Distinct integrin activation pathways for effector and regulatory T cell trafficking and function

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Integrin activation mediates lymphocyte trafficking and immune functions. Conventional T cell (Tconv cell) integrin activation requires Rap1-interacting adaptor molecule (RIAM). Here, we report that Apbβ1p-/- (RIAM-null) mice are protected from spontaneous colitis due to IL-10 deficiency, a model of inflammatory bowel disease (IBD). Protection is ascribable to reduced accumulation and homing of Tconv cells in gut-associated lymphoid tissue (GALT). Surprisingly, there are abundant RIAM-null regulatory T cells (T reg cells) in the GALT. RIAM-null T reg cells exhibit normal homing to GALT and lymph nodes due to preserved activation of integrins αLβ2, αβ1, and αβ7. Similar to Tconv cells, T reg cell integrin activation and immune function require Rap1; however, lamellipodin (Raph1), a RIAM paralogue, compensates for RIAM deficiency. Thus, in contrast to Tconv cells, RIAM is dispensable for T reg cell integrin activation and suppressive function. In consequence, inhibition of RIAM can inhibit spontaneous Tconv cell–mediated autoimmune colitis while preserving T reg cell trafficking and function.

Introduction

The recruitment of leukocytes from the circulation to the gut mucosa plays a critical role in inflammatory bowel disease (IBD; Abraham and Cho, 2009; Adams and Eksteen, 2006; Braus and Elliott, 2009; Economou and Pappas, 2008; Eksteen et al., 2008; Kaser et al., 2010; Khor et al., 2011; Villablanca et al., 2011). Aberrant infiltration of mononuclear phagocytes, neutrophils, and inflammatory lymphocytes is observed in the colonic lamina propia of IBD patients (Caradonna et al., 2000; Smith et al., 2005). Adhesion molecules that mediate gut homing of leukocytes, such as integrin αβ7, have emerged as targets for IBD therapy (Berlin et al., 1995; Cominelli, 2013; Cox et al., 2010).

Leukocyte trafficking to gut and gut-associated lymphoid tissue (GALT) is precisely governed by integrins binding to their ligands, involving integrin αLβ2/intercellular adhesion molecule-1 (ICAM-1, αβ1/vascular cell adhesion molecule-1 (VCAM-1), and αβ7/mucosal addressin cell adhesion molecule-1 (MadCAM-1; Nourshargh et al., 2010; Sun et al., 2014). Integrin αβ7 is a proven therapeutic target in treating IBD (Feagan et al., 2013; Sandborn et al., 2013), but agents that completely block its functions are limited by potential redundancies with other integrins or by side effects (Feagan et al., 2013; Rutgeerts et al., 2013; Sun et al., 2020b). Indeed, in the murine IL-10-deficient spontaneous IBD model, genetic abrogation or antibody blockade of αβ7-mediated homing exacerbated colitis (Sun et al., 2020b). We ascribed this exacerbation to the inhibition of homing of regulatory T cells (T reg cells) to GALT, because T reg cells play an essential role in suppressing intestinal inflammation (Rubtsov et al., 2008) and may therefore serve to limit IBD (Desreumaux et al., 2012; Li et al., 2010; Maul et al., 2005; Neurath, 2014; Wong et al., 2016).

Homing of lymphocytes to GALT requires integrin activation (Sun et al., 2018), a process whereby talin binding to the integrin β cytoplasmic domain causes a marked increase in the integrin’s affinity (Kim et al., 2011). The binding of talin is regulated by the activation of Rap1, leading to its association with talin via the Rap1-interacting adaptor molecule (RIAM) adaptor (Han et al., 2006; Lagarrigue et al., 2015; Lee et al., 2009). This ternary Rap1–RIAM–talin complex can then associate with integrins in the T cell plasma membrane to mediate integrin activation and resulting homing and the formation of immunological synapses (Lagarrigue et al., 2017; Su et al., 2015). Thus, blockade of RIAM could suppress T cell–mediated autoimmune diseases, an idea supported by the reduction in diabetes induced by adoptive transfer of RIAM-null OT1 T cells (Lagarrigue et al., 2017). However, little is known about the role of RIAM in a spontaneous autoimmune disease. This question is of particular interest in the IL-10-deficient IBD model given the dramatic exacerbation of this model by blockade of integrin αβ7 functions (Sun et al., 2020a; Sun et al., 2020b).

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Here, we show, in sharp contrast to the exacerbation of IL-10–deficient colitis by loss of integrin α4β7 function, genetic deletion of RIAM dramatically improves the colitis in this spontaneous autoimmune disease. The amelioration of colitis is due to reduced homing of RIAM-null Tconv cells to GALT. Surprisingly, although Rapi is required for T reg cell function and RIAM is expressed in T reg cells, RIAM is dispensable for T reg cell homing to GALT. Furthermore, T reg cell function requires intact integrin activation (Klapproth et al., 2015); however, RIAM-null T reg cells exhibit intact activation of integrins α4β1, α4β7, and αLβ2 and normal suppressive function. Increased expression of lamellipodin (Lpd), a RIAM parologue, accounts for the lack of RIAM requirement in T reg cells. These data demonstrate that T reg cells have an integrin activation pathway distinct from Tconv cells and identify RIAM as a focus for approaches to suppress the homing and function of Tconv cell cells while sparing T reg cells, thereby shifting the immunological balance in IBD and potentially other autoimmune disorders.

Results
RIAM deficiency protects IL-10–null mice from spontaneous colitis
To investigate the role of RIAM in the development of spontaneous autoimmune disease, we used the IL-10–deficient mouse strain (B6.129P2-Il10<sup>tm1<sup>mgm<sup>kn</sup></sup>) that develops chronic colitis resembling IBD in humans (Kühn et al., 1993; Zhang et al., 2014b). IL-10–deficient mice were crossed with RIAM (Apbb1ip)–deficient mice, and the phenotypes of Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> and Apbb1ip<sup>+/−</sup>Il10<sup>−/−</sup> mice were compared (Fig. 1).

