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Release of Active TGF-β1 from the Latent TGF-β1/GARP Complex on T Regulatory Cells Is Mediated by Integrin β8

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Activated T regulatory cells (Tregs) express latent TGF-β1 on their cell surface bound to GARP. Although integrins have been implicated in mediating the release of active TGF-β1 from the complex of latent TGF-β1 and latent TGF-β1 binding protein, their role in processing latent TGF-β1 from the latent TGF-β1/GARP complex is unclear. Mouse CD4+Foxp3+ Treg, but not CD4+Foxp3- T cells, expressed integrin β8 (Itgb8) as detected by quantitative RT-PCR. Itgb8 expression was a marker of thymically derived (t)Treg, because it could not be detected on Foxp3+Helios- Tregs or on Foxp3+ T cells induced in vitro. Tregs from Itgb8 conditional knockouts exhibited normal suppressor function in vitro and in vivo in a model of colitis but failed to provide TGF-β1 to drive Th17 or induced Treg differentiation in vitro. In addition, Itgb8 knockout Tregs expressed higher levels of latent TGF-β1 on their cell surface consistent with defective processing. Thus, integrin αβ6 is a marker of tTregs and functions in a cell intrinsic manner in mediating the processing of latent TGF-β1 from the latent TGF-β1/GARP complex on the surface of tTregs. The Journal of Immunology, 2014, 193: 000–000.

Activated Foxp3+ T regulatory cells (Tregs) express the latent TGF-β1 binding protein GARP/LRRC32 that is required for expression of latent TGF-β1 on the surface of human and mouse Tregs. In an in vitro culture system in which activated Tregs are used as source of TGF-β1 to drive induced Treg (iTreg) or Th17 differentiation, GARP is required for efficient production of biologically active TGF-β1 (1). Previous studies have indicated, among other mechanisms, that the αv integrins, αβ6 and αβ8, bind the arginine-glycine-aspartic acid (RGD) site in latent TGF-β1 and facilitate the release of biologically active TGF-β1 from the complex of latent TGF-β1 and latent TGF-β1 binding protein (LTBP, the large latent complex). Activation mediated by αβ6 requires the interaction of its cytoplasmic domain with the actin cytoskeleton, which appears to drive the shear forces needed to activate TGF-β1 from large latent complex (2). In contrast, αβ8 has a short cytoplasmic tail that does not interact with the actin cytoskeleton. Some studies have demonstrated αβ6-dependent processing of latent TGF-β1 from the large latent complex requires the coexpression of matrix metalloproteases (MMPs), specifically MMP-14 (or MT1-MMP) (3). It is unknown, however, if this is the only MMP that can function in αβ8-mediated activation of TGF-β1.

Previous studies have focused on processing of latent TGF-β1 from the large latent complex by integrin αβ8 on dendritic cells (DCs). Conditional deletion of Itgb8 from leukocytes or only from DCs resulted in severe inflammatory bowel disease and age-related autoimmunity (4). Deletion of Itgb8 on DCs also resulted in an inability to drive endogenous Th17 differentiation in the gut and in a failure to generate highly pathogenic Th17 cells during experimental autoimmune encephalomyelitis resulting in markedly milder symptoms (5). The role of integrins in processing latent TGF-β1 from the latent TGF-β1/GARP complex is less clear. αβ6, and to a lesser extent αβ8, were shown to activate latent TGF-β1 from 293 cells transfected with αv integrins, pro-TGF-β1, and GARP (6). However, interpretation of these studies was complicated by the fact that TGF-β1 was equally activated in the absence of GARP, probably because of the presence of endogenous LTBP. Nevertheless, it appeared that the ability of αβ6 to activate TGF-β1 from the latent TGF-β1/GARP complex was real because it was preserved in the presence of the ECR3E fragment of LTBP, which inhibits endogenous LTBP, but not GARP. It remains unclear whether similar mechanisms are present in Tregs or platelets that physiologically express GARP.

