UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of Rap1 Binding to Talin1 in Promoting Integrin Activation in T Lymphocytes

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

in

Biology

by

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Professor James Kadonaga

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The Thesis of Qinyi Du is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

I dedicate this thesis to the members of the Ginsberg Lab for their guidance and support.

I dedicate this thesis to my family for their endless love and support. I specially dedicate this thesis to my father who was the best father and friend to me. My love for him will never cease.

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ABSTRACT OF THE THESIS

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Professor Alexandre Gingras, Chair
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Integrins are essential transmembrane adhesion receptors that mediate cell-cell and cell-extracellular matrix adhesions and induce bidirectional signaling across the cell membrane to regulate cell functions in lymphocytes. Ras-related protein 1 (Rap1) is a small GTPase known to regulate the recruitment and tethering of Talin1 to the plasma membrane to initiate integrin activation. Previous studies have shown that Rap1 can bind to Talin1 F0 and F1 domains directly to regulate integrin activation in platelets. However, the function of such interaction remains unclear in integrin activation in T lymphocytes. In our study, we examined mice bearing point mutations in Talin1 F0 and F1 domains, which block Rap1 direct binding to Talin1 without

disturbing Talin1 expression, and found that the direct interaction between Rap1 and Talin1 is pivotal in both CD4+ T cells and regulatory T (Treg) cells integrin activations. Furthermore, by cross-breeding mice bearing F0F1 double mutations with other transgenic mice strains, we also tested whether the binding of Rap1 and Talin1 could compensate for known integrin activation pathways such as the Rap1-RIAM-Talin1 axis in T lymphocytes. We found that the direct interaction between Rap1 and Talin1 is redundant with the Rap1-RIAM/Lamellipodin-Talin1 pathways on T lymphocytes and that the overexpression of Rap1-GTP-interacting adaptor molecule (RIAM) could compensate for the loss of Rap1 and Talin1 direct binding in CD4+ T cells.

INTRODUCTION

Integrins are a superfamily of transmembrane receptors that mediate cell adhesion and induce bidirectional signaling through the cell membrane to regulate cell proliferation, migration and homeostasis. They are heterodimeric consisting of α and β chains which together form 24 different combinations with at least eighteen α subunits and eight β subunits in mammals (Takada, 2007). Integrins are extensively studied for their role in the adaptive immune response including mediating the thymic development, migration, homing and immune synapse formation of T lymphocytes (Bertoni, 2018). Three major types of integrins, β1-integrins (α4β1), β2-integrins (αLβ2), and β7-integrins (α4β7), are found in T lymphocytes. They perform functions by binding to ligands in the extracellular matrix, on the cell surface and soluble protein (Harjunpää, 2019). Particularly, the α4β1 integrin very late antigen 4 (VLA-4) binds to vascular cell adhesion molecule 1 (VCAM-1) to mediate lymphocytes trafficking to inflamed endothelium. Lymphocyte Peyer's patch HEV adhesion molecule 1 (LPAM-1), the α4β7 integrin, binds to mucosal addressin cell adhesion molecule 1 (MAdCAM-1) to mediate T lymphocytes trafficking to gut and gut-associated lymphoid tissues (Bertoni, 2018; Janssen, 1991; Zhi, 2014). In addition to their significance in T lymphocyte development and trafficking, integrins also play an important role in maintaining the suppressive mechanism of Treg cells to regulate effector T cells and preventing autoimmunity. Therefore, integrins are necessary to modulate the functions of CD4+ T and Treg cells (Klann, 2018).

Integrins often remain inactive in a bent-closed conformation with transmembrane domains closely associated and cytoplasmic tails clasped. They can be activated to reorient transmembrane tilt and open the integrin ligand-binding pocket via inside-out signaling (Sun, 2019). During the integrin activation in T lymphocytes, two notable proteins Talin1 and Rap1 are heavily involved.

Talin1 is a cytoskeletal protein (~270 kDa) containing a globular N-terminal head region and a rod domain. The head region includes a FERM (protein 4.1, ezrin, radixin, moesin) domain which is subdivided into F0, F1, F2 and F3 subdomains. The F3 subdomain interacts with the integrin β cytoplasmic tail to disrupt the transmembrane domain (TMD) interaction between α-β subunits and straighten integrins to the active form (Calderwood, 2013; Kim, 2012). Rap1, on the other hand, is a Ras-like small GTP-binding protein subgroup consisting of Rap1A and Rap1B. Previous studies have shown that Rap1 is critical for integrin activation by interacting with the Rap1 effectors RIAM, which recruits Talin1 into integrin cytoplasmic tail in lymphocytes (Klapproth, 2015; Lagarrigue, 2017; Su, 2015). Besides cooperating with effectors, Rap1 was also found to directly bind Talin1 F0 and F1 domains to activate integrins in platelets. Rap1 binds to the Talin1 F0 domain with low affinity and a point mutation (R35E) in the F0 domain could inhibit their interaction. It also binds to the Talin1 F1 domain with a greater affinity and a point mutation (R118E) could inhibit their interaction (Gingras, 2019; Lagarrigue, 2018; Lagarrigue, 2020). However, the possible role of such direct binding between Rap1 and Talin1 F0 and F1 domains remains obscure in lymphocytes integrin activation.

