrins play a key role in T lymphocyte adhesion (Janeway, 2001), and for lymphocyte trafficking to secondary lymphoid organs (Kinashi, 2007). To investigate the effects of Rap1 directly binding to talin1 on integrin-mediated $CD4^+$ T cells functions, we tested the adhesion of talin1(R35E, R118E) $CD4^+$ T cells and WT $CD4^+$ T cells on immobilized ICAM-1, VCAM-1 or MAdCAM-1 by flow chamber assay. Consistent with the defect in integrin activation, loss of Rap1-talin1 directly binding resulted in defective adhesion of $CD4^+$ T cells on all three ligands, either under stimulation of stromal-derived factor 1-α (SDF1-α) or PMA (Fig. 2B). We next tested the role of Rap1-talin1 directly binding on $CD4^+$ T cell migration using a competitive homing assay. $CD4^+$ cells were isolated from $Tln1^{R35E,\ R118E/Flox}$ $CD4^{Cre}$ mice or $Tln1^{wt/Flox}$ $CD4^{Cre}$ and labeled with CFSE and eFluor670 respectively. Equal numbers (1×10^7) of CFSE or eFluor 670-labeled CD4 cells were mixed and then injected intravenously into C57BL/6J mice. The number of CFSE or eFluor 670-labeled cells in mesenteric lymph nodes (MLNs) and peripheral lymph nodes (PLNs) and spleen (SP) were enumerated by flow cytometry. A homing index, defined as the ratio of Talin1(R35E, R118E) $CD4^+$ cells to WT $CD4^+$ cells was determined. Talin1 (R35E, R118E) $CD4^+$ T cells manifested a dramatically defective migration to the PLNs and MLNs (Fig. 2C). However, compared to WT $CD4^+$ T cells, the homing of Rap1-talin1-binding deficient $CD4^+$ T cells to spleen showed no notable changes (Fig. 2C). Taken together, these results indicate that talin1 binding to Rap1 is necessary for regulating $CD4^+$ T cell adhesion and migration to certain secondary lymphoid organs.

Direct binding between Rap1 and talin1 is essential for integrin activation in Treg cells.

Treg cell plays a critical role in maintaining peripheral tolerance, preventing autoimmune diseases and modulating inflammation by suppression of the effector T cells (Dario, 2008). Previous study has shown that integrin activation was required for Treg cell mediated peripheral tolerance and homeostasis (Klann, 2018). To examine the contribution of directly binding between Rap1 and talin1 to integrin activation in Treg cells, we crossed the previously described heterozygous *Tln1*$^{R35E,R118E/flox}$ mice with *Foxp3*^{Cre} mice to specifically mutate talin1 into talin1(R35E, R118E) in Treg cells. The *Tln1*$^{R35E,R118E/flox}$ *Foxp3*^{Cre} mice were viable and healthy. However, the number of neutrophils and lymphocytes slightly increased in comparison with *Tln1*$^{wt/flox}$ *Foxp3*^{Cre} littermates, indicating a mild leukocytosis in the Treg-specific talin1 mutant mice (Fig. 3A). We checked that the talin1(R35E, R118E) Treg cells expressed the same level of Rap1 and talin1 protein as the control group (Fig. 3B). The surface expression of αL, β2, α4, β1 and β7 integrins in talin1(R35E, R118E) Treg cells was similar to the WT Treg cells (Fig. 3C). Overall, these results suggest that *Tln1*$^{R35E,R118E/Flox}$ *Foxp3*^{Cre} mice are suitable for studying the role of directly binding between Rap1 and talin1 in integrin activities in Treg cells.

Rap1 and talin1 are required for Treg cell integrins activation. We examined the importance of Rap1-talin1 directly binding Treg cells integrin activation. The addition of PMA stimulated the binding of soluble ICAM-1, VCAM-1, and MAdCAM-1 to WT Treg cells (Fig. 4A). However, talin1(R35E, R118E) Treg cells exhibited a substantially reduced integrin activation on both of ICAM-1 and MAdCAM-1, indicating that activation of integrins αLβ2 and α4β7 was inhibited in talin1(R35E, R118E) Treg cells (Fig. 4A). Those results indicate that loss of Rap1 directly binding to talin1 substantially disrupt integrin activation in Treg cells.

Rap1 binding to talin1 is required for regulating functions of Treg cells.

Since the β integrin activation was impaired without talin1 binding to Rap1, we wanted to further investigate how does such talin1-binding deficiency affect Treg cell functions. We performed a competitive homing assay to examined the migration ability of talin1(R35E, R118E) Treg cells. We isolated Treg cells from *Tln1*$^{R35E,\ R118E/Flox}$ *Foxp3*^{Cre} *mice* or *Tln1*$^{wt/Flox}$ *Foxp3*^{Cre} and stained with 1 μM and 10 μM eFluor670 respectively. Equal numbers ($1\times10_7$) of different concentrations of eFluor 670-labeled Treg cells were mixed and then injected intravenously into C57BL/6J mice. A homing index, defined as the ratio of talin1(R35E, R118E) Treg cells to WT Treg cells was determined. As we predicted, there was a notable reduction of talin1(R35E, R118E) Treg cells homing to the PLNs and MLNs compared to WT Treg cells, and the homing of talin-binding deficient Treg cells to spleen showed no significant change (Fig. 4B).

We next assessed the suppressive ability of talin1(R35E, R118) Treg cells by performing in vitro suppression assay. Talin1(R35E, R118) and WT Treg cells were isolated and mixed respectively with conventional T cells (responder cells) at different Treg/Responder cell ratios. The proliferation of responder cells was analyzed by flow cytometry. WT Tregs inhibited the proliferation of responder cells substantially. However, consistent with the defect in integrin activation, the suppressive function of talin1(R35E, R118) Tregs was significantly impaired (Fig. 4C). Taken together, these results suggest that blocking the binding of talin1 to Rap1 significantly affected the migration and suppression functions of Tregs.

RIAM is another direct Rap1 effector that mediates integrin activation in T lymphocytes.