As expected, Apbb1ip<sup>+/−</sup>Il10<sup>−/−</sup> mice spontaneously developed diarrhea beginning at the age of 40–50 d under specific pathogen–free conditions. Chronic colitis became more evident in older Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice, as evidenced by diarrhea and bleeding (Fig. 1 D and E), with a significant loss of body weight (Fig. 1 A). More than 95% of these mice subsequently developed rectal prolapse (Fig. 1 B), and half of them died by 100 d of age (Fig. 1 C). In contrast, only approximately one quarter of the Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice developed rectal prolapse (Fig. 1 B), and their body weight increased at the same rate as Il10<sup>−/−</sup> mice (Fig. 1 A); strikingly, only 1 out of 23 Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice died by 100 d (Fig. 1 C). Histologically, IL-10 deficiency led to a severe colitis in Apbb1ip<sup>+/+</sup> mice, with almost complete loss of crypts, dense infiltrates of leukocytes in both mucosa and submucosa, and thickening of the bowel wall (Fig. 1 E). By contrast, the infiltrates in the Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice were much less prominent, and less tissue damage was observed (Fig. 1 E). Blinded histological scoring for inflammatory cell infiltrates and epithelial damage confirmed a reduction in the severity of colitis in Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice compared with Apbb1ip<sup>+/+</sup> controls (Fig. 1 F). Along with increased inflammatory cell infiltration in Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice, colonic expression of proinflammatory cytokines (IL-1β, TNF-α, IL-6, IFN-γ, and IL-17A) was significantly reduced in Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice (Fig. 1 G).

RIAM is dispensable for T reg cell, but not Tconv cell, recruitment to the colon
Since RIAM plays an important role in leukocyte migration (Su et al., 2015), we enumerated CD4<sup>+</sup> T cells in the colonic lamina propria from Apbb1ip<sup>+/−</sup>Il10<sup>−/−</sup> and Apbb1ip<sup>+/−</sup>Il10<sup>−/−</sup> mice. Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice exhibited a marked reduction of Tconv cells in colonic lamina propria relative to Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice; in sharp contrast, there was no reduction in colonic T reg cells in Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice (Fig. 2 A). Accordingly, T reg cells represented a markedly increased fraction of CD4<sup>+</sup> colonic lamina propria T cells in Apbb1ip<sup>−/−</sup>Il10<sup>−/−</sup> mice (Fig. 2 A). Moreover, the fraction of splenic T reg cells in CD4<sup>+</sup> T cells was unaffected by the lack of RIAM (Fig. 2 B). These findings suggest that, in contrast to Tconv cells, RIAM is dispensable for the presence of T reg cells in the colon in IL-10–deficient mice.

We used adoptive transfer (Ostanin et al., 2009; Song-Zhao and Maloy, 2014) to assess whether the beneficial effects of loss of RIAM function on colitis in IL-10–deficient mice might be in part due to reduced Tconv cell function. CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>high</sup> T cells (Tconv cells) from Apbb1ip<sup>+/−</sup> or Apbb1ip<sup>+/−</sup> mice were infused into Rag1<sup>−/−</sup> recipient mice. Rag1<sup>−/−</sup> mice injected with Apbb1ip<sup>+/+</sup> Tconv cells manifested a dramatic loss in body weight after 20–30 d, and half of the mice died by 90 d (Fig. 2, C and D). In contrast, Rag1<sup>−/−</sup> mice injected with Apbb1ip<sup>−/−</sup> Tconv cells maintained body weight, and all mice survived (Fig. 2, C and D). In addition, the expression of colonic IL-1β, TNF-α, IL-6, IFN-γ, and IL-17A were also increased in Rag1<sup>−/−</sup> mice injected with Apbb1ip<sup>−/−</sup>, but not Apbb1ip<sup>+/−</sup>, Tconv cells (Fig. 2 E).

We next tested the role of RIAM in CD4<sup>+</sup> T cell migration using a competitive homing assay. Consistent with previous studies (Klapproth et al., 2015; Lagarrigue et al., 2017; Su et al., 2015), bulk RIAM–deficient CD4<sup>+</sup> T cells showed reduced homing to mesenteric LN (MLNs), Peyer’s patches (PPs), and peripheral LNs (PLNs; Fig. 2 F). Thus, defective migration of RIAM-deficient CD4<sup>+</sup> Tconv cells to the colon can account for their failure to induce colitis in this adoptive T cell transfer model.

RIAM-deficient T reg cells prevent colitis
We next assessed the effect of RIAM deficiency on the ability of T reg cells to prevent intestinal inflammation in the adoptive T cell transfer model (Song-Zhao and Maloy, 2014). Coinjection of WT T reg cells (Apbb1ip<sup>+/+</sup>) and Tconv cells into Rag1<sup>−/−</sup> recipient mice, as expected, prevented colitis, as judged by maintenance of body weight (Fig. 3 A) and prevention of death (Fig. 3 B). Rag1<sup>−/−</sup> mice coinjected with Apbb1ip<sup>−/−</sup> T reg cells and WT Tconv cells also appeared healthy and continued to gain weight, similarly to those coinjected with Apbb1ip<sup>+/+</sup> T reg cells (Fig. 3, A and B). At 90 d, the reduced disease activity in the mice receiving coadministered RIAM–deficient T reg cells was similar to that of those receiving WT T reg cells (Fig. 3 C). The equivalent protection provided by RIAM-deficient T reg cells was confirmed by the marked suppression of colonic proinflammatory cytokines (Fig. 3 D). Thus, the lack of RIAM in T reg cells does not reduce their capacity to prevent adoptive T cell transfer–induced colitis.

RIAM-deficient T reg cells traffic to GALT and suppress T cell proliferation
To further characterize the role of RIAM in T reg cell function, we crossed floxed RIAM mice with Foxp3<sup>YFP-Cre</sup> mice (Rubtsov et al., 2008) to generate Apbb1ip<sup>fl/fl</sup> Foxp3<sup>YFP-Cre</sup> mice in which the
gene encoding RIAM is specifically deleted in T reg cells (Apbb1ip\textsc{TRKO} mice). RIAM expression was undetectable in Apbb1ip\textsc{TRKO} T reg cells (Fig. 4 A) and can be used to trace T reg cells by the presence of YFP (YFP\textsuperscript{+}; Fig. 4 A). Apbb1ip\textsc{TRKO} mice were born at expected frequencies and developed normally, with no overt signs of pathology in comparison with Apbb1ip\textsc{+/+}Foxp3\textsc{YFP}\textsuperscript{-/}Cre littermates (Apbb1ip\textsc{+/+}). Analysis of YFP\textsuperscript{+} T reg cells in MLNs, PPs, and PLNs showed no significant reduction in the abundance of T reg cells in comparison with Apbb1ip\textsc{+/+} littermates (Fig. S1 A). In agreement with previous studies (Klapproth et al., 2015; Lagarrigue et al., 2017; Su et al., 2015), deletion of RIAM in all T cells (Apbb1ip\textsc{CD4KO} mice) showed a dramatic decrease in T cell numbers in LNs and PPs compared with Apbb1ip\textsc{+/+}CD4\textsc{Cre} littermates (Apbb1ip\textsc{+/+}; Fig. S1 B).