The purpose of the current study was to examine how biologically active TGF-β1 is released from the GARP/latent TGF-β1 complex by mouse Tregs. In this study, we demonstrate that integrin αβ6 is a marker of mouse thymically derived (t)Tregs and functions in a cell-intrinsic manner to release active TGF-β1.

Materials and Methods

Mice

C57BL/6 were obtained from the National Center Institute Mouse Repository (Frederick, MD). Foxp3-GFP, C57BL/6-Rag1-/-, and OVA-specific TCR-transgenic OT-II (CD45.1, Rag1-/-) mice were obtained by the National Institute of Allergy and Infectious Diseases (NIAID) and were maintained by Taconic Farms (Germantown, NY) under contract by NIAID. Itgb8flox/flox mice, which have been previously described (7), were obtained from the Mutant Mouse Regional Resource Center (MMRRC stock number 014108-UCD). These mice contain loxP sites within 3’ and 5’ introns of exon 4 of the Itgb8 gene. Lrc432loxP (GARP) and Tgfb1loxP, Rck2loxP (Helios) mice have been described previously (1, 8, 9). Floxed mice were crossed to CD4-CRE mice (Taconic). Helios-GFP reporter mice were developed by Taconic Artemis and will be described in detail at a later date. Helios-GFP reporter mice were crossed to Foxp3-mRFP mice obtained from The Jackson Laboratory (Bar Harbor, ME). OVA-specific TCR-transgenic OT-II mice were obtained from Taconic Farms and bred to...
Foxp3-GFP mice to generate OT-II Foxp3-GFP mice, as described previously (10). All animal protocols used in this study were approved by the NIAID Animal Care and Use Committee.

Inflammatory bowel disease experiments

Inflammatory bowel disease experiments were performed similarly to those described previously (11). Briefly, 4 × 10^6 naive wild-type (WT) CD4^+ T-cells (CD4^+CD25^-CD45RB^-) were transferred into Rag1^-/- mice in the presence or absence of 2 × 10^7 (CD4^+CD25^+CD45RB^-) Tregs from WT or Itgb8 conditional knockout (cKO) mice. Weights were monitored twice weekly for up to 11 wk.

Cell isolation and flow cytometry

For purification of DCs, mouse spleens were fragmented and digested for 30 min at 37°C in the presence of liberase blendzyme II (Roche) and DNase (2 µg/ml) (Roche) in complete medium (modified RPMI 1640 medium supplemented by 10% FBS HyClone, 1% sodium pyruvate, 1% nonessential amino acids, 1% HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. They were labeled with anti-CD11c beads and purified on the AutoFACS Cell Separator (Miltenyi Biotec). T cells from pooled lymph nodes and/or spleens were isolated using CD4 beads.

CD4^+Foxp3^- Tregs and Foxp3^- conventional T cells (Tconvs) were sorted from the pooled lymph nodes of Foxp3-GFP mice. CD4^-CD25^+ Treg and CD4^-CD25^- Tconv also were sorted. All cell sorting was performed on FACSaria flow cytometers (BD Biosciences). Single-cell suspensions were stained using the following Abs according to the manufacturer’s protocol: anti-mouse CD45.1 (A20), CD45.2 (104), CD4 (RM4-5), Foxp3 (FLK-16s), GARP (YGCIC6), and IL-17A (eBio17B7). Purified mouse anti-mouse LAP clone TW7-16B4 was provided by H. Weiner (Harvard Medical School, Boston, MA). The LAP Ab was labeled with SureLight-APC at Columbia Biosciences (Columbia, MD).

For staining of Foxp3, cells were fixed and permeabilized using the Foxp3 fixation/permeabilization staining kit (eBioscience). For staining of cytokines, cells were fixed and permeabilized using the Cytofix/Cytoperm kit from BD Biosciences.

Cytokines and other reagents

Recombinant mouse IL-6 was purchased from BioLegend (San Diego, CA). The MMP inhibitor GM6001 and the GM6001 control were both purchased from Santa Cruz Biotechnology (Dallas, TX).