Since the loss of talin1 binding to Rap1 did not totally block the integrin activation in T lymphocytes, there have to be alternative pathways that regulate integrin activation in T

lymphocytes. Previous studies have shown that RIAM, an effector of Rap1, played a role in leukocyte integrin activation (Lagarrigue, 2016; Su, 2017). A 'canonical' pathway to leukocyte integrin activation involves that, activated Rap1 binds to RIAM, which recruits cytoplasmic talin to the plasma membrane where it binds to integrin β tails and triggers integrin activation (Shattil et al., 2010). To investigate the role of RIAM in the talin1-Rap1 signaling pathway for integrin activation of T lymphocytes, we crossed the previously described *Tln1*$^{R35E,R118E/Flox}$ *CD4*^{Cre} and *Tln1*$^{R35E,R118E/flox}$ *Foxp3*^{Cre} mice with *Apbb1ip*$^{flox/flox}$ mice to produce *Tln1*$^{R35E,\ R118E/Flox}$ *Apbb1ip*$^{flox/flox}$ *CD4*^{Cre} and *Tln1*$^{R35E,R118E/flox}$ *Apbb1ip*$^{flox/flox}$ *Foxp3*^{Cre} mice. The *Tln1*$^{R35E,\ R118E/Flox}$ *Apbb1ip*$^{flox/flox}$ *CD4*^{Cre} and *Tln1*$^{R35E,R118E/flox}$ *Apbb1ip*$^{flox/flox}$ *Foxp3*^{Cre} mice expressed RIAM-deficiency and Rap1-binding deficient talin1 conditionally in CD4^{+} T cells and Treg cells respectively. We accessed the effects of RIAM-deficiency on integrin activation in talin1(R35E, R118E) T lymphocytes. We observed that addition of PMA stimulated the binding of soluble ICAM-1, VCAM-1, and MAdCAM-1 to WT CD4^{+} T cells and Treg cells (Fig. 5A, B). However, RIAM-deficient talin1(R35E, R118E) CD4^{+} T cells and Treg cells showed impaired binding to all three integrin ligands, indicating that activation of integrins αLβ2, α4β1, and α4β7, respectively, was inhibited in talin1(R35E, R118E) CD4^{+} T cells and Treg cells (Fig. 5A, B). We further assessed whether RIAM-deficiency affects the function of CD4^{+} T cells and Treg cells via in vivo competitive homing assay. As expected, the number of RIAM-deficient talin1(R35E, R118E) CD4^{+} T cells and Treg cells homed to PLNs and MLNs sharply reduced compared to the control group and largely accumulated in the spleen (Fig. 5C, D), which indicated that the migration capacity of CD4^{+} T cells and Treg cells were almost blocked by the combination of talin1-binding deficiency and RIAM-deficiency. Overall, the results imply that RIAM also participates in the

Rap1-talin1 dependent pathway for mediating integrin activation and maintaining functions of $CD4_+$ T cells and Treg cells.

FIGURES

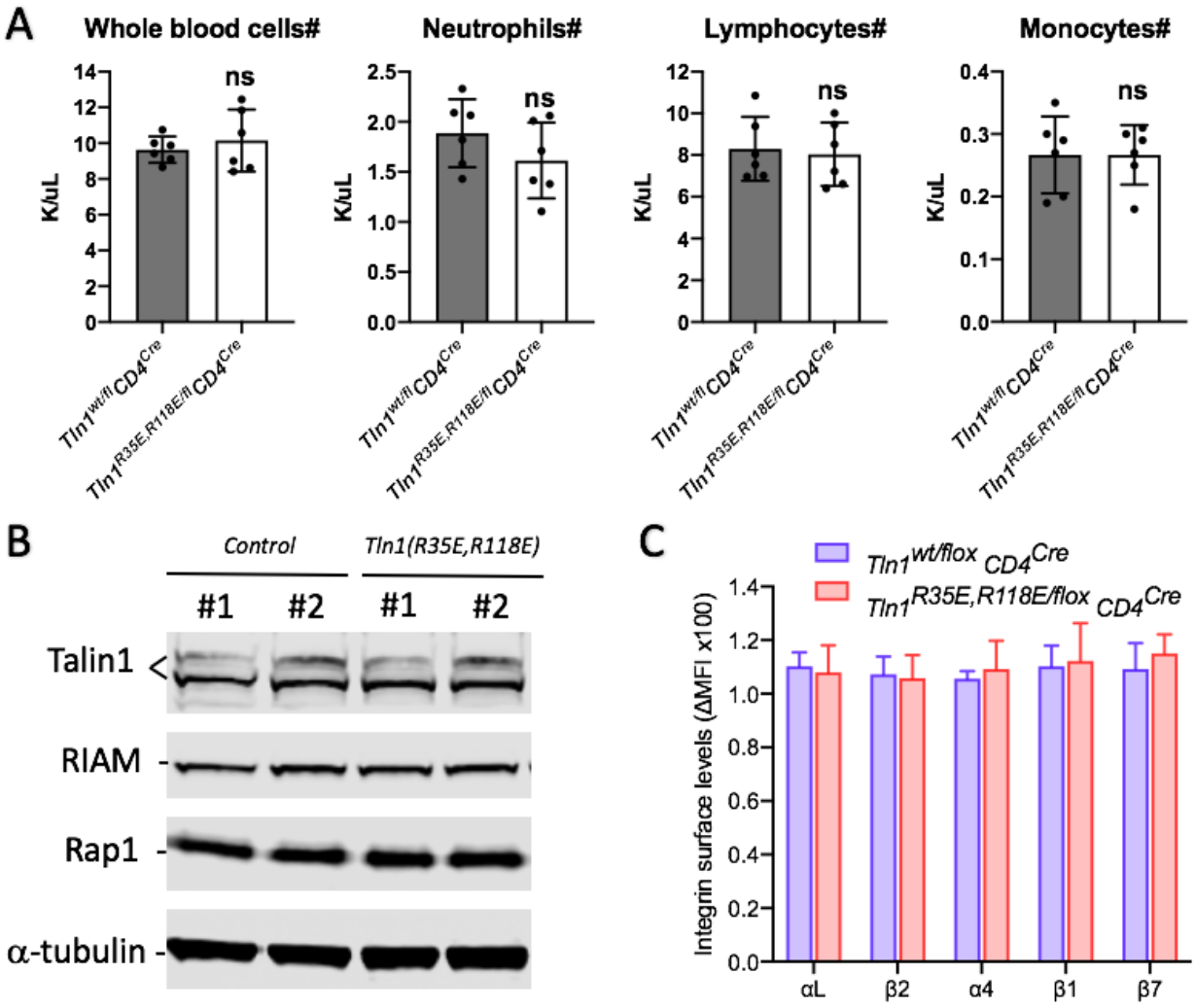

Figure 1. Talin1 and integrins expression on talin1(R35E, R118E) CD4+ T cells. (A) Complete blood count of *Tln1*$^{wt/flox}$ *CD4*^{Cre} and *Tln1*$^{R35E,R118E/flox}$ *CD4*^{Cre} mice. Data represent mean±SEM. Two-tailed t-test.. (B) Expression of talin1, RIAM and Rap1 in talin1(R35E,R118E) CD4+ T cells was assayed by Western blotting. Results are representative of 2 independent experiments, n = 2 mice each time. (C) Surface expression of integrin αL, β2, α4, β1 and β7 in *Tln1*$^{wt/flox}$ *CD4*^{Cre} and *Tln1*$^{R35E,R118E/flox}$ *CD4*^{Cre} mice. Mean fluorescence intensities (MFI) are plotted.