In our study, we were interested in the role of Rap1 and Talin1 direct binding in T lymphocyte integrin activation, especially in CD4+ T and Treg cells. Due to the embryonic lethality of Talin1 F0F1 double-domain mutation, we used the cre-loxP system to create Talin1 (R35E, R118E) mutant mice crossed with either $CD4^{Cre}$ or $Foxp3^{Cre}$ mice (Lagarrigue, 2020). We investigated the α4 integrins, α4β1 and α4β7, and discovered that the inhibition of Rap1 and Talin1 direct binding led to drastic declines in their activation in both CD4+ T and Treg cells compared to control mice. Previous studies have revealed that while the Rap1-RIAM-Talin1 integrin activation pathway is pivotal to CD4+ T and Treg cells, RIAM is dispensable in Treg cells. RIAM,

a member of the Mig-10/RIAM/Lamellipodin (MRL) protein family encoded by APBB1IP, can interact with both Talin and Rap1 to activate integrins (Lee, 2009). Our previous studies also found that lamellipodin (Lpd), another MRL protein and a RIAM paralogue encoded by Raph1, has significantly higher expression on Treg cells and compensates for the loss of RIAM in Treg cell integrin activation (Krause, 2004; Sun, 2021). Based on these results, we decided to further test whether RIAM and Lpd could compensate for Rap1 and Talin1 direct binding in CD4+ T and Treg cell integrin activation. We investigated mice bearing Talin1 (R35E, R118E), RIAM and Lpd mutations and observed more severely impaired integrin activations in CD4+ T and Treg cells. Moreover, we also applied puromycin murine stem cell viruses (pMSCV) encoding RIAM to $Tln1^{R35E,\ R118E/fl}\ CD4^{Cre}$ CD4+ T cells and observed that RIAM overexpression rescued impaired integrin activation in Talin1 (R35E, R118E) CD4+ T cells. In conclusion, the direct binding between Rap1 and Talin1 plays a critical role in integrin activation in CD4+ T and Treg cells, and it serves as an alternative integrin activation pathway in CD4+ T and Treg cells.

RESULTS

Direct binding between Rap1 and Talin1 F0 and F1 domains is critical for CD4+ T cell integrin activation.

Previous studies have shown that the double point mutations in Talin1 F0 (R35E) and F1 (R118E) domains block Rap1 and Talin1 direct binding without disturbing the Talin1 structure or its ability to interact with the integrin β cytoplasmic tail (Gingras, 2019; Lagarrigue, 2018). To investigate the effect of Rap1 and Talin1 direct binding in CD4+ T cell integrin activation, we obtained heterozygous *Tln1*$^{R35E,\ R118E/fl}$ and *Tln1*$^{wt/fl}$ mice by crossing *Tln1*$^{R35E,\ R118E/wt}$ and *Tln1*$^{fl/fl}$ mice. We then crossed *Tln1*$^{R35E,\ R118E/fl}$ and *Tln1*$^{wt/fl}$ mice with *CD4*^{Cre} mice to obtain *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre}, which bear Talin1 R35E and R118E double point mutations specifically in CD4+ T cells, and *Tln1*$^{wt/fl}$ *CD4*^{Cre} mice respectively. *Tln1*$^{wt/fl}$ *CD4*^{Cre} mice were used as the wildtype (WT) control to *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice throughout our study. The cell count numbers of the whole blood cell, neutrophil, lymphocyte and monocyte in peripheral blood collected from *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice were similar to their WT control (Fig. 1A). The protein expression levels of Rap1 and Talin1 in CD4+ T cells collected from *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice were similar to their WT control (Fig. 1B). The surface expression levels of α4, β1 and β7 integrin subunits in CD4+ T cells collected from *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice were similar to their WT control (Fig. 1C). Upon examining their background conditions compared to WT control, we concluded that *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice were viable and suitable for studying the direct binding of Rap1 and Talin1 in CD4+ T cell integrin activation.

We proceeded to test the effect of Rap1 and Talin1 direct binding in CD4+ T cell integrin activation by using soluble ligand binding assay. CD4+ T cells were isolated from *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice and WT control to analyze the binding between α4 integrins, α4β1 and α4β7,

and soluble ligands, VCAM-1 and MAdCAM-1. Phorbol 12-myristate 13-acetate (PMA) was added to stimulate the binding between integrins and soluble ligands. The integrin activation binding to both VCAM-1 and MAdCAM-1 in WT CD4+ T cells were strongly stimulated with PMA. In contrast, in $Tln1^{R35E,\ R118E/fl}$ $CD4^{Cre}$ CD4+ T cells, the integrin activation binding to VCAM-1 declined about 40% and binding to MAdCAM-1 declined about 80% with the stimulation of PMA (Fig. 1D). Therefore, it indicated that the activation of integrin α4β1 and α4β7 was impaired in Talin1 (R35E, R118E) CD4+ T cells, suggesting that the loss of Rap1 and Talin1 direct binding negatively affects CD4+ T cell integrin activation.

Rap1 and Talin1 F0 and F1 domains direct binding plays a significant role in Treg cell integrin activation.

Previous study has shown that integrins are pivotal in maintaining the suppressive functions of Treg cells (Klann, 2018). Therefore, we investigated the role of Rap1 and Talin1 direct binding in Treg cell integrin activation. We crossed $Tln1^{R35E,\ R118E/fl}$ and $Tln1^{wt/fl}$ mice with $Foxp3_{Cre}$ mice to obtain $Tln1^{R35E,\ R118E/fl}$ $Foxp3^{Cre}$, which bear R35E and R118E double point mutations specifically in Treg cells, and $Tln1^{wt/fl}$ $Foxp3^{Cre}$ mice respectively. $Tln1^{wt/fl}$ $Foxp3^{Cre}$ mice were used as the WT control to $Tln1^{R35E,\ R118E/fl}$ $Foxp3^{Cre}$ mice throughout our study. The cell count numbers of the whole blood cell, neutrophil and lymphocyte in peripheral blood collected from $Tln1^{R35E,\ R118E/fl}$ $Foxp3^{Cre}$ mice were slightly increased compared to their WT control (Fig. 2A). Such increases in neutrophil and lymphocyte numbers indicated a possible mild leukocytosis in $Tln1^{R35E,\ R118E/fl}$ $Foxp3^{Cre}$ mice. The protein expression levels of Rap1 and Talin1 in Treg cells collected from $Tln1^{R35E,\ R118E/fl}$ $Foxp3^{Cre}$ mice were similar to their WT control (Fig. 2B). The surface expression levels of α4, β1 and β7 integrin subunits in Treg cells

