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Justin P. Edwards, Angela M. Thornton and Ethan M. Shevach

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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 knockout mice expressed 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 αβ8 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 online version of this article contains supplemental material.

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. Itgb8−/− 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. Ltre3.2fl/fl (GARP) and Tgfb1fl/fl mice have been described previously (1, 8, 9). Fosq2Ko (Helios) 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

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, αvβ6 and αvβ8, 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 αvβ6 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, αvβ8 has a short cytoplasmic tail that does not interact with the actin cytoskeleton. Some studies have demonstrated αvβ6-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 remains unclear whether similar mechanisms are present in Tregs or platelets that physiologically express GARP. It remains unclear whether similar mechanisms are present in Tregs or platelets that physiologically express GARP. The 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 αvβ6 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 αvβ8 is a marker of mouse thymically derived (t)Tregs and functions in a cell-intrinsic manner to release active TGF-β1.


**Results**

**Integrin αβ6 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 αβ6 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 αβ6 and αβ6 by Tregs. Sorted CD4Foxp3+ expressed substantially higher levels of Itgb6 than CD4Foxp3+ Tconvs by qRT-PCR (Fig. 1A). Neither cell type expressed Itgb6 (data not shown). Unfortunately, we could not determine the expression of β6 on a single-cell basis, because no Ab is currently available for staining and FACS analysis. The expression of Itgb6 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 β6 including β1, β3, β5, and β6 (15).

Itgb6 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). Itgb6 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 Itgb6 was higher in Tregs than in CD103+ DCs (relative to GAPDH). As previously described (17), the level of Itgb6 was substantially higher in CD103+ DCs from

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**FIGURE 1.** Integrin αβ6 is expressed by Foxp3+ Tregs. (A) Sorted CD4Foxp3+ (GFP+) and CD4Foxp3+ (GFP−) were measured for Itgb6 and Foxp3 message as measured by qRT-PCR. Data are expressed relative to CD4Foxp3+ cells. (B and C) Sorted CD4Foxp3+ and CD4Foxp3+ from CD4 cKOs of Tgb1 and Lrc32 and their CRE− littermate controls were measured for Itgb6 message. Data are expressed relative to WT CD4+ CD25+ cells. (D) Sorted CD4Foxp3+ (GFP+) and CD4Foxp3+ (GFP−) from spleen, peripheral lymph nodes, or mesenteric lymph nodes were measured for Itgb6 message as measured by qRT-PCR. (E) Sorted CD4Foxp3+ and CD4Foxp3+ (GFP−) (fresh or after overnight stimulation with plate-bound anti-CD3-IL-2), CD11b+CD103+, and CD11b+ CD103+ DCs from the mesenteric LNs, and CD11b+ or CD11b− DCs were measured for Itgb6 message. DCs were first gated on Thy1.2 CD19+ CD11c+I-Aβ− 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 iTregs also express Igfb8 (1). iTregs were generated from naive 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 Igfb8 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 Igfb8, 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 peripherally derived Tregs, respectively, the relative expression of Igfb8 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 Igfb8 (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 Igfb8, because Tregs sorted from HcZ/2(Helios)Ikzf2 CD4-CRE cKO mice maintained their expression of Igfb8 (Fig. 2D).

Igfb8 is required for clearance of TGF-β1 from the GARP/latent-TGF-β1 complex

To investigate the function of Igfb8 on Tregs, we bred CD4-cKOs of Igfb8, using the previously described Igfb8GFPF1 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⁻ littermates. The percentages of CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ T cells in the periphery were also identical in CRE⁺ and CRE⁻ littermates (Supplemental Fig. 2, right panels).

We hypothesized that if αIβ8 is involved in activation of TGF-β1 from the GARP/latent-TGF-β1 complex, that in the absence of Igfb8, latent-TGF-β1 would accumulate on the surface of Tregs. Freshly isolated Tregs from WT and Igfb8 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. Igfb8 cKO mice also expressed higher levels of GARP and GARP/LAP double-positive cells (Fig. 3B).