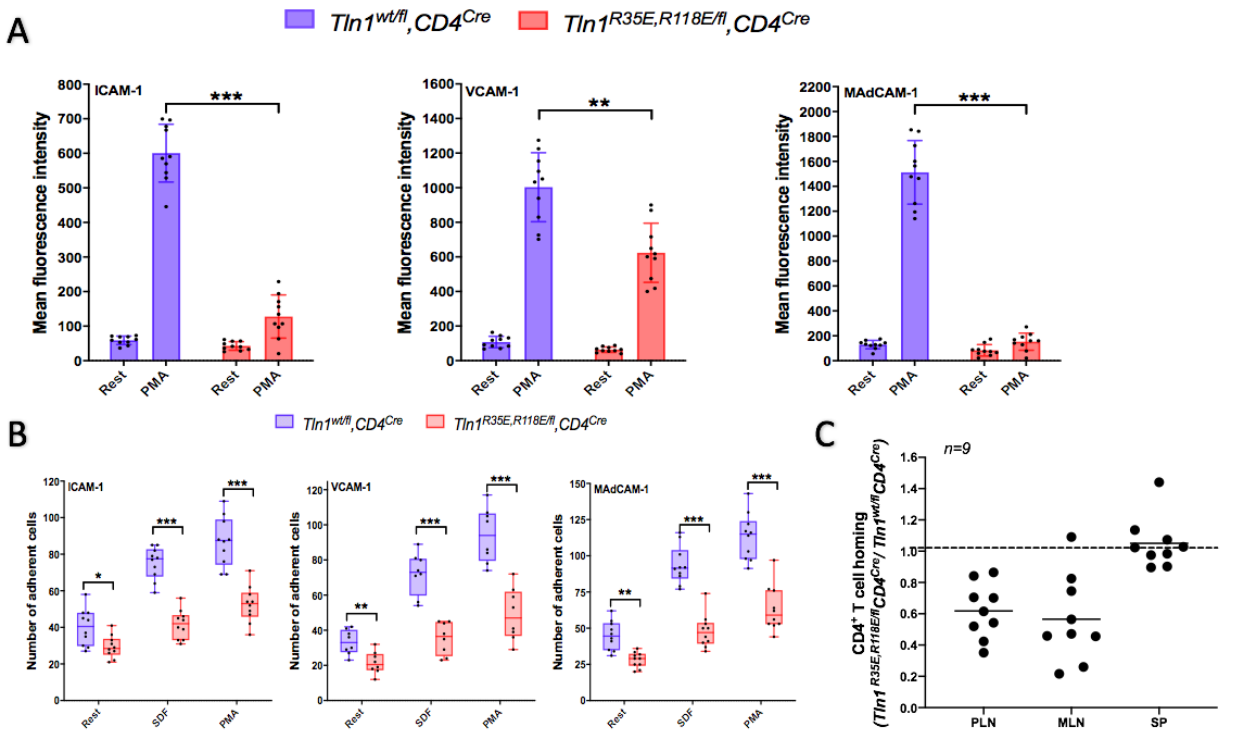

Figure 2. Talin1 binding to Rap1 plays an important role in maintaining integrin activation and functions of CD4+ T cells. (A) Binding of soluble ICAM-1, VCAM-1 or MAdCAM-1 to CD4+ T cells isolated from *Tln1*$^{wt/flox}$ *CD4*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *CD4*^{Cre} mice in the presence or absence of PMA (100 nM). Data represent mean±SEM (n=10). One-way ANOVA with Bonferroni post test. (B) Adhesion to ICAM-1, VCAM-1 or MAdCAM-1 of CD4+ T cells isolated from *Tln1*$^{wt/flox}$ *CD4*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *CD4*^{Cre} mice under no stimulation, SDF stimulation and PMA stimulation. Data represent mean±SEM (n=8). One-way ANOVA with Bonferroni post test. (C) In vivo competitive homing of CD4+ T cells to different lymphoid tissues. CD4+ T cells were isolated from either *Tln1*$^{wt/flox}$ *CD4*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *CD4*^{Cre} mice, differentially labeled and mixed prior to injection into C57BL/6 mice. CD4+ T cells homed into different lymphoid organs were analyzed by flow cytometry 3 hours after injection. The ratio of talin1(R35E,R118E) CD4+ T cells to WT CD4+ T cells from different lymphoid organs were shown (*n*=9). Data represent mean±SEM. MLN, mesenteric lymph node; PLN, peripheral lymph node; SP, spleen. One-way ANOVA with Bonferroni post test. *P<0.05; **P<0.01, ***P<0.001.