collected from *Tln1*$^{R35E,\ R118E/fl}$ *Foxp3*^{Cre} mice were similar to their WT control (Fig. 2C). Upon examining their background conditions compared to WT control, we concluded that *Tln1*$^{R35E,\ R118E/fl}$ *Foxp3*^{Cre} mice were viable and suitable for studying the direct binding of Rap1 and Talin1 in Treg cell integrin activation.

We proceeded to test the effect of Rap1 and Talin1 direct binding in Treg cell integrin activation by using soluble ligand binding assay. Treg cells were isolated from *Tln1*$^{R35E,\ R118E/fl}$ *Foxp3*^{Cre} mice and WT control to analyze the binding between α4 integrins, α4β1 and α4β7, and soluble ligands, VCAM-1 and MAdCAM-1. The integrin activation binding to both VCAM-1 and MAdCAM-1 in WT Treg cells were strongly stimulated with PMA. In contrast, in *Tln1*$^{R35E,\ R118E/fl}$ *Foxp3*^{Cre} Treg cells, the integrin activation binding to VCAM-1 showed no significant difference while binding to MAdCAM-1 declined about 60% with the stimulation of PMA (Fig. 2D). It indicated that the activation of integrin α4β7, but not α4β1, was impaired in Talin1 (R35E, R118E) Treg cells, suggesting that the loss of Rap1 and Talin1 direct binding negatively affects integrin activation in Treg cells.

Loss of Rap1 and Talin1 direct binding along with RIAM and Lpd deficiency inhibit CD4+ T and Treg cell integrin activation

Upon analyzing previously obtained data, we found that the inhibition of Rap1 and Talin1 direct binding does not fully block integrin activation in CD4+ T and Treg cells. Previous studies have shown that RIAM and Lpd, two Rap1 effectors, could interact with Rap1 and Talin1 to activate integrin in T lymphocytes (Rap1/RIAM/Talin signaling pathway) (Su, 2015; Sun, 2021). We wanted to examine whether this alternative pathway compensates for Rap1 and Talin1 direct binding in CD4+ T and Treg cells. We crossed *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} and *Tln1*$^{R35E,\ R118E/fl}$

Foxp3^{Cre} mice with *Apbb1ip*$^{fl/fl}$ mice to obtain *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *CD4*^{Cre}, which express Rap1-Talin1 binding and RIAM deficiency in CD4+ T cells, and *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Foxp3*^{Cre} mice, which express Rap1-Talin1 binding and RIAM deficiency in Treg cells, respectively. We then crossed *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *CD4*^{Cre} and *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Foxp3*^{Cre} mice with *Raph1*$^{fl/fl}$ mice to obtain *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Raph1*$^{fl/fl}$ *CD4*^{Cre}, which express Rap1-Talin1 binding, RIAM and Lpd deficiency in CD4+ T cells, and *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Raph1*$^{fl/fl}$ *Foxp3*^{Cre} mice, which express Rap1-Talin1 binding, RIAM and Lpd deficiency in Treg cells, respectively. Mice produced were viable and appropriate for further study.

We examined the effect of loss of Rap1-Talin1 binding, RIAM and Lpd in CD4+ T cells isolated from *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Raph1*$^{fl/fl}$ *CD4*^{Cre} mice. *Tln1*$^{wt/fl}$ *CD4*^{Cre} mice were used as the WT control. The integrin activation binding to both VCAM-1 and MAdCAM-1 in WT CD4+ T cells were stimulated with PMA. In contrast, in *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Raph1*$^{fl/fl}$ *CD4*^{Cre} CD4+ T cells, the integrin activation binding to VCAM-1 showed about 50% reduction while binding to MAdCAM-1 showed almost fully inhibition with the stimulation of PMA (Fig. 3A, B). It indicated that the activation of integrins α4β1 and α4β7 was further impaired in Talin1(R35E, R118E)/RIAM/Lpd-KO CD4+ T cells compared to *Tln1*$^{wt/fl}$ *CD4*^{Cre} CD4+ T cells, suggesting that Rap1 and Talin1 direct binding is redundant with known alternative integrin activation pathways performed by RIAM or Lpd in CD4+ T cells.

We then examined the effect of loss of Rap1-Talin1 binding, RIAM and Lpd in Treg cells isolated from *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Raph1*$^{fl/fl}$ *Foxp3*^{Cre} mice. *Tln1*$^{wt/fl}$ *Foxp3*^{Cre} mice were used as the WT control. *Tln1*$^{L325R/fl}$ *Foxp3*^{Cre} mice were used as another control which selectively inhibit Talin1 from activating integrins in Treg cells as previously described (Klann, 2018). The

integrin activation binding to both VCAM-1 and MAdCAM-1 in WT Treg cells were stimulated with PMA. In contrast, in *Tln1*$^{R35E,\ R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Raph1*$^{fl/fl}$ *Foxp3*^{Cre} Treg cells, the integrin activation binding to VCAM-1 showed about 50% reduction while binding to MAdCAM-1 showed almost fully inhibition with the stimulation of PMA compared to WT control. Both VCAM-1 and MAdCAM-1 binding results were similar to the binding decreases observed in Talin1 (L325R) Treg cells. (Fig. 3C, D). It indicated that the activation of integrins α4β1 and α4β7 was further impaired in Talin1 (R35E, R118E)/RIAM/Lpd-KO Treg cells, which were consistent with the integrin activation in Talin1 (L325R) Treg cells, suggesting Rap1-Talin1 direct binding and Rap1/RIAM/Talin pathway are the main integrin activation pathways in T cells.