Talin plays a critical role in T reg cell-mediated maintenance of immune homeostasis (Klann et al., 2017). T reg cell–specific deletion of talin results in spontaneous lymphocyte activation associated with autoimmune failure to thrive resembling that observed in Foxp3\textsuperscript{-/-} mice (Lahl et al., 2007) due to numerical and functional deficiencies of T reg cells in the periphery. Since RIAM plays a key role in talin-dependent Tconv cell integrin activation (Han et al., 2006), we investigated whether RIAM was also important for this T reg cell function. Examination of the resting (CD44\textsc{loCD62Lhi}) and previously activated (CD44\textsc{hiCD62Llo}) CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell
compartments suggested that lack of T reg cell RIAM did not affect T lymphocyte activation in Apbb1ipTRKO mice (Fig. 4 B). We next assessed whether expression of RIAM was required for T reg cell suppressive functions. Using an in vitro suppression assay, we observed that T reg cells lacking RIAM were able to suppress Tconv cell proliferation (Fig. 4 C). Analysis of anti-inflammatory cytokines in RIAM-deficient T reg cells revealed no significant defect in the production of IL-10 or TGF-β (Fig. 4 D). We examined the role of RIAM in T reg cell migration using a competitive homing assay. Both WT and RIAM-deficient T reg cells homed equally well to MLNs, PPs, PLNs, and spleen (SP; Fig. 4 E). Taken together, these data show that RIAM is dispensable for T reg cell cytokine production, migration, and suppressive function.

RIAM is dispensable for T reg cell integrin activation

Leukocyte homing depends on integrin function (Hogg et al., 2011); in particular, T reg cell suppression is dependent on the integrin αLβ2–ICAM-1 interaction (Tran et al., 2009). RIAM is essential for activation of B and T cell integrins (Klapproth et al., 2015; Lagarrigue et al., 2017; Su et al., 2015), firm adhesion to ICAM-1 and VCAM-1, and lymphocyte trafficking to secondary lymphoid organs. Because of the surprising preservation of both the suppressive function and homing of RIAM-null T reg cells, we assessed RIAM’s importance for T reg cell integrin activation. Addition of PMA stimulated similar binding of soluble ICAM-1, IL-6, IFN-γ, and IL-17A in distal colon tissues from individual groups of mice in C and D. Results are normalized to GAPDH. n = 3–5 mice per group. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a one-way ANOVA with Bonferroni posttest. * P < 0.05; ** P < 0.01; *** P < 0.001. Apbb1ip+/+; Apbb1ip+/+Il10−/− mice, Apbb1ip−/−; Apbb1ip+/+Il10−/− mice, (C and D) Adoptive transfer of 1 × 106 CD4+CD25−/CD45RB+ T conv cells from Apbb1ip+/+ or Apbb1ip−/− mice into Rag1−/− mice. (C) Changes in body weight. Data are shown as a percentage of the original weight. (D) Survival ratio. The number of mice in each group is indicated. n = 3–5 mice per group. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a one-way ANOVA with Bonferroni posttest. (E) in vivo competitive homing of CD4+ T cells to different lymphoid tissues. CD4+ T cells were isolated from either Apbb1ip+/+ or Apbb1ip−/− mice, differentially labeled, and mixed before injection into C57BL/6 mice. CD4+ T cells homed into different lymphoid organs were analyzed by flow cytometry 3 h after injection. The ratio of Apbb1ip+/+CD4+ T cells to Apbb1ip−/−CD4+ T cells from different lymphoid organs is shown (n = 14). n = 3–4 mice per group. Data are representative of four independent experiments. Data represent mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001. Apbb1ip−/− Tconv, mice injected with Apbb1ip−/− Tconv; Apbb1ip+/+ Tconv, mice injected with Apbb1ip−/− Tconv.
Figure 3. RIAM-deficient T reg cells prevent adoptive T cell transfer–induced colitis. 1 × 10^6 CD4^+CD25^−CD45RB^hi Tconv cells isolated from WT C57BL/6 mice were injected into Rag1^{−/−} mice in the presence or absence of 2 × 10^5 CD4^+CD25^−CD45RB^hi T reg cells isolated from Apbb1ip^{−/−} or Apbb1ip^{−/−} mice. (A and B) Changes in body weight (A) and survival ratio (B) are shown. Changes in body weight are shown as a percentage of the original weight. The number of mice in each group is indicated. n = 3–5 mice per group. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a two-way ANOVA with Bonferroni posttest. (C) IBD Dai. n = 3–5 mice per group. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a one-way ANOVA with Bonferroni posttest. RNA expression analysis of IL-1β, TNF-α, IL-6, IFN-γ, and IL-17A in distal colon tissue. Results are normalized to GAPDH. n = 2–4 mice per group. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a one-way ANOVA with Bonferroni posttest. ***, P < 0.001. Apbb1ip^{+/+} T reg, mice injected with WT Tconv cells; Apbb1ip^{−/−} T reg; Apbb1ip^{−/−} T reg, mice injected with WT Tconv cells and Apbb1ip^{−/−} T reg cells; MFI, mean fluorescence intensity; No T reg, mice injected with WT Tconv cells. The Apbb1ip^{−/−} T reg cell group and Apbb1ip^{−/−} T reg cell group were compared with the “No T reg” group or each other in C and D.

Talin and Rap1 are required for T reg cell function

Lymphocyte integrin activation is triggered by receptors such as chemokine, B cell, or T cell receptors (Abram and Lowell, 2009). A “canonical” pathway to lymphocyte integrin activation involves activation of Rap1 GTPase (Su et al., 2015); active Rap1 subsequently binds RIAM, which recruits cytoplasmic talin to the plasma membrane, where it binds to integrin β tails and triggers integrin activation (Han et al., 2006; Shattil et al., 2010). Because RIAM was dispensable for T reg cell integrin activation, we next examined the role of Rap1 in these cells.