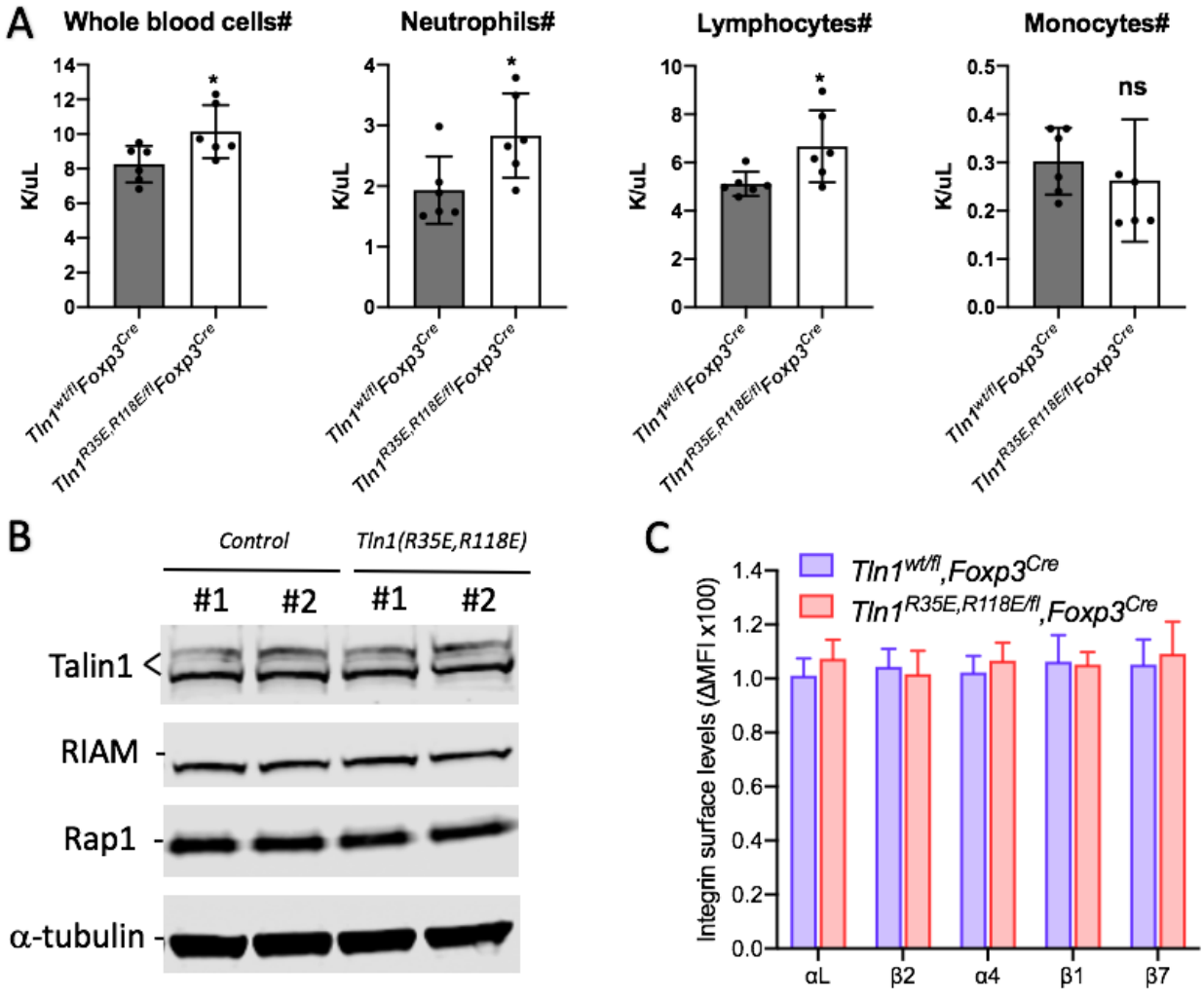

Figure 3. Talin1 and integrins expression on talin1(R35E, R118E) Treg cells. (A) Complete blood count of *Tln1*$^{wt/flox}$ *Foxp3*^{Cre} and *Tln1*$^{R35E,R118E/flox}$ *Foxp3*^{Cre} mice. Data represent mean±SEM. Two-tailed t-test.*P<0.05. (B) Expression of talin1, RIAM and Rap1 in talin1(R35E,R118E) Treg cells was assayed by Western blotting. Results are representative of 2 independent experiments, n = 2 mice each time. (C) Surface expression of integrin αL, β2, α4, β1 and β7 in *Tln1*$^{wt/flox}$ *Foxp3*^{Cre} and *Tln1*$^{R35E,R118E/flox}$ *Foxp3*^{Cre} mice. Mean fluorescence intensities (MFI) are plotted.

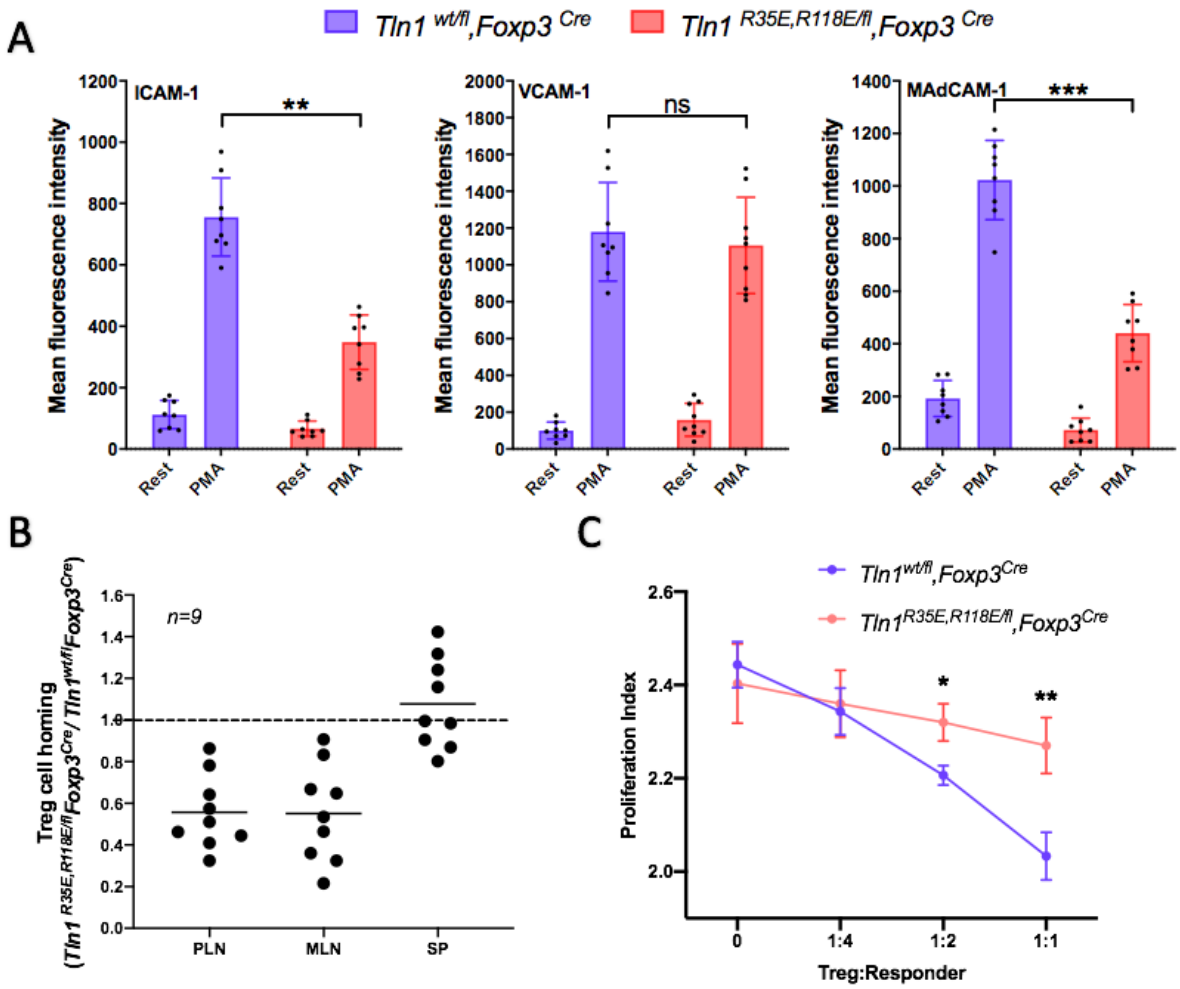

Figure 4. Direct binding between talin1 and Rap1 is required for maintaining Treg cells function. (A) Binding of soluble ICAM-1, VCAM-1 or MAdCAM-1 to Treg cells isolated from *Tln1*$^{wt/flox}$ *Foxp3*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *Foxp3*^{Cre} mice in the presence or absence of PMA (100 nM). Data represent mean±SEM (n=10). One-way ANOVA with Bonferroni post test. (B) In vivo competitive homing of Treg cells to different lymphoid tissues. Treg cells were isolated from either *Tln1*$^{wt/flox}$ *Foxp3*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *Foxp3*^{Cre} mice, differentially labeled and mixed prior to injection into C57BL/6 mice. Treg cells homed into different lymphoid organs were analyzed by flow cytometry 3 hours after injection. The ratio of talin1(R35E,R118E) Treg cells to WT Treg cells from different lymphoid organs is shown (*n*=9). Data represent mean±SEM. MLN, mesenteric lymph node; PLN, peripheral lymph node; SP, spleen. One-way ANOVA with Bonferroni post test.(C) Treg suppression function. Treg cells isolated from CD45.2 congenic *Tln1*$^{wt/flox}$ *Foxp3*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *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.