RIAM overexpression compensates for loss of Rap1 and Talin1 direct binding in integrin activation in CD4+ T cells

To further confirm our results described above, we infected proliferating Talin1 (R35E, R118E) mutant CD4+ T cells with MSCV-IRES-GFP encoding RIAM and GFP or GFP alone and then analyzed activation on GFP+ cells by flow cytometry (Han, 2006; Lee, 2009). CD4+ T cells isolated from *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice were transduced with pMSCV-EGFP and pMSCV-RIAM-EGFP respectively. CD4+ T cells isolated from *Tln1*$^{wt/fl}$ *CD4*^{Cre} mice were transduced with pMSCV-EGFP as the WT control. The integrin activation binding to both VCAM-1 and MAdCAM-1 in WT CD4+ T cells were stimulated with PMA. *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} CD4+ T cells infected with pMSCV-EGFP binding to VCAM-1 showed about 50% reduction while their binding to MAdCAM-1 showed about 75% reduction with the stimulation of PMA compared with WT control respectively. In contrast, the binding of *Tln1*$^{R35E,\ R118E/fl}$

CD4^{Cre} CD4+ T cells infected with pMSCV-RIAM-EGFP to both VCAM-1 and MAdCAM-1 showed no significant difference with the stimulation of PMA compared to WT control (Fig. 4). It indicated that the overexpression of RIAM in Talin1 (R35E, R118E) CD4+ T cells rescued the drastic decrease in integrin activation caused by the loss of Rap1-Talin1 binding, suggesting that the Rap1/RIAM/Talin signaling pathway can compensate for the loss of Rap1 binding to Talin1.

FIGURES

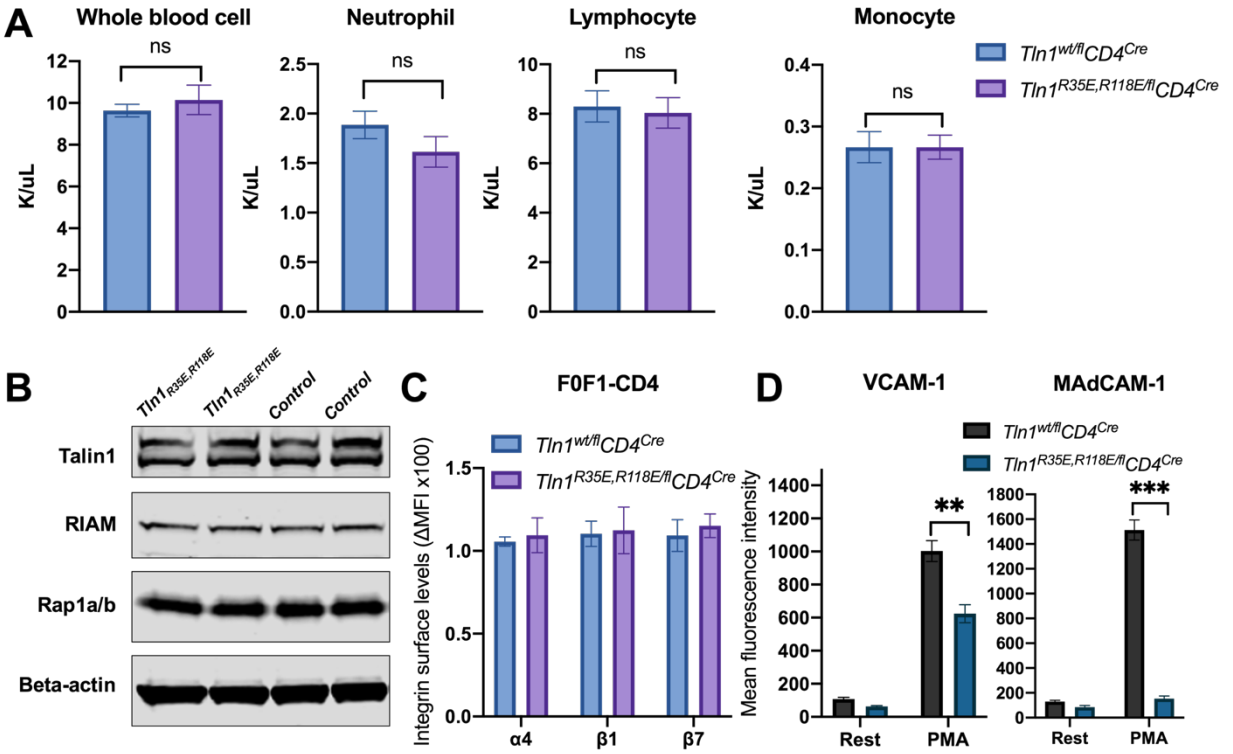

Figure 1. Rap1 and Talin1 direct binding is critical for integrin activation in CD4+ T cells. (A) Whole blood cell, neutrophil, lymphocyte and monocyte counts collected from *Tln1*$^{wt/fl}$ *CD4*^{Cre} control and *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice. Data represent mean ± SEM, analyzed by one-way ANOVA with Bonferroni post-test. (B) Expression of Talin1, RIAM and Rap1 in *Tln1*$^{R35E,\ R118E/fl}$ CD4+ T cells presented by Western blotting. n = 2. (C) Surface expression of integrin subunits α4, β1 and β7 in *Tln1*$^{wt/fl}$ *CD4*^{Cre} control and *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice. Mean fluorescence intensities are quantified. (D) Binding of soluble ligand VCAM-1 or MAdCAM-1 to CD4+ T cells isolated from *Tln1*$^{wt/fl}$ *CD4*^{Cre} control and *Tln1*$^{R35E,\ R118E/fl}$ *CD4*^{Cre} mice with or without PMA stimulation. Data represent mean ± SEM, analyzed by one-way ANOVA with Bonferroni post-test. n = 10. Not significant, $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