Igfb8 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 Igfb8 is required for suppression of T effector cell proliferation in vitro. Using the traditional T cell suppression assay, Tregs from both Igfb8 cKO and WT floxed littermates 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 Igfb8 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 Igfb8 cKO Tregs, we used an in vitro culture system in which activated Tregs are cultured with naive T cells under activating conditions (splenic DCs with soluble anti-CD3) in the

*FIGURE 2. (A) Naive CD4⁺ T cells (CD44hiCD62Lhi) from Foxp3-GFP mice were cultured with plate-bound anti-CD3 (iTreg PB-anti-CD3) or DCs and soluble anti-CD3 (iTreg DCs + soluble anti-CD3). Naive CD4⁺ cells were also sorted from OT-II Foxp3-GFP mice and cultured with splenic DCs and cognate peptide (iTreg OT-II w/ DCs + peptide). In all conditions, cells were cultured in the presence of recombinant TGF-β1 and IL-2 for 4 d. For the same experiment, naïve T cells (TcNorms) or CD4⁺ GFP⁺ (Foxp3⁺) cells were cultured on plate-bound anti-CD3 with IL-2 for 4 d. (B) Sorted CD4⁺GFP⁺ (Helios⁺)RFP⁺(Foxp3⁻), CD4⁺GFP⁺ RFP⁻, and CD4⁺GFP⁻RFP⁻ cells from double-reporter mice were measured for Igfb8 message. (C) Sorted CD4⁺GFP⁺ (Helios⁺)RFP⁻(Foxp3⁻), CD4⁺GFP⁻RFP⁺, and CD4⁺GFP⁻RFP⁻ cells from double-reporter mice were stimulated with plate-bound anti-CD3+IL-2 for 3 d then rest overnight in IL-2. The cells were then measured for Igfb8 message by qRTPCR. (D) Sorted CD4⁻CD25⁻ and CD4⁻CD25⁺ from CD4 cKOs of HcZ/2 and their CRE⁻ littermate controls were measured for Igfb8 message. (A), data are expressed relative to T conventional cell population; in (B), data are expressed relative to Helios⁺Foxp3⁻ cells; in (C), data are expressed relative to expanded (CD4⁺Foxp3⁻) non-Treg population; and in (D), data are expressed relative to WT CD4⁺CD25⁻ cells.
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 could result in the release of biologically active 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 αvβ8 expressed on one cell could result in the activation of TGF-β1 from the GARP/latent-TGF-β1 complex on a distinct cell (Fig. 5C, 5D). Previous studies indicated 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...
GM6001. 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 αvβ8-mediated activation of latent TGF-β1 activation from the GARP/latent TGF-β1 complex is dependent on MMPs.

Helios−/low Tregs are less efficient at producing biologically active TGF-β1

Because we found that Helios+ Tregs expressed substantially higher levels of Itgb8 (Fig. 2B), we wished to assess the ability of the Helios+ versus Helios−/low 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+ Tregs were more efficient at driving Th17 differentiation (Fig. 6A, 6C) than Helios−/low cells. This result is consistent with the higher level of expression of Itgb8 on the Helios+ cells. It is not due to an inability of the Helios−/low cells to express latent TGF-β1 on their surface, because total Tregs activated in vitro when gated on the Helios−/low or Helios+ subsets both have similar levels of latent TGF-β1 on their surface (Fig. 6D). It also remains possible that Helios−/low Tregs can process the latent-TGF-β1 by an αvβ8-independent mechanism. Surprisingly, when the cocultures were...
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 Itgb8 KO Tregs (Fig. 5A), it is unlikely that low levels of Itgb8 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-β1−/− mice as inflammation does not develop in TGF-β1−/− 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 integrins are unable to restore biologically active TGF-β1 expression on leukocytes, particularly CD103+ DCs, to the level of Tregs expressing TGF-β1 (4). In this report, we have demonstrated that Itgb8 on Tregs is constitutive and only modestly upregulated during the course of T cell activation, expression of Itgb8 on Tregs was constitutive and only modestly upregulated during activation.

Thus far, mice with selective deficiency of GARP on T 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 Tregs 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 Itgb8 and that Treg intrinsic expression of Itgb8 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
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