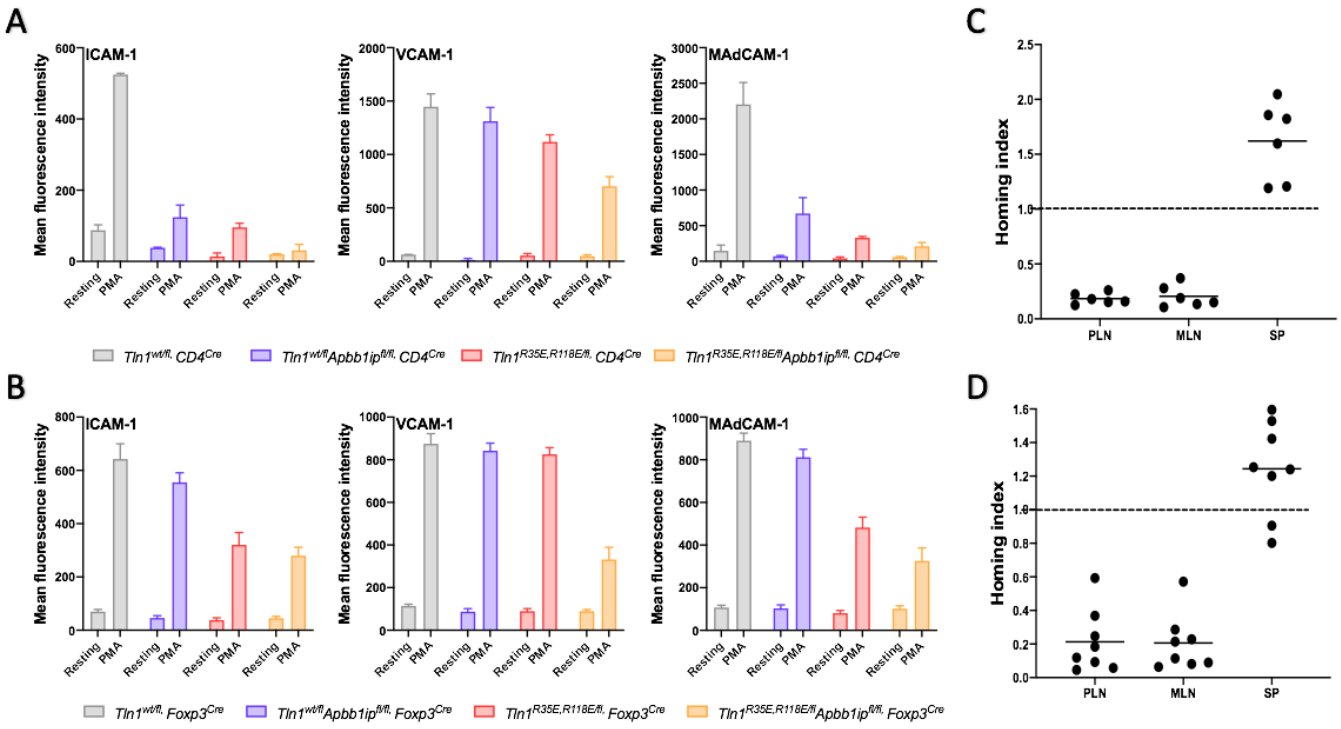

Figure 5. RIAM also plays a role in integrin activation in Treg cells and CD4+ T cells. (A, B) Binding of soluble ICAM-1, VCAM-1 or MAdCAM-1 to CD4+ T cells isolated from *Tln1*$^{wt/flox}$ *CD4*^{Cre}, *Tln1*$^{R35E,R118E/flox}$ *CD4*^{Cre}, *Tln1*$^{wt/flox}$ *Apbb1ip*$^{flox/flox}$ *CD4*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *Apbb1ip*$^{flox/flox}$ *CD4*^{Cre} mice (A) and to Treg cells isolated from *Tln1*$^{wt/flox}$ *Foxp3*^{Cre}, *Tln1*$^{R35E,R118E/flox}$ *Foxp3*^{Cre}, *Tln1*$^{wt/flox}$ *Apbb1ip*$^{flox/flox}$ *Foxp3*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *Apbb1ip*$^{flox/flox}$ *Foxp3*^{Cre} mice (B) in the presence or absence of PMA (100 nM). Data represent mean±SEM (n=10). One-way ANOVA with Bonferroni post test. (C, D) In vivo competitive homing of CD4+ T cells and Treg cells to different lymphoid tissues. CD4+ T cells were isolated from either *Tln1*$^{wt/flox}$ *Apbb1ip*$^{flox/flox}$ *CD4*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *Apbb1ip*$^{flox/flox}$ *CD4*^{Cre} mice (C); Treg cells were isolated from either *Tln1*$^{wt/flox}$ *Apbb1ip*$^{flox/flox}$ *Foxp3*^{Cre} or *Tln1*$^{R35E,R118E/flox}$ *Apbb1ip*$^{flox/flox}$ *Foxp3*^{Cre} mice (D), differentially labeled and mixed prior to injection into C57BL/6 mice. CD4+ T cells and Treg cells homed into different lymphoid organs were analyzed by flow cytometry 3 hours after injection. The ratio of talin1(R35E,R118E)/RIAM deficient CD4+ T cells to WT CD4+ T cells (C) and the ratio of talin1(R35E,R118E)/RIAM deficient Tregs to WT Tregs (D) from different lymphoid organs were shown (*n*=7).

Discussion

Integrin activation is essential for regulating the functions of many cell types, including matrix remodeling, cell migration and many cell-to-cell interactions (Calderwood, 2004). Among the studies on the signaling pathway of integrin activation, platelet integrins are the best-characterized system, and platelet integrin αIIbβ3 had been studied most extensively for talin-mediated integrin activation (Harburger, 2009), but few studies have focused on the integrin activation in lymphocytes. All of these signaling pathways require the Rap1 activation, which leads to talin1 binding to the integrin β cytoplasmic domain as the final step of integrin activation (Ebisun, 2010: Calderwood, 2015). Here we analyzed mice bearing talin1 mutations in two Rap1-binding sites talin1(R35E, R118E) and utilized these mice to explore the connection between Rap1 and talin1 in $CD4_+$ T cells and Treg cells. Previous studies have shown that the Rap1 binding sites on F0 and F1 domain of talin1 could account for integrin activation in platelets (Lagarrigue, 2018; Gingras, 2019). Our results demonstrated that disabling both Rap1-binding sites on talin1 induced severe reductions on integrin activation in both $CD4_+$ T cells and Treg cells and thereby caused dramatic defects in maintaining normal functions in those cells, suggesting that talin1 is a principle direct effector of Rap1 that mediates integrin activation in T lymphocytes.