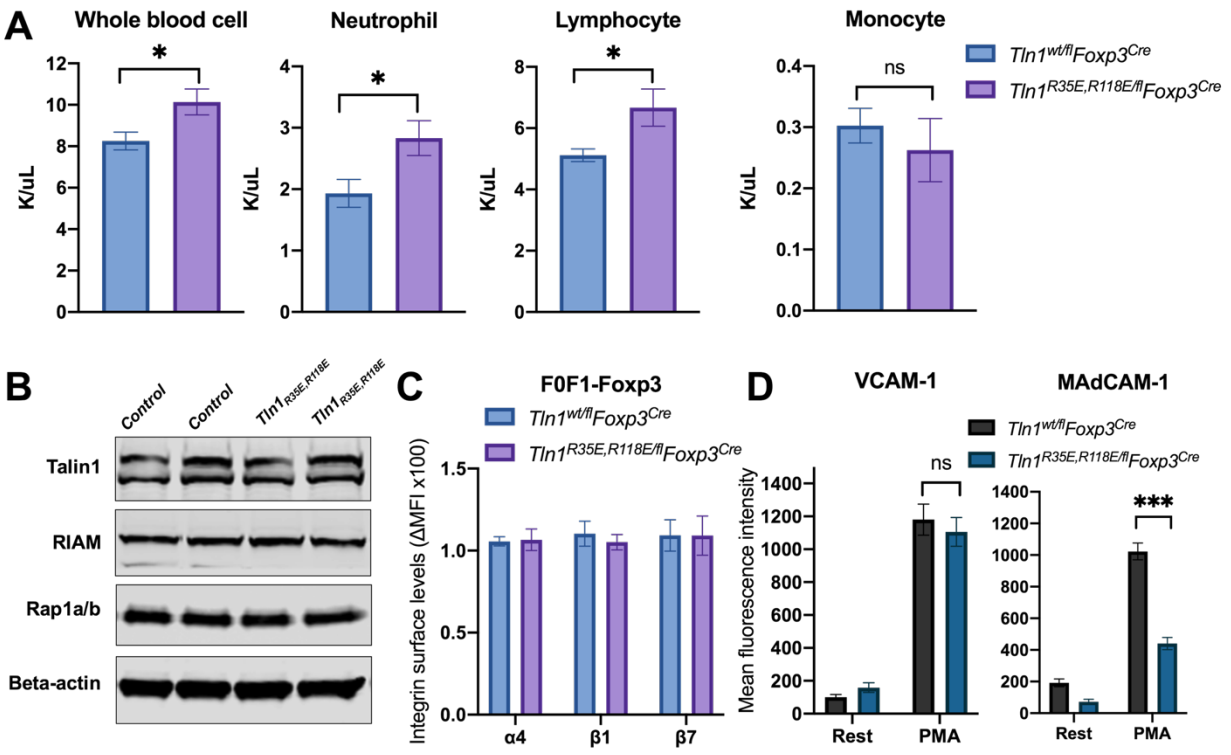

Figure 2. Rap1 and Talin1 direct binding is critical for integrin activation in Treg cells. (A) Whole blood cell, neutrophil, lymphocyte and monocyte counts collected from *Tln1*$^{wt/fl}$ *Foxp3*^{Cre} control and *Tln1*$^{R35E,\ R118E/fl}$ *Foxp3*^{Cre} mice. Data represent mean ± SEM, analyzed by one-way ANOVA with Bonferroni post-test. (B) Expression of Talin1, RIAM and Rap1 in *Tln1*$^{R35E,\ R118E/fl}$ Treg cells presented by Western blotting. n = 2. (C) Surface expression of integrin subunits α4, β1 and β7 in *Tln1*$^{wt/fl}$ *Foxp3*^{Cre} control and *Tln1*$^{R35E,\ R118E/fl}$ *Foxp3*^{Cre} mice. Mean fluorescence intensities are quantified. (D) Binding of soluble ligand VCAM-1 or MAdCAM-1 to Treg cells isolated from *Tln1*$^{wt/fl}$ *Foxp3*^{Cre} control and *Tln1*$^{R35E,\ R118E/fl}$ *Foxp3*^{Cre} mice with or without PMA stimulation. Data represent mean ± SEM, analyzed by one-way ANOVA with Bonferroni post-test. n = 10. Not significant, $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