We crossed Rapla^{b/b}, Raplb^{b/b}, and Foxp3^{YM-Cre} mice to generate Rapla^{b/b}, Raplb^{b/b}, and Foxp3^{YM-Cre} mice in which both Rapla and Raplb were deleted selectively in T reg cells (Rapla,^{b-TREKO}). Strikingly, male Rapla,b^{TREKO} mice developed systemic autoimmunity indicated by runting, dermatitis, lymphocytosis, and splenomegaly, ultimately resulting in death by 2–3 mo of age (Fig. S3, A–D), similar to T reg cell–specific talin1 knockout (Tln1^{TRKO}) mice or those expressing an integrin activation defective talin1 (L325R; Tln1^{L325R/TRKO}; Klann et al., 2018). Rapla,b-deficient T reg cells from either male or healthy female mice exhibited impaired binding to all three integrin ligands (Fig. 6A). In the in vitro suppression assay, T reg cells lacking Rapla,b did not suppress proliferation of Tconv cells (Fig. 6E). In addition, these mice exhibited increased percentages of CD4^+ T cells that displayed an activated (CD44^hiCD62L^lo) phenotype; thus, Rapla,b-deficient T reg cells result in inappropriate T cell activation in Rapla,b^{TREKO} mice (Fig. 6B) associated with systemic autoimmunity. Taken together, these results indicate that unlike RIAM, Rapl is indispensable for integrin activation in T reg cells.

Talin binding to the β integrin subunit is the final step in integrin activation (Kim et al., 2011), and, as shown above, Rapla,b^{TREKO} mice developed a profound defect in integrin activation. We therefore compared the effects of Rapla and Raplb deletion to talin1 deletion on T reg cell integrin activation. Rapla,b-deficient T reg cells phenocopied the integrin activation defect in T reg cells from Tln1^{TRKO} mice (Fig. 6A). T cells from Tln1^{TRKO} or Rapla,b^{TREKO} mice, which contain WT Tconv cells and Rapla,b- or talin-deficient T reg cells, exhibited similar relative abundance of CD44^hiCD62L^lo activated T cells (Fig. 6B). We then adoptively transferred CD4^+ T cells from Tln1^{TRKO} or Rapla,b^{TREKO} mice into Rag1^{−/−} mice. Both groups of mice showed a dramatic loss in body weight as early as 2–3 wk (Fig. 6C), and half of both groups of mice died by 10 wk (Fig. 6D) as a result of severe colitis. The suppressive activity of Rapla,b- and talin1-null T reg cells was impaired to a similar degree (Fig. 6E). Thus, Rapla,b represents the principal upstream signaling pathway mediating talin-dependent integrin activation and functions in T reg cells.

Lpd compensates for the loss of RIAM in T reg cells function

RIAM is dispensable for T reg cell functions, leading us to ask whether another protein could serve as a bridge between Rap1 and talin1. Lpd is a RIAM paralogue that plays an important role in cell migration (Krause et al., 2004). We found Lpd was more impaired (Fig. 5, C and D). Thus, RIAM is dispensable for activation of multiple classes of integrin on T reg cells.
highly expressed in T reg cells than in Tconv cells (Fig. 7 A). To explore the function of Lpd and RIAM, we crossed floxed Lpd mice or/and floxed RIAM mice with Foxp3YFP-Cre mice to specifically delete Lpd alone or in combination with RIAM in T reg cells (Raph1TRKO and Raph1,Apbb1ipTRKO). Both Raph1TRKO and Raph1,Apbb1ipTRKO mice exhibited leukocytosis, with Raph1,Apbb1ipTRKO mice exhibiting even higher levels of neutrophils and lymphocytes in peripheral blood (Fig. S3 E). Lpd-deficient T reg cells exhibited reduction in their binding to ICAM-1 and MAdCAM-1, but not to VCAM-1. Lpd/RIAM-deficient T reg cells were even more profoundly affected, exhibiting an ~75% reduction in ICAM-1 and MAdCAM-1 binding and an ~40% decrease in VCAM-1 binding (Fig. 7 B). Thus, Lpd compensates for the loss of RIAM in T reg cell integrin activation, and Lpd and RIAM are partially redundant. Consistent with the defect in integrin activation, the suppressive function of Lpd-deficient T reg cells was impaired and the suppressive function of Lpd/RIAM–deficient T reg cells reduced to an even greater extent (Fig. 7, C and D). In sharp contrast, deletion of Lpd in Tconv cells had a negligible effect on PMA-stimulated binding of soluble ICAM-1, VCAM-1, or MAdCAM-1, indicating that the activation of integrins αLβ2, α4β1, and α4β7 is preserved in Lpd-deficient CD4+ T cells (Fig. S4). As expected (Klapproth et al., 2015; Lagarrigue et al., 2017; Su et al., 2015), RIAM-deficient CD4+ T cells exhibited a profound reduction in their binding to ICAM-1 and MAdCAM-1, but not to VCAM-1. Taken together, these results show that both RIAM and Lpd are partially redundant for integrin activation and suppressive function in T

![Figure 4. RIAM-deficient T reg cells exhibit intact suppressive activity and migration. (A) RIAM expression in T reg cells from Apbb1ip+/+ or Apbb1ipTRKO mice. (B) Representative expression of CD44 and CD62L in splenic CD4+ (upper panels) and CD8+ (lower panels) T cells from Apbb1ip+/+ and Apbb1ipTRKO mice. The percentage of CD44hiCD62Llo resting T cells is shown on the right. Data are representative of four independent experiments. (C) T reg cell suppressive activity. T reg cells isolated from CD45.2 congenic Apbb1ip+/+ or Apbb1ipTRKO mice were mixed with responder cells at the indicated T reg cell/responder cell ratios. Responder cells are CFSE-labeled CD45.1 congenic C57BL/6 CD4+CD25− naive T cells activated by anti-CD3 (5 µg/ml), anti-CD28 (5 µg/ml), and IL-2. CFSE populations gated on CD45.1-cells were analyzed by flow cytometry at 72 h to determine the proliferation index using FlowJo software. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a two-tailed t test. (D) Intracellular expression of IL-10 and TGFβ1 of GFP+ T reg cells from Apbb1ip+/+ or Apbb1ipTRKO mice. Splenocytes were stimulated ex vivo with PMA and ionomycin in the presence of monensin (IL-10) or brefeldin A (TGFβ1) for 4 h at 37°C. Cells were fixed and permeabilized before staining (n = 6). Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a two-tailed t test. (E) In vivo competitive homing of T reg cells to different lymphoid tissues. YFP+ T reg cells were sorted from Apbb1ip+/+ or Apbb1ipTRKO mice. Lymphoid organs were isolated 3 h after injection of T reg cells before flow cytometry analysis. The ratio of Apbb1ip+/+ T reg cells (from Apbb1ipTRKO mice) to Apbb1ip+/+ T reg cells (from Apbb1ip+/+ mice) within various lymphoid tissues is shown (n = 12). n = 4 mice per group. Data are representative of three independent experiments. Data represent mean ± SEM. Apbb1ip+/+, Apbb1ip+/+;Foxp3YFP-Cre mice; Apbb1ipTRKO, Apbb1ipTRKO;Foxp3YFP-Cre mice; TRKO, T reg cell–specific RIAM knockout. Sun et al. Journal of Experimental Medicine 6 of 13

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reg cells. Lpd makes a greater contribution to T reg cell integrin activation and consequently can compensate for loss of RIAM.