In vitro suppression assay

In vitro suppression assays were performed as described previously (12).

In vitro T cell differentiation

To induce Th17 or iTreg differentiation using activated Tregs as a source of biologically active TGF-β1, CD4^-Foxp3^- T cells from Foxp3-GFP mice were activated and expanded using plate-bound anti-CD3 (1 µg/well, 24-well plate) with IL-2 (100 U/ml) for 2–3 d, followed by overnight culture with IL-2 alone. Cells were generally >90–95% Foxp3^- after expansion. Tregs were washed and mixed 1:1 with naive CD45.1 OT-II cells (RAG1^-/-) in the presence of recombinant mouse IL-6 (10 ng/ml) and stimulated with splenic DCs and soluble anti-CD3 for 4 d. Similar experiments were performed using exogenous IL-2 (100 U/ml) and activated Tregs to drive iTreg differentiation. Th17-differentiated T cells were stimulated with the Cell Stimulation Cocktail and Protein Transport Inhibitors (eBioscience).

In other experiments, iTregs were differentiated to analyze the expression of Itgb8. Naive CD4^- T cells (CD44^hiCD62L^hi) from Foxp3-GFP mice were cultured with plate-bound anti-CD3 or DCs and soluble anti-CD3. Naive CD4^- cells were also sorted from OT-II Foxp3-GFP mice and cultured with splenic DCs and cognate peptide. In all conditions, cells were cultured in the presence of recombinant TGF-β1 and IL-2. After 4 d of culture, CD4^-GFP (Foxp3^-) cells were sorted and immediately subject to RNA isolation.

mRNA isolation, cDNA production, and real-time PCR

Tregs and conventional CD4^- T cells were sorted and immediately subjected to RNA extraction or stimulated overnight with IL-2 and plate-bound anti-CD3 then subjected to RNA extraction using TRIzol reagent. The contaminating DNA was then removed by DNase I treatment. The SuperScript II First-Strand Synthesis Supermix for quantitative real-time PCR (qRT-PCR; Invitrogen Life Technologies) was used to generate cDNA. Real-time PCR was performed with the ABI Prism7900HT, using the Kapa Probe Fast Universal qPCR Kit and TaqMan Probes for GAPDH, ITGB8, and Foxp3.

Results

Integrin αbβ8 is a marker of Foxp3^- Tregs

Previous studies from our group and others have demonstrated that activated Foxp3^- Tregs express latent TGF-β1 on their cell surface bound to the tethering molecule, GARP (1, 13, 14). Although αbβ8 on DCs has been shown to mediate the release of active TGF-β1 from the large latent complex, we have previously shown that even in the absence of DCs, Tregs are able to release active TGF-β1, primarily from the latent TGF-β1/GARP complex (1). We therefore decided to examine the potential expression and function of αbβ8 and αbβ8 by Tregs. Sorted CD4^-Foxp3^- expressed substantially higher levels of Itgb8 than CD4^-Foxp3^- Tconvs by qRT-PCR (Fig. 1A). Neither cell type expressed Itgb6 (data not shown). Unfortunately, we could not determine the expression of β8 on a single-cell basis, because no Ab is currently available for staining and FACS analysis. The expression of Itgb8 was not dependent on the expression of TGF-β1 or GARP (Fig. 1B, 1C). However, both cell populations expressed integrin α4 (CD51) either freshly isolated or after activation with anti-CD3 and IL-2 (Supplemental Fig. 1). CD51, however, is well known to be able to pair with multiple integrin β-chains in addition to β8 including β1, β3, β5, and β6 (15).