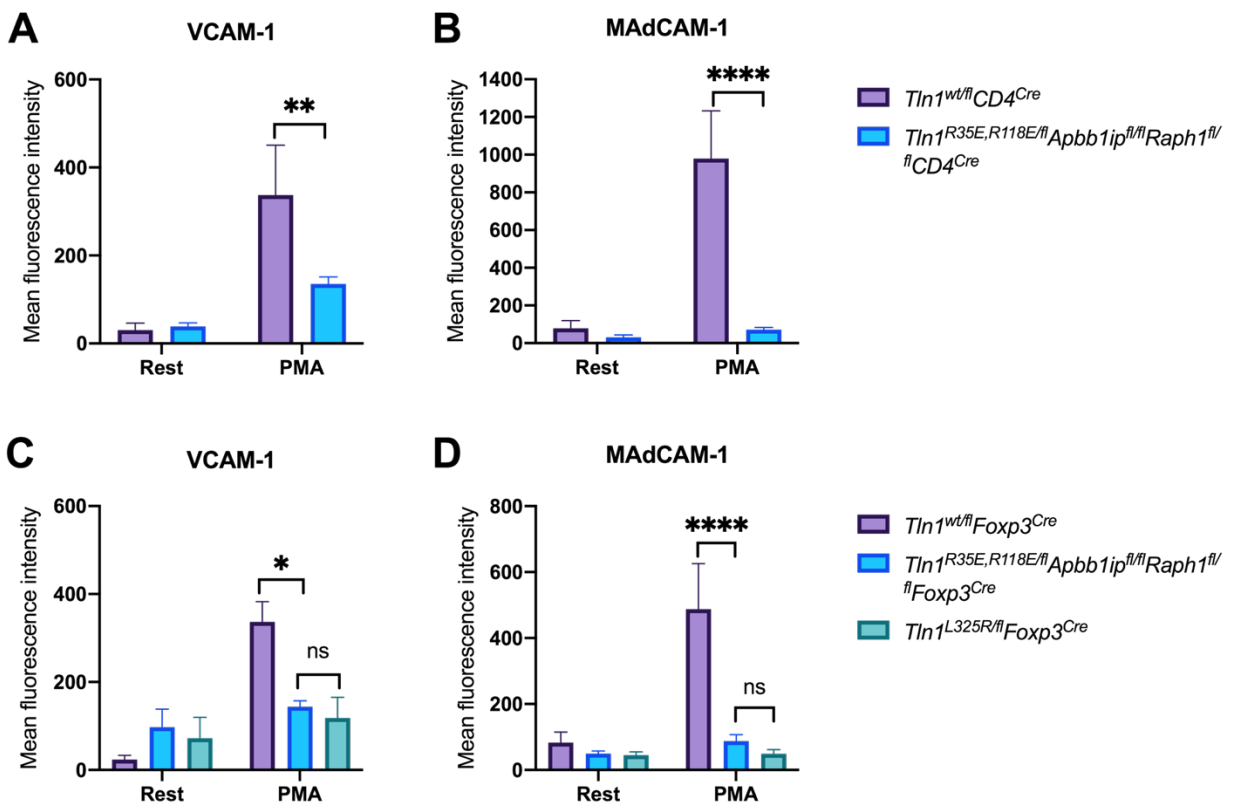

Figure 3. Loss of Rap1 and Talin1 direct binding along with RIAM and Lpd deficiency inhibit integrin activation in CD4+ T and Treg cells. (A and B) Binding of soluble ligand VCAM-1 or MAdCAM-1 to CD4+ T cells isolated from *Tln1*$^{wt/fl}$ *CD4*^{Cre} control and *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Raph1*$^{fl/fl}$ *CD4*^{Cre} mice with or without PMA stimulation. Data represent mean ± SEM, analyzed by two-way ANOVA with Bonferroni post-test. n = 9. (C and D) Binding of soluble ligand VCAM-1 or MAdCAM-1 to Treg cells isolated from *Tln1*$^{wt/fl}$ *Foxp3*^{Cre} control, *Tln1*$^{L325R/fl}$ *Foxp3*^{Cre} control and *Tln1*$^{R35E, R118E/fl}$ *Apbb1ip*$^{fl/fl}$ *Raph1*$^{fl/fl}$ *Foxp3*^{Cre} mice with or without PMA stimulation. Data represent mean ± SEM, analyzed by two-way ANOVA with Bonferroni post-test. n = 8. Not significant, p >0.05; *, 0.01< p <0.05; **, 0.001< p <0.01; ***, p <0.001; ****, p <0.0001.

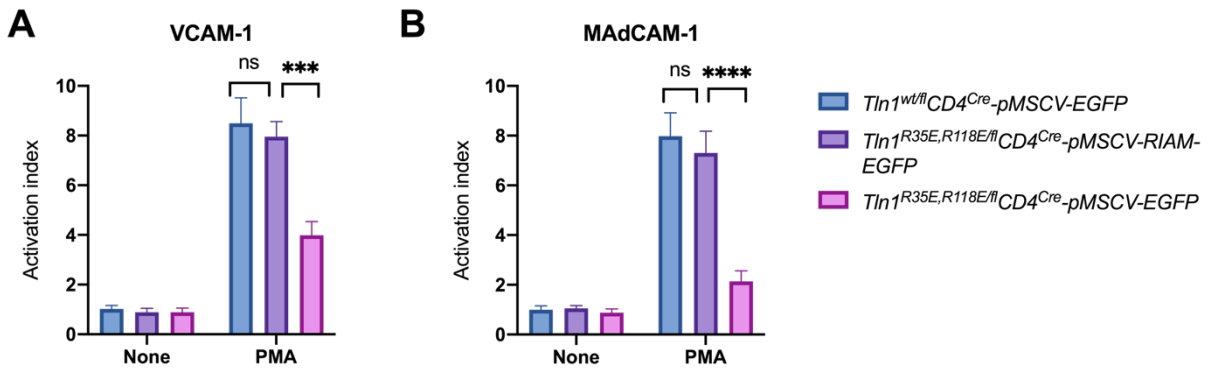

Figure 4. Overexpression of RIAM compensates for loss of Rap1 and Talin1 direct binding in *Tln1*$^{R35E, R118E/fl}$ *CD4*^{Cre} CD4+ T cell integrin activation. CD4+ T cells collected from *Tln1*$^{wt/fl}$ *CD4*^{Cre} control and then transduced with pMSCV-EGFP control; from *Tln1*$^{R35E, R118E/fl}$ *CD4*^{Cre} and then transduced with pMSCV-EGFP control; from *Tln1*$^{R35E, R118E/fl}$ *CD4*^{Cre} and then transduced with pMSCV-RIAM-EGFP. n = 4. (A and B) Binding of soluble ligand VCAM-1 or MAdCAM-1 to control or *Tln1*$^{R35E, R118E/fl}$ *CD4*^{Cre} CD4+ T cells collected and transduced as described above with or without PMA stimulation. Data represent mean ± SEM, analyzed by two-way ANOVA with Bonferroni post-test. n = 4. Not significant, $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