The structure-guided point mutant (R35E) in F0 and (R118E) in F1 domain of talin1 only blocked the direct interaction between Rap1 and talin1 without interfering the binding between talin1 and other proteins (Lagarrigue, 2018; Gingras, 2019). Based on our studies, lack of Rap1 binding to talin1 did not completely block integrin activation in T lymphocytes (Fig. 2A and Fig. 4A). Previous studies have shown that RIAM mediates Rap1 dependent talin-induced integrin activation (Lagarrigue, 2016) and RIAM is essential for efficient lymphocyte adhesion and trafficking to secondary lymphoid organs (Lafuente, 2004; Klapproth, 2015). To investigate the

role of RIAM in integrin activation in talin1(R35E, R118E) lymphocytes, we deleted RIAM in both talin1(R35E, R118E) $CD4^+$ T cells or Treg cells. RIAM-deficient $CD4^+$ T cells showed a significant reduction in binding to ICAM-1 and MAdCAM-1 but no effect on cells binding to VCAM-1, which were similar to the results of talin1(R35E, R118E) $CD4^+$ T cells. However, talin1(R35E, R118E) $CD4^+$ T cells with RIAM-deficiency were even more profoundly affected exhibiting ~90% reduction in ICAM-1 and MAdCAM-1 binding and even a ~50% decrease in VCAM-1 binding (Fig. 5A). Although RIAM is dispensable for Treg cells binding to all the three integrin ligands (Fig. 5B), talin1(R35E, R118E) Treg cells with RIAM-deficiency exhibited slightly more decrease than talin1(R35E, R118E) Treg cells on binding to ICAM-1 and MAdCAM-1 and unexpected 50% reduction on binding to VCAM-1 (Fig. 5B). Consistent with the defect in integrin activation, the migration capacity of talin1(R35E, R118E) $CD4^+$ T cells and Treg cells with RIAM-deficiency were impaired and reduced to an even greater extent (Fig. 2C, Fig. 4B and Fig. 5C, D). These results indicate that both Rap1-talin1 directly binding and the classic Rap1/RIAM/talin1 axis existed to mediate integrin activation in T lymphocytes. However, it's not clear whether those two signaling pathways could compensate each other. Future studies could be done to uncover the results.

Disabling the Rap1 binding sites on talin1 impaired the activation of multiple classes of integrins on T lymphocytes, including β1, β2 and β7 integrins. However, the defects were various. Talin1(R35E, R118E) $CD4^+$ T cells or Treg cells binding to ICAM-1 and MAdCAM-1 exhibited more reductions than cells binding to VCAM-1. One possibility is that integrin β1 might have a higher affinity with talin1 to mediate cells binding to VCAM-1. Previous solution nuclear magnetic resonance (NMR) study that estimated the dissociation constants (Kd) of talin F3-β cytoplasmic tail to be in the μM range with the highest affinity for β1D, followed by β7 and β3

cytoplasmic tail (Anthis, 2009; Anthis, 2010; Anthis, 2009$_{2nd}$). However, surface plasmon resonance (SPR) estimated results were different (Calderwood, 2002; O'Connor, 2001; Bouaouina, 2008). The variable affinities probably arise from different technologies, different talin fragments, differences in talin and β cytoplasmic tail sequences, and sample preparations. Another possibility of why integrin β1 was affected less might be that integrin β1 employed an alternative pathway that can compensate for the Rap1-talin1-directly-binding deficiency. Lamellipodin (Lpd) is a member of the Mig-10/RIAM/lamellipodin (MRL) family of adaptor proteins that can bind to Ras family GTPases including Rap1 (Coló, 2012; Hansen, 2015). Lpd contains talin binding motifs and can form a complex with integrins and talin (Lagarrigue, 2015). Previous studies have shown Lpd plays an important role in cell migration (Chang, 2013). However, its role in Rap1-dependent integrin activation has remained obscure. Besides Rap1-talin1 directly binding and RIAM, integrin β1 might also utilize Lpd as the bridge of Rap1 and talin1 to mediate integrin activation. To prove this, experiments of deleting Lpd in talin1(R35E, R118E) CD4$_+$ T cells and Treg cells with or without RIAM-deficiency need to be done in the future.

The study of integrin activation in leukocytes has been subdivided into more and more groups, given the fact that different cell types utilized a unique mechanism for integrin activation. The same signaling pathway could play a different role in different cell types. Different signaling pathways could exist in one cell type. Our findings suggest the important role of Rap1 binding to talin1 F0 and F1 domains on integrin activation in CD4$_+$ T cells and Treg cells, which is similar to the role of Rap1-talin1 interaction in platelets. However, RIAM, as the Rap1 effector in T cells, also plays critical roles in integrin activation in CD4$_+$ T cells and Treg cells, which is dispensable for platelets integrin activation. Our future studies will continue working on finding other

alternative effectors of Rap1 and further exploring the mechanism of integrin activation employed differently by $CD4^+$ T cells and Treg cells.

Materials and Methods

Antibodies and reagents

The following Antibodies were from BioLegend: CD4 (GK1.5), 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 CD4 T cell isolation kit was from BioLegend. Liberase TL (Research Grade) and DNAseI 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 based on Sun's method (Sun, 2011).

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) and C57BL/6 (CD45.2) mice were from The Jackson Laboratory. *Abpp1ip*$^{flox/flox}$, *Tln1*$^{flox/flox}$, *CD4*^{Cre}, *Foxp3*$^{Cre\text{-}YFP}$ mice have been described previously (Su, 2015; Klapproth, 2015; Rubtsov, 2008). *Tln1*$^{wt/flox}$ and *Tln1*$^{R35E,R118E/flox}$ mice were obtained from Dr. Frederic Lagarrigue.

Mononuclear cells were isolated from mesenteric lymph node (MLN), peripheral lymph node (PLN) and spleen (SP) colonic as previously described (Berlin, 1995). Cell counting with

immunofluorescence cytometry was performed using an Accuri C6 Plus and FACSCalibur (BD Biosciences).

Blood counts

Peripheral blood was collected from the retro-orbital plexus and transferred to tubes containing K_+.EDTA. Cell counts were performed using a Hemavet 950FS Hematology System programmed with mouse-specific settings (Drew Scientific). All samples were tested in duplicate, and the mean for each animal was plotted.

Western blotting

Washed $CD4_+$ T cell and Treg cells were pelleted by centrifugation at 700 g for 5 min at room temperature and then lysed in Laemmli sample buffer. Lysates were subjected to a 4-20% gradient SDS-PAGE. Antibody directed against talin1 (8d4) was from Novus Biologicals. Antibody against α-tubulin (B-5-1-2) was from Sigma-Aldrich. The appropriate IRDye/Alexa Fluor-coupled secondary antibodies were from LI-COR. Nitrocellulose membranes were scanned using an Odyssey CLx infrared imaging system (LI-COR) and blots were processed using Image Studio Lite software (LI-COR).

Flow cytometry

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,

$5\times10_6$ cells were washed and resuspended in HBSS containing 0.1% BSA and 1 mM Ca_{2+}/Mg_{2+}, before incubation with integrin ligands for 30 min at room temperature 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) before Foxp3 staining.