Discussion

RIAM is abundant in hematopoietic cells (Watanabe et al., 2008), and previous studies found that RIAM is essential for efficient leukocyte adhesion and proper trafficking of B and T cells to secondary lymphoid organs (Su et al., 2015), because RIAM mediates Ralp-dependent talin-induced integrin activation (Han et al., 2006; Lee et al., 2009). To investigate the role of RIAM in a spontaneous autoimmune disease, we used the IL-10-deficient mouse model (Kühn et al., 1993; Zhang et al., 2014a). Here, we found the expression of RIAM-deficient T reg cells exhibited normal homing to secondary lymphoid organs (Su et al., 2015), because efficient leukocyte adhesion and proper trafficking of B and T cells to secondary lymphoid organs was associated with preservation of activation of αβ2, α4β7, and α4β1 integrins. As is true in other leukocytes, T reg cell integrin activation and function were dependent on talin (Yamahashi et al., 2015) and Rap1 GTPase (Su et al., 2015). Lpd, a RALGDS family of adaptor proteins which contain an RA domain (Bailly, 2015), is a member of the Mig-10/RIAM/Lpd family of adaptor proteins which contain an RA domain (Bailly, 2015) that can bind to Ras family GTPases, including Rap1. Although Lpd kines was also preserved in RIAM-null T reg cells (Fig. S5). Lpd is higher in T reg cells than Tconv cells and, consequently, its role in Rap1-dependent integrin activation has been questioned (Zhang et al., 2014a). Here, we found the expression of Lpd is higher in T reg cells than Tconv cells and other leukocytes. Earlier work had indicated that Tconv cells used ZAP-70 to proliferate but that T reg cell suppressive function is ZAP-70 independent (Au-Yeung et al., 2010); thus, there are other differences in regulatory pathways between T reg cells and Tconv cells. Inhibiting RIAM can suppress the trafficking and function of lymphocytes and neutrophils while sparing T reg cells, thereby shifting the immunological balance to ameliorate IBD and potentially other autoimmune and inflammatory diseases.

RIAM is dispensable for integrin activation in T reg cells and thus for T reg cells function. This was true for all three classes of leukocyte integrins (β1, β2, and β7), as assessed by agonist-induced binding of ligands for αβ2 (ICAM-1), α4β1 (VCAM-1), and α4β7 (MAdCAM-1). Integrin activation in response to chemo- kines was also preserved in RIAM-null T reg cells (Fig. S5). Lpd (Krause et al., 2004) is a member of the Mig-10/RIAM/Lpd family of adaptor proteins which contain an RA domain (Bailly, 2004) that can bind to Ras family GTPases, including Rap1. Although Lpd plays an important role in cell migration (Coló et al., 2012; Krause et al., 2004; Lagarrigue et al., 2015; Law et al., 2013), contains talin binding motifs (Lee et al., 2009), and can form a complex with integrins and talin (Lagarrigue et al., 2015), its role in Rap1-dependent integrin activation has been questioned (Zhang et al., 2014a). Here, we found the expression of Lpd is higher in T reg cells than Tconv cells and, consequently, that Lpd can compensate for the lack of RIAM in both integrin activation and T reg cell function (Figs. 6 and 7), thereby establishing the biological significance of Lpd in this critical regulator of the immune response. In the absence of Lpd, RIAM contributes to T reg cell function, because Lpd-RIAM double-deficient T reg cells exhibited a greater defect in T reg cell function, including impaired integrin activation and reduced...
Bodies have encountered serious mechanism-based toxicities, et al., 2002) all validate this principle. The latter two anti-α4β7 integrin antibodies, efalizumab anti-α4β7 integrin (Feagan et al., 2003; Rice et al., 2013), natalizumab anti-α4β1 integrin (Sandborn et al., 2009), and vedolizumab anti-α4β7 integrin (Fontes et al., 2013) have been approved by the FDA for the treatment of inflammatory bowel disease (IBD) and multiple sclerosis (MS), respectively. Indeed, the success of vedolizumab anti-α4β7 integrin in IBD (Feagan et al., 2003), natalizumab anti-α4β1 integrin in multiple sclerosis (Rice et al., 2005), and efalizumab anti-αLβ2 integrin in psoriasis (Dredick et al., 2002) all validate this principle. The latter two antibodies have encountered serious mechanism-based toxicities, such as progressive multifocal leukoencephalopathy. In the case of integrin αLβ2, blockade of integrin activation can exert a therapeutic effect while reducing mechanism-based toxicities (Petrich et al., 2007a). Here, we show that the loss of Tconv cell integrin activation due to lack of RIAM can ameliorate a spontaneous model of IBD. Importantly, earlier studies had shown that loss of α4β7 integrin function in T reg cells can have a deleterious effect in IBD (Sun et al., 2020b; Zhang et al., 2016). In addition, we conclude that, in spite of RIAM’s important role in Tconv cells, it is dispensable for T reg cell integrin activation and function; hence, RIAM inhibition is a candidate for fine tuning the immune response to inhibit effector T cells while sparing T reg cells.

Materials and methods
Antibodies and reagents
The following antibodies were from BioLegend: CD3 (17A2, 2C11), CD4 (GK1.5), CD8 (53–6.7), CD44 (1M7), CD62L (MEL-14), B220 (RA3-6B2), CD29 (HMβ1-1), CD18 (M18/2), β7 (FIB504), Foxp3 (MF-14), CD28 (37.51), IL-10 (JES5-16E3), and TGF-β1 (TW7-16B4). Secondary Alexa Fluor–labeled antibodies were purchased from Thermo Fisher Scientific.