DISCUSSION

For the past decades, the integrin activation pathways in different cell types have been extensively studied. In T lymphocytes, the interactions between Rap1 effectors, such as RIAM and Lpd, and Talin1 have been identified and are essential for activating integrins and maintaining cell functions (Lagarrigue, 2016; Sun, 2021). Nevertheless, the possibility of new alternative integrin activation pathways still exists. Recent studies showed that the direct binding between Rap1 and Talin1 F0 and F1 domains is critical for integrin activation in platelets (Lagarrigue, 2020). By detecting soluble ligand binding to activated integrins, we found that Rap1 and Talin1 F0F1 domains direct binding also plays a vital role in T lymphocyte integrin activation. The direct inhibition of Rap1 binding to Talin1 F0F1 domains caused significant decreases in both α4β1 and α4β7 activation in CD4+ T cells (Fig. 1D). It also caused a significant decrease in α4β7 activation but not in α4β1 activation in Treg cells (Fig. 2D). Yet the failure to completely block integrin activation in both CD+ T and Treg cells suggested that there could be alternative mechanisms that compensate for Rap1-Talin1 direct binding. Here we further examined the impact of inhibiting Rap1-Talin1 direct binding along with deleting two known Rap1 effectors, RIAM and Lpd. Both α4β1 and α4β7 activations were severely impaired in CD+ T cells that the activation of α4β7 was almost fully inhibited. Both α4β1 and α4β7 activations showed drastic decreases in Treg cells that the reduction level of α4β1 activation was similar to its Talin1 (L325R) control (the inhibition of Talin1 and integrin β tail binding) while the almost fully inhibition of α4β7 activation was similar to its Talin1 (L325R) control as well (Fig. 3). Although the reduction levels of α4β1 activation in Talin1 (R35E, R118E) and Talin1 (R35E, R118E)/RIAM/Lpd-KO CD4+ T cells were comparable, it could be explained by previous studies which indicated that RIAM and Lpd were dispensable for α4β1 activation in

CD4+ T cells. Additionally, previous studies have also shown that α4β7 integrin activation was impaired in RIAM/Lpd-KO CD4+ T cells, while α4β1 and α4β7 integrin activations were impaired in RIAM/Lpd-KO Treg cells (Klapproth, 2015; Sun, 2020). We were able to collect sufficient CD4+ T cells with Rap1-Talin1 binding deficiency to test whether the overexpression of RIAM could compensate for the loss of Rap1-Talin1 binding. The defect CD4+ T cells exhibited normal activation of α4β1 and α4β7 compared to their WT counterparts after overexpressing RIAM (Fig. 4). We therefore concluded that the direct binding between Rap1 and Talin1 not only plays a vital role in α4 integrin activation but also serves as an alternative pathway to other known activation pathways in T lymphocytes. However, although there are three major types of integrins, αLβ2, α4β1 and α4β7, expressed on T lymphocytes, our study focused mainly on the relationship between Rap1 and Talin1 direct binding and the activation of α4 integrins by far (Bertoni, 2018). Further studies could be done to test whether Rap1 and Talin1 direct binding has a similar effect on the activation of αLβ2 in CD4+ T and Treg cells. By examining the activation of all three major T lymphocyte integrins, we could gain a better understanding of how significant Rap1 and Talin1 direct binding is in T cell integrin activation.

Furthermore, despite MAdCAM-1 binding to α4β7 was almost fully blocked in Rap1-Talin1 binding, RIAM and Lpd deficient CD4+ T and Treg cells, we still observed a reduced level of VCAM-1 binding to α4β1 which indicated the possibility of additional integrin activation pathways other than the ones we presented here. Some possible speculations could be that there are other unknown Rap1 effectors interacting with Talin, or there are pathways exclude the presence of Talin to activate VLA-4. One of the possible candidates which might be important to VLA-4 activation in T lymphocytes is regulator for cell adhesion and polarization enriched in lymphoid tissues (RAPL). RAPL is a 30 kDa Rap1-binding protein that could couple

Rap1 to integrin activation without involving Talin1 in lymphocytes. It was known to function as a downstream effector of Rap1 to induce cell polarization and clustering of LFA-1 (αLβ2) at the leading edge. RAPL could bind to activated Rap1 after T-cell receptor (TCR) or chemokine stimulation which brings RAPL closer to the cell membrane to directly bind the αL cytoplasmic domain of LFA-1 (Katagiri, 2003; Zhang, 2012). Previous studies have shown that RAPL-deficient T lymphocytes had impaired adhesion to VCAM-1 with the stimulation of chemokine ligand 21 (CCL21) despite having a normal surface expression of VLA-4. RAPL-deficient T lymphocytes also showed defective abilities of homing to peripheral lymph nodes and trafficking to spleens which require functioning LFA-1 and VLA-4 (Katagiri, 2004). The adhesion of a human T cell line to VCAM-1 through VLA-4 was inhibited by the deficiency of Rap1 and RAPL, but not RIAM (Parmo-Cabañas, 2007). Although their results indicated that RAPL is involved in α4β1 activation, the actual mechanism of how RAPL interacts with α4β1 remains obscure. Future studies could be done to determine how RAPL-involved pathway works to activate VLA-4 and whether it could compensate for Rap1 to Talin1 direct binding or Rap1 effector dependent pathways in T lymphocytes.