Itgb8 expression on Tregs was not dependent on the lymphoid tissue from which the Tregs were isolated (Fig. 1D) and was only modestly increased upon activation (Fig. 1E). Itgb8 has been shown to be expressed in CD103^+ DCs in the mesenteric lymph nodes or gut-associated lymphoid tissues (16, 17). We found that the level of expression of Itgb8 was higher in Tregs than in CD103^+ DCs (relative to GAPDH). As previously described (17), the level of Itgb8 was substantially higher in CD103^+ DCs from

FIGURE 1. Integrin αbβ8 is expressed by Foxp3^- Tregs. (A) Sorted CD4^-Foxp3^- (GFP^-) and CD4^-Foxp3^- (GFP^-) were measured for Itgb8 and Foxp3 message as measured by qRT-PCR. Data are expressed relative to CD4^-Foxp3^- cells. (B and C) Sorted CD4^-CD25^- and CD4^-CD25^+ from CD4 cKOs of Itgb8 and Lrrc32 and their CRE^- littermate controls were measured for Itgb8 message. Data are expressed relative to WT CD4^-CD25^+ cells. (D) Sorted CD4^-Foxp3^- (GFP^-) and CD4^-Foxp3^- (GFP^-) from spleen, peripheral lymph nodes, or mesenteric lymph nodes were measured for Itgb8 message as measured by qRT-PCR. (E) Sorted CD4^-Foxp3^- (GFP^-) and CD4^-Foxp3^- (GFP^-) from CD11c^-elle^-CD11b^- DCs were measured for Itgb8 message. DCs were first gated on Thy1.2 CD19^-CD11c^-I-A^- Ab^- cells.
the mesenteric lymph nodes than in CD103⁺ DCs or DC populations from the spleen (Fig. 1E).  

**Integrin β8 is expressed primarily by Helios⁺Foxp3⁺ Tregs**

Because we have previously shown that iTregs express the latent TGF-β1/GARP complex, it was of interest to determine whether iTreg also express Itgb8 (1). iTregs were generated from naïve CD4⁺Foxp3⁺ T cells from Foxp3-GFP or OT-II Foxp3-GFP mice. iTregs were generated using plate-bound anti-CD3, splenic DCs with soluble anti-CD3, or splenic DCs with OVA peptide (OT-II cells) cultured for 4 d with IL-2 and TGF-β1 and then sorted for Foxp3⁺ (GFP⁺) cells. Tregs and Foxp3⁻ cells stimulated with plate-bound anti-CD3+IL-2 were cultured in parallel and sorted for GFP⁺ or GFP⁻ cells, respectively. iTregs generated with plate-bound anti-CD3 did not express substantial levels of Itgb8 nor did those generated using DCs with peptide. Tregs cultured in parallel maintained their expression, whereas cultured Foxp3⁻ cells did not show substantial expression. Interestingly, iTregs generated using splenic DCs and soluble anti-CD3 did show some expression of Itgb8, whereas those generated using more physiologically relevant conditions with peptide did not. The level of expression by iTregs generated using splenic DCs and soluble anti-CD3 was still lower than cultured Tregs (Fig. 2A). As we have previously proposed (9) that Foxp3⁺Helios⁺ and Foxp3⁺Helios⁻ Tregs may represent iTreg and peripheral derived Tregs, respectively, the relative expression of Itgb8 was determined for each of these populations. CD4⁺Foxp3⁺Helios⁺, CD4⁺Foxp3⁺Helios⁻, and CD4⁺Foxp3⁻Helios⁻ cells were isolated from a Helios(GFP)/Foxp3(RFP) double-reporter mouse. Only the CD4⁺Foxp3⁺Helios⁺ cells expressed substantial levels of Itgb8 (Fig. 2B). Furthermore, this difference in expression was maintained even after activation and expansion for 4 d in culture (Fig. 2C). Helios did not control the expression of Itgb8, because Tregs sorted from IκBz(Helios)⁺ Fl/Fl CD4-CRE cKO mice maintained their expression of Itgb8 (Fig. 2D).