Figure 6. Loss of T reg cell Rap1α,β recapitulates the talin-null phenotype. (A) Binding of soluble ICAM-1, VCAM-1, or MadCAM-1 to YFP+ T reg cells isolated from Rap1α,b−/−, Rap1α,bTRKO, TLN1+−, or TLN1TRKO mice in the presence or absence of PMA (100 nM). Data are representative of five independent experiments. Data represent mean ± SEM. Significant differences were determined using a one-way ANOVA with Bonferroni posttest. (B) Relative abundance of splenic CD4+ T cells from Rap1α,b−/− mice, Rap1α,bTRKO mice, TLN1−− mice, or TLN1TRKO mice. The ratio of CD44+CD62L− T cells over CD44−CD62L+ T cells is shown. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a one-way ANOVA with Bonferroni posttest. (C and D) 2 × 10⁶ CD4+ T cells isolated from Rap1α,b−/−, Rap1α,bTRKO, TLN1+−, or TLN1TRKO mice were injected into Rap1−− mice to induce colitis. Changes in body weight (C) and survival ratio (D) are shown. The number of mice in each group is indicated. n = 2–4 mice per group. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a two-way ANOVA with Bonferroni posttest. (E) T reg cell suppressive capability. T reg cells isolated from CD45.2 congenic Rap1α,b−/−, Rap1α,bTRKO, TLN1+−, or TLN1TRKO mice were mixed with responder cells at the indicated T reg cell/responder cell ratios. Responder cells are CFSE-labeled CD45.1+ congenic C57BL/6 CD4+CD25− naive T cells 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 72 h to determine the proliferation index using Flowjo software. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a two-way ANOVA with Bonferroni posttest. * P < 0.05; ** P < 0.01; *** P < 0.001. Rap1α,bTRKO, Rap1α,b−/−, Rap1α,bWT/Rap1α,bWT,Foxp3YFP-Cre mice; Rap1α,bTRKO, Rap1α,b−/−, Rap1α,bWT/Rap1α,bWT,Foxp3YFP-Cre mice; TLN1+−, TLN1−−/−, Foxp3YFP-Cre, TLN1TRKO, TLN1−−/−, Foxp3YFP-Cre mice; TRKO, T reg cell–specific RIAM knockout.
from Jackson ImmunoResearch. Foxp3 transcription factor fixation/permeabilization kit was purchased from eBioscience. CFSE and eFluor 670 were purchased from Invitrogen and BioLegend respectively. PMA and piroxicam were from Sigma. CFSE and eFluor 670 were purchased from Invitrogen and BioLegend respectively. PMA and piroxicam were from Sigma.

Mouse colitis models

Il10−/− mice spontaneously develop a chronic IBD under specific pathogen–free conditions. The phenotypes of chronic colitis in Il10−/− mice (C57BL/6 genetic background) became more evident at 10–12 wk. In a mixed C57BL/6–129/SvEv genetic background, the phenotypes of chronic colitis appeared earlier, at 6–8 wk.

Because Il10−/− mice develop spontaneous colitis, which has negative consequences on their capacity to breed, we separately crossed the mice as Apbbip+/+Il10−/− × Apbbip+/+Il10−/− and Apbbip+/+Il10−/− × Apbbip+/+Il10−/− to generate Apbbip+/+Il10−/− and Apbbip+/+Il10−/−, respectively. Sex-matched Apbbip+/+Il10−/− and Apbbip+/+Il10−/− mice were mixed together in a same cage starting at 3–4 wk. For piroxicam treatment, mice were administered piroxicam (200 ppm in diet, every day) for 2 wk and euthanized 3 wk after piroxicam treatment ended (Holgersen et al., 2014). For the adoptive T cell transfer model, 8–10-wk-old

Figure 7. Lpd plays an important role in T reg cell function. (A) RNA expression of Raph1 in Tconv cells and T reg cells. Results are normalized to GAPDH. Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a two-tailed t test. (B) Binding of soluble ICAM-1, VCAM-1, or MAdCAM-1 to YFP+ T reg cells isolated from Rap1fl/fl, Apbb1ip+/+, Raph1TRKO, or Rap1fl/fl, Apbb1ipTRKO mice. Mice were from The Jackson Laboratory. (C) and (D) T reg cell suppressive capability. T reg cells isolated from CD45.2 congenic Rap1fl/fl, Apbb1ip+/+, Raph1TRKO, or Rap1fl/fl, Apbb1ipTRKO mice were mixed with responder cells at the indicated T reg cell/responder cell ratios. Responder cells are CFSE-labeled CD45.1 congenic C57BL/6.CD4+CD25− naïve T cells activated by anti-CD3 (5 µg/ml), anti-CD28 (5 µg/ml), and IL-2. CFSE populations gated on CD45.1− cells were analyzed by flow cytometry (C) at 72 h to determine the proliferation index using FlowJo software (D). Data are representative of three independent experiments. Data represent mean ± SEM. Significant differences were determined using a two-way ANOVA with Bonferroni posttest. (E) Integrin activation signaling pathway in T reg cells. In T reg cells, agonist stimulates Rap1 binding to Lpd and RIAM, leading talin binding to integrin β tail to activate integrins. *, P < 0.05; **, P < 0.1; ***, P < 0.001. Raph1fl/fl, Apbb1ip+/+, Raph1TRKO, Raph1fl/fl, Apbb1ipTRKO, Rap1fl/fl, Apbb1ip+/+, Foxp33TP-Cre, Raph1TRKO, Raph1fl/fl, Apbb1ipTRKO, Rap1fl/fl, Apbb1ip+/+, Foxp33TP-Cre, Raph1TRKO mice; Rap1fl/fl, Apbb1ip+/+, Foxp33TP-Cre mice; Rap1fl/fl, Apbb1ipTRKO, Rap1fl/fl, Apbb1ip+/+, Foxp33TP-Cre, Raph1TRKO mice, Raph1TRKO, Raph1fl/fl, Apbb1ipTRKO, Rap1fl/fl, Apbb1ip+/+, Foxp33TP-Cre, Raph1TRKO mice; Rap1fl/fl, Apbb1ipTRKO, Rap1fl/fl, Apbb1ip+/+, Foxp33TP-Cre, Raph1TRKO mice. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, and 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), and MAdCAM-1−/− mice have been described previously (Fontenot et al., 2005; Sun et al., 2014). Cell counting with immunofluorescence was performed using an Accuri C6 Plus and FACSCalibur (BD Biosciences).
mice were used. 5 × 10^6 CD4+CD25+ CD45RB\textsuperscript{high} Tconv cells from C57BL/6 mice were injected intraperitoneally into Rag\textsuperscript{1/2} mice in the presence or absence of 1 × 10^6 CD4+CD25+CD45RB\textsuperscript{low} T reg cells derived from the indicated mice (0.2 ml PBS each recipient). Only comparison between littermates was considered.