Flow chamber assay

Coverslips were coated with three kinds of integrin ligands in coating buffer (PBS, 10 mM NaHCO3, pH 9.0) for 1 h and then blocked for 1 h with casein (1%) at room temperature. Coverslips were then sealed to polydimethylsiloxane chips. 1 dyn/cm wall shear stress was applied to the cells during the experiment by adjusting the pressure between the inlet well and the outlet reservoir. Cells were diluted to $5 \times 10_6$ cells/ml in HBSS (with 1 mM Ca_{2+}/ Mg_{2+}) and immediately perfused through the flow chamber. Bound cells were observed using a Keyence BZX-700 all-in-one fluorescence microscope, and adhesive interactions between the flowing cells and the coated ligands were assessed by manually tracking the motions of individual cells.

In vivo competitive lymphocyte homing

Competitive homing assay utilized high and low dose cell tracker was as described (Haeryfar, 2008). YFP_+ Treg cells were sorted with a FACSAria 2 (BD Biosciences) from *Tln1*$^{wt/flox}$ *Foxp3*^{Cre} and *Tln1*$^{R35E,R118E/flox}$ *Foxp3*^{Cre} mice and labeled with 1μM and 10 μM of eFluor670,

resulting in readily discriminated cell populations. Equal numbers (1×10^7) of differentially-labelled Tregs were mixed and then intravenously injected into C57BL/6 recipient mice. Lymphoid organs were harvested 3 hours after injection and isolated cells were analyzed by flow cytometry. The ratio of *Tln1*$^{R35E,R118E/flox}$ *Foxp3*^{Cre} (eFluor670^{high}) to *Tln1*$^{wt/flox}$ *Foxp3*^{Cre} Tregs (eFluor670^{low}) from different lymphoid organs are shown. For competitive homing assay of talin1(R35E, R118E) CD4$^+$ T cells, CD4$^+$ T cells were isolated by MojoSort™ mouse CD4 T cell isolation kit (BioLegend) from *Tln1*$^{wt/flox}$ *CD4*^{Cre} and *Tln1*$^{R35E,R118E/flox}$ *CD4*^{Cre} mice and labeled with 1μM of CFSE and eFluor670, respectively. MLN, mesenteric lymph node; PLN, peripheral lymph node; SP, spleen.

Treg cell suppression assays

CD4$^+$CD25$^-$ T cells (Responder cells) were isolated from spleens of C57BL/6 (CD45.1) WT mice with the CD4$^+$ T cell negative isolation kit (Biolegend); a biotin-conjugated anti-CD25 (PC61; BioLegend) Ab was included to deplete Treg cells. YFP$^+$ Treg cells were sorted with a FACSAria 2 (BD Biosciences). Responder cells were labelled with CFSE and cocultured with Treg cells (8:1, 4:1, 2:1 and 1:1 ratios) in the presence of 5 μg/ml immobilized anti-CD3 (2C11) and *IL2* for 4 days at 37°C. Proliferation index was calculated by FlowJo v10.

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 or Two-tailed t-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.

This thesis is part being prepared for submission for publication of the material. Sun, Hao; Lagarrigue, Frederic; Gingras, Alexandre; Lopez-Ramirez, Miguel; Tan, Boyang; Ginsberg, Mark H. The thesis author will be an author of the paper.

REFERENCES

Abram, C. L., & Lowell, C. A. (2009). The ins and outs of leukocyte integrin signaling. Annual review of immunology, 27, 339–362. https://doi.org/10.1146/annurev.immunol.021908.132554

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002.

Anthis, N. J., Haling, J. R., Oxley, C. L., Memo, M., Wegener, K. L., Lim, C. J., Ginsberg, M. H., & Campbell, I. D. (2009). Beta integrin tyrosine phosphorylation is a conserved mechanism for regulating talin-induced integrin activation. The Journal of biological chemistry, 284(52), 36700–36710. https://doi.org/10.1074/jbc.M109.061275

Anthis, N. J., Wegener, K. L., Critchley, D. R., & Campbell, I. D. (2010). Structural diversity in integrin/talin interactions. Structure (London, England : 1993), 18(12), 1654–1666. https://doi.org/10.1016/j.str.2010.09.018

Anthis, N. J., Wegener, K. L., Ye, F., Kim, C., Goult, B. T., Lowe, E. D., Vakonakis, I., Bate, N., Critchley, D. R., Ginsberg, M. H., & Campbell, I. D. (2009). The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. The EMBO journal, 28(22), 3623–3632. https://doi.org/10.1038/emboj.2009.287

Berlin, C., Bargatze, R. F., Campbell, J. J., von Andrian, U. H., Szabo, M. C., Hasslen, S. R., Nelson, R. D., Berg, E. L., Erlandsen, S. L., & Butcher, E. C. (1995). alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell, 80(3), 413–422. https://doi.org/10.1016/0092-8674(95)90491-3

Bertoni, A., Alabiso, O., Galetto, A. S., & Baldanzi, G. (2018). Integrins in T Cell Physiology. International journal of molecular sciences, 19(2), 485. https://doi.org/10.3390/ijms19020485

Bouaouina, M., Lad, Y., & Calderwood, D. A. (2008). The N-terminal domains of talin cooperate with the phosphotyrosine binding-like domain to activate beta1 and beta3 integrins. The Journal of biological chemistry, 283(10), 6118–6125. https://doi.org/10.1074/jbc.M709527200

Calderwood D. A. (2004). Integrin activation. Journal of cell science, 117(Pt 5), 657–666. https://doi.org/10.1242/jcs.01014

Calderwood D. A. (2015). The Rap1-RIAM pathway prefers β2 integrins. Blood, 126(25), 2658–2659. https://doi.org/10.1182/blood-2015-09-668962

Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujioka, Y., Liddington, R. C., & Ginsberg, M. H. (2002). The phosphotyrosine binding-like domain of talin activates

integrins. The Journal of biological chemistry, 277(24), 21749–21758. https://doi.org/10.1074/jbc.M111996200

Chang, Y. C., Zhang, H., Brennan, M. L., & Wu, J. (2013). Crystal structure of Lamellipodin implicates diverse functions in actin polymerization and Ras signaling. Protein & cell, 4(3), 211–219. https://doi.org/10.1007/s13238-013-2082-5

Coló, G. P., Lafuente, E. M., & Teixidó, J. (2012). The MRL proteins: adapting cell adhesion, migration and growth. European journal of cell biology, 91(11-12), 861–868. https://doi.org/10.1016/j.ejcb.2012.03.001

Ebisuno, Y., Katagiri, K., Katakai, T., Ueda, Y., Nemoto, T., Inada, H., Nabekura, J., Okada, T., Kannagi, R., Tanaka, T., Miyasaka, M., Hogg, N., & Kinashi, T. (2010). Rap1 controls lymphocyte adhesion cascade and interstitial migration within lymph nodes in RAPL-dependent and -independent manners. Blood, 115(4), 804–814. https://doi.org/10.1182/blood-2009-03-211979