**Itgb8 is required for clearance of TGF-β1 from the GARP/latent-TGF-β1 complex**

To investigate the function of Itgb8 on Tregs, we bred CD4-cKOs of Itgb8, using the previously described Itgb8Fl/Fp mice (7). When bred with the CD4-CRE, the percentages of thymocyte subpopulations (Supplemental Fig. 2, upper panels), including the percentages of Foxp3⁺ cells within the CD4 single-positive population (Supplemental Fig. 2, lower panels), were identical in CRE⁺ and CRE⁻ littersmates. The percentages of CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ T cells in the periphery were also identical in CRE⁺ and CRE⁻ littersmates (Supplemental Fig. 2, right panels).

We hypothesized that if αββ₁ is involved in activation of TGF-β1 from the GARP/latent-TGF-β1 complex, that in the absence of Itgb8, latent-TGF-β1 would accumulate on the surface of Tregs. Freshly isolated Tregs from WT and Itgb8 cKO mice displayed similar low levels of cell-surface-latent-TGF-β1, whereas after activation, cKO Tregs displayed significantly higher levels of latent-TGF-β1 than activated WT Tregs (Fig. 3A, 3B). Because resting Tregs express higher level of GARP than latent TGF-β1, it is likely that the levels of latent TGF-β1 are probably limiting in forming the expression of the GARP/latent-TGF-β1 complex on the cell surface. Itgb8 cKO mice also expressed higher levels of GARP and GARP/LAP double-positive cells (Fig. 3B).

**Itgb8 is not required for Treg-mediated suppression in vitro or in vivo in a transfer model of colitis**

We next determined whether Treg expression of Itgb8 is required for suppression of T effector cell proliferation in vitro. Using the traditional T cell suppression assay, Tregs from both Itgb8 cKO and WT floxed littersmates equally suppressed proliferation with increasing ratios of Treg to effector T cells (Fig. 4A). This was not unexpected, however, because it has previously been shown by our group and others that neither TGF-β1 nor GARP expression is required for in vitro suppression (1, 8). Next, we determined whether Itgb8 plays a role in Treg-mediated suppression in the transfer model of colitis. Transfer of WT CD4⁺CD25⁻CD45RB⁺ T cells resulted in significant weight loss in the recipients; both WT and cKO Tregs were equally capable of protecting from disease (Fig. 4B). Importantly, 75 d posttransfer, both WT and cKO Tregs equally maintained Foxp3⁺ cells in the mesenteric lymph nodes (Fig. 4C, 4D). This result is consistent with a previous report (18), which demonstrated that TGF-β1 was required for suppression of inflammatory bowel disease in this model but that the source of the TGF-β1 was not the Treg.  

**ITGB8 expression by Tregs is required for the bioavailability of active TGF-β1 from Tregs**

To evaluate the availability of biologically active TGF-β1 released from WT and Itgb8 cKO Tregs, we used an in vitro culture system in which activated Tregs are cultured with naïve T cells under activating conditions (splenic DCs with soluble anti-CD3) in the
presence of IL-6 or IL-2 to drive Th17 or iTreg differentiation, respectively. We have previously shown that TGF-β1 derived from the TGF-β1/GARP complex plays a major role as the source of TGF-β1 required for differentiation of Th17 cells or iTreg (1). WT, but not Itgb8 cKO, Tregs, promoted the differentiation of Th17 or iTreg as measured by IL-17A and Foxp3 expression, respectively (Fig. 5A, 5B). The same result was observed when the Tregs were only activated overnight prior to being cultured with naive cells (data not shown). To determine whether the GARP/latent-TGF-β1 complex and αvβ8 needed to be expressed on the same cells or whether αvβ8 expressed on one cell could result in the activation of TGF-β1 from the GARP/latent-TGF-β1 complex on a different cell, we cultured Tregs from GARP (Lrrc32) cKO or Itgb8 cKO mice separately or together with naive T cells in the Th17 differentiation assay. Neither GARP nor Itgb8 cKO cells alone could efficiently drive Th17 differentiation, however when mixed they were able to substantially restore Th17 differentiation, indicating that integrin αvβ8 expressed by human epithelial cell lines required the coexpression of an MMP, in particular MMP-14 (3), to activate latent TGF-β1 from the large latent complex. The contribution of MMP-14 was demonstrated by the use of the MMP inhibitor