Overall, our study has demonstrated the importance of Rap1 and Talin1 direct binding to integrin activation in T lymphocytes, notably in CD4+ T and Treg cells. Integrin activation serves a pivotal role in maintaining T cell functions including thymic development, migration, homing, immune synapse formation and specifically the suppressive function of Treg cells. However, the loss of Rap1 and Talin1 direct binding caused by Talin1 (R35E, R118E) mutation might not have similar impacts on other cell types. For instance, in platelets with Talin1 (R35E, R118E) mutation, the loss of Rap1 and Talin1 interaction led to decreases in integrin activation, aggregation and hemostatic plug formation, while cannot fully account for defective

thrombocytopoiesis, granule secretion and Phosphatidylserine (PS) exposure (Lagarrigue, 2020). In another study focusing on Talin1 F0 (K15, R30 and R35) mutation, the partial inhibition of Rap1 and Talin1 interaction reduced integrin activation, adhesion efficiency, extravasation and phagocytosis in neutrophils; it also reduced integrin activation but did not influence adhesion or spreading in macrophages (Bromberger 2018). We can hereby get a glimpse of how Rap1-Talin1 direct signaling pathway could be cell specific by activating varied integrins and supporting different cell functions depending on cell types. In the future, we can dive deeper into understanding the cell specific role of Rap1 and Talin1 binding pathway in integrin activation and other subsequent cell functions.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies were from BioLegend: CD3 (17A2, 2C11), CD4 (GK1.5), CD28 (37.51), CD29 (HMβ1−1), CD49d (R1-2), β7 (FIB504), IL2 (TCGF) and Foxp3 (MF-14). Secondary Alexa Fluor-labelled antibodies were from Jackson ImmunoResearch. eFluor 670 was from Biolegend. PMA was from Sigma-Aldrich. MojoSort mouse CD4 T cell isolation kit was from BioLegend. Liberase TL (Research Grade) and DNAseI were from Roche. Recombinant mouse VCAM-1-Fc was from R&D Systems. Recombinant mouse MAdCAM-1-Fc was purified by ProteinA beads as previously described (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 following federal regulations as well as institutional guidelines and regulations on animal studies. All mice were housed in specific pathogen-free conditions. *Abpp1ip*$^{fl/fl}$, *Tln1*$^{fl/fl}$, *Raph1*$^{fl/fl}$, *CD4*^{Cre} and *Foxp3*$^{Cre\text{-}YFP}$ mice have been described previously (Klapproth, 2015; Miller, 2016; Rubtsov, 2008; Su, 2015). *Tln1*$^{wt/fl}$ and *Tln1*$^{R35E,\ R118E/fl}$ mice were obtained from Dr. Frederic Lagarrigue. Mononuclear cells were isolated from spleen (SP) as previously described (Berlin, 1995). Cell counting with immunofluorescence cytometry was performed using an Accuri C6 Plus and FACSCalibur (BD Biosciences).

Blood count

Peripheral blood was collected from the retro-orbital plexus and transferred to tubes containing K^+ EDTA. Cell counting was performed by a Hemavet 950FS Hematology System programmed with mouse-specific settings (Drew Scientific).

Western blotting

$CD4^+$ T and Treg cells isolated from mouse tissues were washed and 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 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 stained with conjugated antibody for 30 min at 4°C. Cells were washed twice before flow cytometry analysis using an 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+}, before incubation with integrin ligands for 30 min at 37°C with or without 100 nM PMA. Cells were then incubated with AlexFluor647-conjugated anti-human IgG (1:200) for 30 min at 4°C.

Cell transfection

HEK293T cells were seeded 70-90% confluent at transfection on plate and incubated for 4 days at 37°C before retroviral supernatant collection. Lipofectamine 3000 transfection kit was from Invitrogen. Retroviral DNA and P300 reagent were pre-mixed and incubated in Reduced Serum Medium without serum (Opti-MEM) for 5 min at room temperature, before mixed and incubated with Lipofectamine 3000 reagent for 20 min at room temperature. DNA-reagents complexes were then added to plate seeded with HEK293T cells.

Cell transduction

Cells isolated using Mojo system were washed and resuspended in RPMI medium at 10^6 cells/mL and incubated at 37°C and 5% CO_2 on plate overnight, after added with CD3, CD28 antibodies and IL-2. Retroviral supernatant was harvested from transfected HEK293T cells and filtered to remove cellular debris. RetroNectin reagent was from Takara. RetroNectin was coated on plate with PBS and incubated for 2 h at room temperature. The RectroNectin-coated plate was washed and ready for use, after incubated in PBS with 2% BSA for 30 min at room temperature. Spinoculation was performed at 2000g and 32°C for 2 h after adding retroviral supernatant on plate. Cells were added on plate in fresh RPMI with IL-2 and incubated at 37°C and 5% CO_2 overnight after removing media. Fresh or thawed retroviral supernatant was added carefully without disturbing plate bottom after aspirating old media. Spinoculation was performed at 2000g and 32°C for 1 h. Cells were incubated at 37°C and 5% CO_2 for 3 days with constant addition of fresh media with IL-2 before analyzed by soluble ligand binding assay.

Statistical Analysis

Statistical analysis was performed using PRISM software (version 8.00, 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 captions. The resulting *P* values are indicated as follows: NS: not significant, $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

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