Mouse body weight was measured daily, and values are shown as a percentage of the original weight. During the duration of the experiment, we assessed the clinical progression of colitis by daily blinded scoring a disease activity index (DAI) by two independent investigators. The DAI is the combined score of body weight loss, stool consistency, and rectal bleeding and prolapse as follows: (1) weight loss: 0 (no loss), 1 (1–5%), 2 (5–10%), 3 (10–20%), or 4 (>20%); (2) stool consistency: 0 (normal), 1 (soft), 2 (very soft), or 3 (diarrhea); (3) rectal bleeding: 0 (none), 1 (red), 2 (dark red), or 3 (gross bleeding); and (4) rectal prolapse: 0 (none), 1 (signs of prolapse), 2 (clear prolapse), or 3 (extensive prolapse). Mice were sacrificed at week 15.

**Histology**

Formalin-fixed, paraffin-embedded Swiss-rolled colon sections of 4-mm thickness were mounted on glass slides and followed by H&E staining or periodic acid–Schiff staining. Images were acquired with a Nanozoomer 2.0HT Slide Scanner (Hamamatsu). Blinded histological scoring was performed by two investigators based on the method described previously (Erben et al., 2014), and total scoring range is 0–12 (Table 1).

**Flow cytometry**

Cells isolated from mouse tissues were washed and resuspended in PBS containing 0.1% BSA and stained with conjugated antibody for 30 min at 4°C. Then, 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\textsuperscript{2+}/Mg\textsuperscript{2+} before incubation with integrin ligands for 30 min at 37°C in presence with or without 100 nM PMA. Cells were then incubated with Alexa Fluor 647-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 Sciences) and permeabilized with the Foxp3 transfection factor fixation/permeabilization kit (eBioscience) before IL-10, TGF-β1, and Foxp3 staining.

**Static cell adhesion assay**

Coverslips were coated with integrin ligand in coating buffer (PBS and 10 mM NaHCO\textsubscript{3}, pH 9.0) overnight at 4°C. The coverslips were then rinsed with PBS and free binding sites were blocked with 2% BSA in coating buffer for 1 h at 37°C. Splenocytes were added onto coverslips, and adhesion was allowed for 1 h at 37°C. Coverslips were next washed with PBS and fixed with 2% paraformaldehyde at room temperature for 15 min. Bound cells were observed using a Keyence BZ-X700 all-in-one fluorescence microscope with CFI Plan Apo λ4× fluorescent objective (Nikon Plan Apochromat, NA 0.2) or CFI Plan Apo λ10× fluorescent objective (Nikon Plan Apochromat, NA 0.45), which was operated with a 2/3-inch, 2.83-million-pixel monochrome charge-coupled device (colorized with LC filter) at 25°C.

| Score | Description                          |
|-------|--------------------------------------|
| 0     | Normal                               |
| 1     | Hyperproliferation, irregular crypts, goblet cell loss |
| 2     | Mild to moderate crypt loss (10–50%) |
| 3     | Severe crypt loss (50–90%)           |
| 4     | Complete crypt loss, surface epithelium intact |
| 5     | Small to medium-sized ulcer (<10 crypt widths) |
| 6     | Large ulcer (>10 crypt widths)       |

**Infiltration with inflammatory cells**

| Mucosa   | Score | Description               |
|----------|-------|---------------------------|
| 0        | None  | None                      |
| 1        | Mild infiltration     | Mild to moderate infiltration and/or edema |
| 2        | Severe infiltration  | Severe infiltration       |

| Submucosa | Score | Description               |
|-----------|-------|---------------------------|
| 0         | None  | None                      |
| 1         | Mild to moderate infiltration and/or edema | Mild to moderate infiltration and/or edema |
| 2         | Severe infiltration  | Severe infiltration       |

| Muscularis/serosa | Score | Description                |
|-------------------|-------|---------------------------|
| 0                  | Not involved | Not involved               |
| 1                  | Involved   | Involved                   |

Total scoring range: 0–12.

**T reg cell suppression assays**

CD4+CD25− T cells (responder cells) were isolated from SPs 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) antibody was included to deplete T reg cells. YFP− T reg cells were sorted with a FACSAria 2 (BD Biosciences). Responder cells were labeled with CFSE and cocultured with T reg cells (8:1, 4:1, 2:1, and 1:1 ratios) in the presence of 5 µg/ml immobilized antibodies against CD3 (2C11) and CD28 (37.51) and 10 µg/ml eFluor670, respectively, resulting in readily discriminated cell populations. Equal numbers (1 × 10^7) of differentially labeled T reg cells were mixed and then intravenously injected objective (Nikon Plan Apochromat, NA 0.2) or CFI Plan Apo λ10× fluorescent objective (Nikon Plan Apochromat, NA 0.45), which was operated with a 2/3-inch, 2.83-million-pixel monochrome charge-coupled device (colorized with LC filter) at 25°C.

**In vivo competitive lymphocyte homing**

The competitive homing assay used high- and low-dose cell tracker as described previously (Haeryfar et al., 2008). YFP− T reg cells were sorted with a FACSARia 2 (BD Biosciences). YFP− T reg cells were labeled with CFSE and cocultured with T reg cells (8:1, 4:1, 2:1, and 1:1 ratios) in the presence of 5 µg/ml immobilized antibodies against CD3 (2C11) and CD28 (37.51) and IL2 for 4 d at 37°C. The proliferation index was calculated by FlowJo v10.
Table 2. Sequences of primers used for real-time quantitative PCR analyses

| Primer | Direction | Sequence (5′–3′) |
|--------|-----------|-----------------|
| IL-1β  | Forward   | AGTGGGATCCAAAGCAATAC |
|        | Reverse   | CTCACCTTGGCTTCTTCT |
| TNF-α  | Forward   | AGTGGACGGCCTGTAGGCCC |
|        | Reverse   | GAGGTTGACTTCTCCTGGAT |
| IL-6   | Forward   | CTGCAAGAGACTCCATCCAGT |
|        | Reverse   | GAAGTGGAGGAAGGGCTTG |
| IFN-γ  | Forward   | CTCTTCTACTGGCTTTTCT |
|        | Reverse   | TTCTTCCACATCTATGCCACTT |
| GAPDH  | Forward   | CCAGGTGTCTCTCTGGGACTT |
|        | Reverse   | CCTGTGCTAGCGGCTTACA |

into C57BL/6 recipient mice. Lymphoid organs were harvested 3 h after injection, and isolated cells were analyzed by flow cytometry. The ratio of Apbb1ip+/− T reg cells (eFluor670high) to Apbb1ip+/− T reg cells (eFluor670low) from different lymphoid organs is shown. For the competitive homing assay of RIAM-deficient CD4+ T cells, CD4+ T cells were isolated by MojoSort mouse CD4 T cell isolation kit (BioLegend) from Apbb1ip+/− or Apbb1ipΔAMS mice and labeled with 1 µM CFSE and eFluor670, respectively.