**FIGURE 3.** Activated Itgb8-deficient Tregs accumulate latent TGF-β1 on their surface. (A and B) Freshly isolated cells from pooled lymph nodes and enriched CD4+ cells from Itgb8 cKO mice (CD4-CRE) or CRE littermates were stimulated for 48 h with plate-bound anti-CD3 and IL-2 and then stained for CD4, Foxp3, LAP (or IC), and GARP (or IC). (A) Representative histograms for LAP (latent TGF-β1) from CD4+Foxp3+ cells. (B) Graphic representations indicating LAP and GARP mean fluorescence intensity on CD4+Foxp3+ cells as well as percentage of LAP and GARP double-positive cells.

**FIGURE 4.** Tregs from Itgb8 cKO mice suppress normally in vitro and in vivo. (A) T cell suppression assays using sorted CD4+CD25− cells from WT mice in the presence or absence of increasing ratios of Itgb8 WT and KO CD4+CD25+ cells, as described previously (12). (B) WT CD4+CD25+CD45RBhi T cells were transferred into Rag1−/− mice in the presence or absence of CD4+CD25hiCD45RBlow Tregs from WT or Itgb8 cKO mice and monitored for weight loss for up to 11 wk. Indicated are the average weight changes from five mice in each group. (C and D) At the end of the experiment, mesenteric lymph nodes were stained for CD4 and Foxp3. (C) Representative lymph node samples. (D) The percentage of CD4+ cells that were Foxp3+ in individual mice.
Thus far, we have not observed any effect of this inhibitor over a wide dose range on Treg-mediated Th17 induction up to 20 μM, as compared with the vehicle control or GM6001-control (Fig. 5E). It is important to note, however, that it is unknown which MMPs are expressed by Tregs and whether \( \alpha_v \beta_8 \)-mediated activation of latent TGF-β1 activation from the GARP/latent TGF-β1 complex is dependent on MMPs.

**Helios<sup>−/low</sup> Tregs are less efficient at producing biologically active TGF-β1**

Because we found that Helios<sup>+</sup> Tregs expressed substantially higher levels of \( \text{Itgb8} \) (Fig. 2B), we wished to assess the ability of the Helios<sup>+</sup> versus Helios<sup>−/low</sup> Tregs to drive Th17 differentiation in the same experimental setup as used in Fig. 5. When using plate-bound anti-CD3 to activate the cells in the culture, Helios<sup>+</sup> Tregs were more efficient at driving Th17 differentiation (Fig. 6A, 6C) than Helios<sup>−/low</sup> Tregs. This result is consistent with the higher level of expression of \( \text{Itgb8} \) on the Helios<sup>+</sup> cells. It is not due to an inability of the Helios<sup>−/low</sup> cells to express latent TGF-β1 on their surface, because total Tregs activated in vitro when gated on the Helios<sup>−/low</sup> or Helios<sup>+</sup> subsets both have similar levels of latent TGF-β1 on their surface (Fig. 6D). It also remains possible that Helios<sup>−/low</sup> Tregs can process the latent-TGF-β1 by an \( \alpha_v \beta_8 \)-independent mechanism. Surprisingly, when the cocultures were