Gingras, A. R., Lagarrigue, F., Cuevas, M. N., Valadez, A. J., Zorovich, M., McLaughlin, W., Lopez-Ramirez, M. A., Seban, N., Ley, K., Kiosses, W. B., & Ginsberg, M. H. (2019). Rap1 binding and a lipid-dependent helix in talin F1 domain promote integrin activation in tandem. The Journal of cell biology, 218(6), 1799–1809. https://doi.org/10.1083/jcb.201810061

Haeryfar, S. M., Hickman, H. D., Irvine, K. R., Tscharke, D. C., Bennink, J. R., & Yewdell, J. W. (2008). Terminal deoxynucleotidyl transferase establishes and broadens antiviral CD8+ T cell immunodominance hierarchies. Journal of immunology (Baltimore, Md. : 1950), 181(1), 649–659. https://doi.org/10.4049/jimmunol.181.1.649

Han, J., Lim, C. J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D. A., Puzon-McLaughlin, W., Lafuente, E. M., Boussiotis, V. A., Shattil, S. J., & Ginsberg, M. H. (2006). Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. Current biology : CB, 16(18), 1796–1806. https://doi.org/10.1016/j.cub.2006.08.035

Hansen, S. D., & Mullins, R. D. (2015). Lamellipodin promotes actin assembly by clustering Ena/VASP proteins and tethering them to actin filaments. eLife, 4, e06585. https://doi.org/10.7554/eLife.06585

Harburger, D. S., & Calderwood, D. A. (2009). Integrin signalling at a glance. Journal of cell science, 122(Pt 2), 159–163. https://doi.org/10.1242/jcs.018093

Hogg, N., Patzak, I., & Willenbrock, F. (2011). The insider's guide to leukocyte integrin signalling and function. Nature reviews. Immunology, 11(6), 416–426. https://doi.org/10.1038/nri2986

Humphries, J. D., Byron, A., & Humphries, M. J. (2006). Integrin ligands at a glance. Journal of cell science, 119(Pt 19), 3901–3903. https://doi.org/10.1242/jcs.03098

Janeway CA Jr, Travers P, Walport M. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001.

Kim, C., Ye, F., & Ginsberg, M. H. (2011). Regulation of integrin activation. Annual review of cell and developmental biology, 27, 321–345. https://doi.org/10.1146/annurev-cellbio-100109-104104

Kinashi T. (2007). Integrin regulation of lymphocyte trafficking: lessons from structural and signaling studies. Advances in immunology, 93, 185–227. https://doi.org/10.1016/S0065-2776(06)93005-3

Klann, Jane & Kim, Stephanie & Remedios, Kelly & He. (2018). Integrin Activation Controls Regulatory T Cell–Mediated Peripheral Tolerance. The Journal of Immunology. 200. ji1800112. 10.4049/jimmunol.1800112.

Klapproth, S., Sperandio, M., Pinheiro, E. M., Prünster, M., Soehnlein, O., Gertler, F. B., Fässler, R., & Moser, M. (2015). Loss of the Rap1 effector RIAM results in leukocyte adhesion deficiency due to impaired β2 integrin function in mice. Blood, 126(25), 2704–2712. https://doi.org/10.1182/blood-2015-05-647453

Klapproth, S., Sperandio, M., Pinheiro, E. M., Prünster, M., Soehnlein, O., Gertler, F. B., Fässler, R., & Moser, M. (2015). Loss of the Rap1 effector RIAM results in leukocyte adhesion deficiency due to impaired β2 integrin function in mice. Blood, 126(25), 2704–2712. https://doi.org/10.1182/blood-2015-05-647453

Lafuente, E. M., van Puijenbroek, A. A., Krause, M., Carman, C. V., Freeman, G. J., Berezovskaya, A., Constantine, E., Springer, T. A., Gertler, F. B., & Boussiotis, V. A. (2004). RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. Developmental cell, 7(4), 585–595. https://doi.org/10.1016/j.devcel.2004.07.021

Lagarrigue, F., Gingras, A. R., Paul, D. S., Valadez, A. J., Cuevas, M. N., Sun, H., Lopez-Ramirez, M. A., Goult, B. T., Shattil, S. J., Bergmeier, W., & Ginsberg, M. H. (2018). Rap1 binding to the talin 1 F0 domain makes a minimal contribution to murine platelet GPIIb-IIIa activation. Blood advances, 2(18), 2358–2368. https://doi.org/10.1182/bloodadvances.2018020487

Lagarrigue, F., Kim, C., & Ginsberg, M. H. (2016). The Rap1-RIAM-talin axis of integrin activation and blood cell function. Blood, 128(4), 479–487. https://doi.org/10.1182/blood-2015-12-638700

Lagarrigue, F., Vikas Anekal, P., Lee, H. S., Bachir, A. I., Ablack, J. N., Horwitz, A. F., & Ginsberg, M. H. (2015). A RIAM/lamellipodin-talin-integrin complex forms the tip of

sticky fingers that guide cell migration. Nature communications, 6, 8492. https://doi.org/10.1038/ncomms9492

Mor, A., Dustin, M.L. and Philips, M.R. (2007), Small GTPases and LFA-1 reciprocally modulate adhesion and signaling. Immunological Reviews, 218: 114-125. doi:10.1111/j.1600-065X.2007.00538.x

O'Connor, K. L., & Mercurio, A. M. (2001). Protein kinase A regulates Rac and is required for the growth factor-stimulated migration of carcinoma cells. The Journal of biological chemistry, 276(51), 47895–47900. https://doi.org/10.1074/jbc.M107235200

Reedquist, K. A., Ross, E., Koop, E. A., Wolthuis, R. M., Zwartkruis, F. J., van Kooyk, Y., Salmon, M., Buckley, C. D., & Bos, J. L. (2000). The small GTPase, Rap1, mediates CD31-induced integrin adhesion. The Journal of cell biology, 148(6), 1151–1158. https://doi.org/10.1083/jcb.148.6.1151

Rubtsov, Y. P., Rasmussen, J. P., Chi, E. Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W. R., Jr, Muller, W., & Rudensky, A. Y. (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity, 28(4), 546–558. https://doi.org/10.1016/j.immuni.2008.02.017

Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. Nat Rev Mol Cell Biol. 2010;11(4):288-300. doi:10.1038/nrm2871

Su, W., Wynne, J., Pinheiro, E. M., Strazza, M., Mor, A., Montenont, E., Berger, J., Paul, D. S., Bergmeier, W., Gertler, F. B., & Philips, M. R. (2015). Rap1 and its effector RIAM are required for lymphocyte trafficking. Blood, 126(25), 2695–2703. https://doi.org/10.1182/blood-2015-05-644104

Sun, H., Wu, Y., Qi, J., Pan, Y., Ge, G., & Chen, J. (2011). The CC' and DE loops in Ig domains 1 and 2 of MAdCAM-1 play different roles in MAdCAM-1 binding to low- and high-affinity integrin alpha4beta7. The Journal of biological chemistry, 286(14), 12086–12092. https://doi.org/10.1074/jbc.M110.208900

Vignali, D., Collison, L. & Workman, C. How regulatory T cells work. Nat Rev Immunol 8, 523–532 (2008). https://doi.org/10.1038/nri2343