Real-time quantitative PCR analyses
Total RNA was 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 µg total RNA of colon using SuperScript III First-Strand synthesis and oligo-dT primers according to the manufacturer’s protocol (Thermo Fisher Scientific). Kappa 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 2) according to the manufacturer’s protocol. The 2−ΔΔCT method was used for analysis, and data were normalized to GAPDH. Control values (WT mice or Rag−/− mice injected with PBS) were set to 1 for comparisons.

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 a two-tailed t test, one-way ANOVA, or two-way ANOVA followed by Bonferroni posttest as indicated in the figure legends. The resulting P values are indicated as follows: NS, P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001. Plotted data show the mean ± SEM of three to five independent experiments.

Online supplemental material
Fig. S1 shows RIAM-deficient T reg cells have a similar number and percentage of T reg cells in lymphoid organs compared with WT T reg cells. Fig. S2 shows RIAM-deficient T cells or T reg cells have similar integrin expression levels compared with WT cells. Fig. S3 shows that mice with T reg cell-specific deletion of Rapla,b have spontaneous lethal inflammation and shows blood cells counts in these T reg cell-specific gene-depleted mice. Fig. S4 shows the role of Lpd in Tconv cells. Fig. S5 shows integrin activation in response to chemokines in T reg cell-specific gene-depleted mice.

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Figure S1. Apbb1ipTRKO mice show a similar number and percentage of T reg cells in lymphoid organs. (A and B) The percentage of T reg cells in MLNs, PPs, PLNs, and SPLs from Apbb1ip+/+ (Apbb1ipwt/wt,Foxp3YFP-Cre) or Apbb1ipTRKO mice (n = 6; A), and Apbb1ip+/+ (Apbb1ipwt/wt,CD4Cre) or Apbb1ipCD4KO mice (n = 6; B), n = 3 mice per group. Data are representative of two independent experiments. Data represent mean ± SEM. **, P < 0.01; ***, P < 0.001; two-tailed t test. Apbb1ip+/+, Apbb1ip+/+; Apbb1ipTRKO, Apbb1ipTRKO mice; CD4KO, CD4+ T cell–specific knockout; SPL, spleen, TRKO, T reg cell–specific RIAM knockout.
Figure S2. Integrin expression in RIAM-null T cells and T reg cells. (A and B) Surface expression of integrin β1, β2, β7, and intracellular expression of RIAM in Apbb1ip<sup>+/+</sup> (Apbb1ip<sup>wt/wt</sup>,CD4<sup>Cre</sup>) or Apbb1ip<sup>CD4KO</sup> (Apbb1ip<sup>fl/fl</sup>,CD4<sup>Cre</sup>) mice (A) and Apbb1ip<sup>+/+</sup> (Apbb1ip<sup>wt/wt</sup>,Foxp3<sup>YFP-Cre</sup>) or Apbb1ip<sup>TRKO</sup> (Apbb1ip<sup>fl/fl</sup>,Foxp3<sup>YFP-Cre</sup>) mice (B). Representative histograms are displayed. Data are representative of three independent experiments. Apbb1ip<sup>+/+</sup>, Apbb1ip<sup>wt/wt</sup>,Foxp3<sup>YFP-Cre</sup> mice or Apbb1ip<sup>+/+</sup>,CD4<sup>+</sup> T cell–specific knockout; TRKO, T reg cell–specific RIAM inactivation.
Figure S3. **Phenotype of Rap1a,1b TRKO mice, and complete blood counts of these T reg cell-specific gene-depleted mice.** (A–C) Representative morphology (A) and organs (SP, thymus [Thy], and LNs, including aortic LN [aLN], brachial LN [bLN], mLN, and inguinal LN [iLN]; B) from male Rap1a,1b+/+ and Rap1a,1b TRKO mice. (C) Survival of Rap1a,1b+/+ and Rap1a,1b TRKO mice. (D and E) Complete blood counts of Rap1a,1b+/+, Rap1a,1b TRKO, TLN1+/+, or TLN1 TRKO mice (D) and Raph1,Apbb1ip+/+, Raph1 TRKO, and Raph1,Apbb1ip+/+ mice (E). n = 3 mice per group. Data are representative of two independent experiments. Data represent mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; one-way ANOVA with Bonferroni posttest.

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Integrin activation in T reg cells

Figure S4. **Lpd-deficient Tconv cells show normal integrin activation.** Binding of soluble ICAM-1, VCAM-1, or MadCAM-1 to splenic T cells isolated from Raph1,Apbb1ip+/+, Raph1CD4KO, Apbb1ipCD4KO, or Raph1,Apbb1ipCD4KO mice in the presence or absence of PMA (100 nM). Data are representative of three independent experiments. Data represent mean ± SEM. ***, P < 0.001, one-way ANOVA with Bonferroni posttest.

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Figure S5. **Chemokine-induced integrin activation in T reg cells.** Binding of soluble ICAM-1, VCAM-1, or MAdCAM-1 to YFP+ T reg cells isolated from WT, Apbb1ipTRKO, Raph1TRKO, Rap1a,1bTRKO, TLN1+/+, and TLN1TRKO mice in the presence or absence of different chemokines (1 µM). Data are representative of five independent experiments. Data represent mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; one-way ANOVA with Bonferroni posttest. MFI, mean fluorescence intensity; Apbb1ipTRKO, Apbb1ipfl/fl,Foxp3YFP-Cre mice; Raph1TRKO, Raph1fl/fl,Foxp3YFP-Cre mice; Rap1a,bTRKO, Rap1afl/fl,Rap1bfl/fl,Foxp3YFP-Cre mice; TLN1TRKO,TLN1fl/fl,Foxp3YFP-Cre mice; TRKO, T reg cell–specific RIAM knockout; WT, Foxp3YFP-Cre mice.