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**FIGURE 5.** Treg-mediated Th17 differentiation requires activation of latent TGF-β1 via integrin \( \alpha_v \beta_8 \). (A) Naive CD45.1<sup>+</sup> OT-II cells from Rag1<sup>−/−</sup> mice were activated by soluble anti-CD3 in the presence of IL-6, splenic DCs, and preactivated CD4<sup>+</sup>CD25<sup>+</sup> T cells from \( \text{Itgb8} \) cKO mice (CD4-CRE) or CRE<sup>+</sup> littermates. Cells were reactivated with Cell Stimulation Cocktail and Protein Transport Inhibitors and then stained for CD4, CD45.1, CD45.2, and IL-17A. The percentage of IL-17A<sup>+</sup> cells derived from the CD4<sup>+</sup>CD45.1<sup>+</sup>CD45.2<sup>−</sup> cells in the culture are shown in each panel. (B) Same as in (A), except in the presence of IL-2 (100 U/ml). Culture was stained for CD4, CD45.1, CD45.2, and intracellularly for Foxp3. (C and D) Same culture set up as in (A), except using preactivated CD4<sup>+</sup>CD25<sup>+</sup> T cells from \( \text{Itgb8} \) or \( \text{Lrrc32} \) cKO mice or CRE<sup>+</sup> littermates. In the right panel, half of the CD4<sup>+</sup>CD25<sup>+</sup> T cells were from \( \text{Itgb8} \) cKO mice and half were from \( \text{Lrrc32} \) cKO mice. (D) Bar graph indicates the average (± SD) of duplicates within the experiment from (C). (E) Same as in (A), except using sorted CD4<sup>+</sup>Foxp3<sup>+</sup> cells from Foxp3-GFP reporter mice and plate-bound anti-CD3 to activate the cells in the culture. Cells were cultured in the presence of a vehicle control (DMSO), the MMP-inhibitor GM6001, or the GM6001 control and measured for IL-17A as in (A).
activated using soluble anti-CD3 with splenic DCs, which both the Helios+ and Helios−/low Tregs were equally capable of driving Th17 differentiation (Fig. 6B, 6C). Because splenic DCs were unable to restore biologically active TGF-β1 production from Ighb8 KO Tregs (Fig. 5A), it is unlikely that low levels of Ighb8 on the DCs contribute to TGF-β1 activation, but instead, it is possible that the DCs act as a platform to force a close proximity between the Tregs and naive T cells, thus resulting in a decreased threshold for TGF-β1 concentrations. As result, lower levels of biologically active TGF-β1 produced by Helios−/low (Fig. 6A, 6C) are sufficient to drive a full response in the presence of DCs (Fig. 6B, 6C).

Discussion

TGF-β1 is critical to the maintenance of immune homeostasis as deletion of TGF-β1 results in a generalized inflammatory syndrome and autoimmunity. T and B lymphocytes play a critical role in disease development in TGF-β−/− mice as inflammation does not develop in TGF-β−/− mice on a SCID background (19).

Almost every cell type expresses TGF-β1 receptors, but TGF-β1 is always produced in an inactive form associated with LAP that prevents its binding to its receptor. Activation of TGF-β1 is closely regulated so that its effects can be mediated in an appropriate environment. A number of mechanisms have been proposed for the activation of TGF-β1 including plasmin, matrix metalloproteases, lysosomal proteases, and thrombospondin. Recent studies have demonstrated that physiologically, integrins are the key activators of TGF-β1. The strongest evidence in favor of this is that mice with a single point mutation in the RGD sequence of LAP that cannot bind to integrins phenotypically copy mice with a global deficiency of TGF-β1 (20).

Although 6 of the 24 integrins can bind latent TGF-β1 via the RGD sequence in LAP, only αvβ6, αvβ5, αvβ6, and αvβ8 have been demonstrated to liberate active TGF-β1. A number of studies have shown that αvβ6 and αvβ8 are the key activators of TGF-β1 in vivo. Integrin αvβ8 is primarily expressed in epithelial cells and β6−/− mice develop a lung and skin inflammation and pulmonary emphysema (21). The cytoplasmic domain of αvβ8 connects to the actin cytoskeleton and αvβ8-mediated TGF-β1 activation is controlled by cell contraction (2). In contrast, αvβ8 is widely expressed by many different cell types including neurons, astrocytes, airway epithelial cell, fibroblasts, DCs, and T cells. The cytoplasmic domain of αvβ8 does not connect to the actin cytoskeleton. It appears that the role of this integrin is to present latent TGF-β1 to a membrane bound protease resulting in release of active TGF-β1 (3). It is not clear whether αvβ8 uses different mechanisms to activate TGF-β1 in different cell types.

Although most cell types are capable of secreting latent TGF-β1 bound to LTBP as the large latent complex, Tregs and platelets express a unique TGF-β1 binding protein, GARP that targets latent TGF-β1 to the cell surface (1, 13, 14). Within the immune system, αvβ6 expression on leukocytes, particularly CD103+ DCs, appears to be critical in generating active TGF-β1 from the large latent complex (4). In this report, we have demonstrated that Ighb8 is selectively expressed on Treg and mediates the release of active TGF-β1 from the latent TGF-β1/GARP complex. In contrast to the latent TGF-β1/GARP complex whose expression is markedly upregulated during the course of T cell activation, expression of Ighb8 on Tregs was constitutive and only modestly upregulated during activation.

Thus far, mice with selective deficiency of GARP on T-cells cells show no evidence of spontaneous autoimmune disease or inflammation in any organ. Similarly, mice with a deletion of β8 in CD4+ T cells did not have a phenotype (4). In contrast, mice lacking αv or β6 only on DCs develop systemic autoimmunity and colitis and may also have a defect in generating Tregs (4, 16). In addition, mice with a myeloid cell–specific β8 deficiency exhibit a reduction in Th17 cells and are protected from experimental autoimmune encephalomyelitis (5). Importantly, the same myeloid cell must both present the Ag and express the integrin. It is unclear why the expression of αvβ8 on cells on DCs is so critical for the maintenance of immune homeostasis, while loss of αvβ8 expression on Treg does not lead to a defect in immune regulation, at least in the steady state. One possibility is that the number of DCs that express αvβ8 is much higher than the number of αvβ8 expressing Tregs and that they are much more broadly distributed in the body. However, this is clearly not the case because αvβ8 expression is highly restricted to intestinal CD103+ DC, and αvβ8 expression is not detected on splenic DC (16). The most likely explanation for the different phenotypes of β8−/− DC and T cells is that DCs are constantly exposed to the large latent complex at sites where they access it in the extracellular matrix. Furthermore, in the gut, potentiation of Th17 induction may be modulated by the intestinal microflora and Treg induction can be enhanced by the production of retinoic acid by the CD103+ DC. In contrast, although Tregs express relatively high levels of αvβ8 (Fig. 1), Tregs only express the latent TGF-β1/GARP complex when ac-
tivated and the conditions leading to expression of this complex in vivo have yet to be defined.

It remains unclear why Tregs should express a second pathway for the delivery and activation of TGF-β1 on their cell surface. We have demonstrated that Treg-derived active TGF-β1 can play a role both in the differentiation of iTreg and Th17 cells. Tregs may primarily exert their effects via recognition of self-antigens during the process of priming and differentiation of naïve T cells on the surface of Ag-presenting DCs. It is likely that not all DCs express Il6b8 and that Treg intrinsic expression of Il6b8 may be required to facilitate activation of Treg-expressed latent TGF-β1 during the priming/differentiation of naïve T cells. Depending on the makeup of the inflammatory environment (IL-2 versus IL-6), Treg-derived active TGF-β1 would then promote infectious tolerance [existing Tregs driving de novo Treg differentiation (22)] by the generation of iTreg or promote the induction of potentially regulatory or pathogenic Th17 cells.

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Disclosures
The authors have no financial conflicts of interest.

References
Figure S1: CD4^+Foxp3^+ Tregs express αv integrin. Freshly isolated T-cells from pooled lymph nodes or enriched CD4^+ T-cells stimulated overnight with plate bound anti-CD3+IL-2 were stained for CD4, Foxp3, and CD51 (αv integrin, or isotype control).
Figure S2: *Itgb8* conditional knockout mice have normal thymic and Treg development. *Itgb8*^{F/F} mice were crossed to CD4-CRE. (Left) Thymocytes and (Right) cells from pooled lymph nodes were stained for CD4, CD8, and Foxp3. Percentage given for Foxp3+ cells is from the CD4^+CD8^− gate.