β7 Integrin Inhibition Can Increase Intestinal Inflammation by Impairing Homing of $\text{CD}25^{hi}\text{FoxP3}^+$ Regulatory T Cells

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SUMMARY
This work proposes that the combination of defective homing and reduced intrinsic regulatory T cell function synergize to reduce regulatory T-cell function sufficiently in the gut to counteract the protective effect of β7 deficiency on homing of conventional T cells, thus exacerbating intestinal inflammation.

BACKGROUND & AIMS: Integrin α4β7 mediates lymphocyte trafficking to the gut and gut-associated lymphoid tissues, a process critical for recruitment of effector lymphocytes from the circulation to the gut mucosa in inflammatory bowel disease (IBD) and murine models of intestinal inflammation. Antibody blockade of β7 integrins generally is efficacious in IBD; however, some patients fail to respond, and a few patients can experience exacerbations. This study examined the effects of loss of β7 integrin function in murine models of IBD.

METHODS: In a mouse IBD model caused by lack of interleukin 10, a cytokine important in CD25hiFoxP3+ regulatory T cell (Treg) function, genetic deletion of β7 integrin or antibody blockade of α4β7-mucosal addressin cell adhesion molecule-1 (MAdCAM-1) interaction paradoxically exacerbated colitis.lovulation of leukocytes from the blood stream to the intestine via cell adhesion and transmigration through blood vessel walls, leading to massive infiltration of mononuclear phagocytes, neutrophils, and intestinal T cells into the intestinal lamina propria.

RESULTS: Loss of β7 impaired the capacity of Tregs homing to the gut and therefore suppress intestinal inflammation in an adoptive T-cell transfer model; however, the intrinsic suppressive function of β7-deficient Tregs remained intact, indicating that the β7 deficiency selectively impacts gut homing. Deletion of β7 integrin did not worsen colitis in an acute dextran sodium sulfate model in which Treg number and function were normal.

CONCLUSIONS: In Integrin subunit beta (Itgb)7−/− mice, loss of β7-dependent Treg homing to gut-associated lymphoid tissues combined with loss of intrinsic Treg function exacerbated intestinal inflammation. These results suggest that IBD patients with reduced CD25hiFoxP3+ Treg numbers or function or lack of interleukin 10 could be at risk for failure of α4β7 blocking therapy. 

Keywords: Integrin β7 Blockade; Inflammatory Bowel Disease; Regulatory T Cells; Gut-Associated Lymphoid Tissue.

Abbreviations used in this paper: CD, Crohn’s disease; CFSE, carboxyfluorescein succinimidyl ester; DSS, dextran sodium sulfate; GALT, gut-associated lymphoid tissue; GFP, green fluorescent protein; IBD, inflammatory bowel disease; Itgb, Integrin subunit beta; IL, interleukin; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MLN, mesenteric lymph node; PCR, polymerase chain reaction; PLN, peripheral lymph node; PP, Peyers’s patch; SP, spleen; Tconv, conventional T cells; TGF, transforming growth factor; Th, helper T cell; Treg, regulatory T cell; UC, ulcerative colitis; WT, wild-type.

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Results

**β7 Deficiency Exacerbates Spontaneous Colitis in Il10⁻/⁻ Mice**

Integrin α4β7 mediates trafficking of Tconv to gut and GALT and antibody blockade of α4β7 benefits many patients with either CD or UC. To assess the role of β7 integrin in the development of chronic intestinal inflammation in a mouse model, we used an IL10-deficient mouse strain (B6.129P2-Il10tm1Cgn/J) that develops chronic colitis resembling IB in human beings. IL10-deficient mice were crossed with β7-deficient mice, and the phenotypes of Integrin subunit beta (Itgb7)⁻/⁻Il10⁻/⁻ mice were compared with Itgb7⁺/+Il10⁻/⁻ mice.

As expected, Itgb7⁺/+Il10⁻/⁻ mice spontaneously developed diarrhea beginning at the age of 60–70 days under specific pathogen-free conditions in our animal facility. On average, the mice did not lose weight; however, 6 of 22 mice died by 70 days (Figure 1B), and 7 of 22 mice developed rectal prolapse. Unexpectedly, β7 deficiency exacerbated disease in Il10⁻/⁻ mice as judged by dramatic weight loss accompanied by severe diarrhea and rectal bleeding (Figure 1A and C). Furthermore, Itgb7⁻/⁻Il10⁻/⁻ mice almost uniformly developed rectal prolapse (Figure 1D) and more than two thirds of them died by approximately 70 days (Figure 1B). Itgb7⁻/⁻Il10⁻/⁻ mice were more anemic than integrin β7–replete Itgb7⁺/+Il10⁻/⁻ animals (Figure 1E). The exacerbated colitis in Itgb7⁻/⁻Il10⁻/⁻ mice was confirmed by significantly increased crypt distortion, mucosal ulceration, and infiltration of immune cells, compared with Itgb7⁻/⁻/Il10⁻/⁻ mice (Figure 1F and G). In addition, colonic expression of proinflammatory cytokines was increased significantly in Itgb7⁻/⁻Il10⁻/⁻ mice compared with Itgb7⁺/+Il10⁻/⁻ mice (Figure 2).

To assess the contribution of β7 integrin further in mediating leukocyte recruitment and subsequent damage to the gut mucosa, we used piroxicam administration as a method to synchronize development of colitis in IL10-deficient mice. We administered piroxicam to IL10-null mice orally for 2 weeks. By 35 days after piroxicam initiation, approximately 80% of Itgb7⁺/+Il10⁻/⁻ mice died. In contrast, only approximately 20% of Itgb7⁻/⁻Il10⁻/⁻ mice died (Figure 1H). Thus, β7 deficiency exacerbated both spontaneous and induced IBD in IL10-deficient mice, a surprising result in light of the benefits of blockade of α4β7 in many IBD patients.
colonic Tregs in Itgb7<sup>-/-</sup>Il10<sup>-/-</sup> mice. Moreover, the number of Tregs in mesenteric lymph nodes (MLNs) also was reduced significantly in Itgb7<sup>-/-</sup>Il10<sup>-/-</sup> mice. However, the Treg numbers in peripheral lymph nodes (PLNs) was similar to Itgb7<sup>+/+</sup>Il10<sup>-/-</sup> mice (Figure 6). Thus, in this colitis model, the absence of β7 causes a profound reduction

**Figure 1.** Loss of β7 expression exacerbated spontaneous colitis induced by IL10 deficiency. Changes in (A) body weight, (B) survival ratio, (C) stool consistency and rectal bleeding, and (D) rectal prolapse occurrence in Itgb7<sup>+/+</sup>Il10<sup>-/-</sup> mice (n = 22) and Itgb7<sup>-/-</sup>Il10<sup>-/-</sup> mice (n = 25). Changes in body weight are shown as a percentage of the original weight. The stool consistency was as follows: 0 (normal), 1 (soft), 2 (very soft), and 3 (diarrhea); the rectal bleeding score was as follows: 0 (none), 1 (red), 2 (dark red), and 3 (gross bleeding). Data represent means ± SEM. Two-way analysis of variance with the Bonferroni posttest. (E) Concentration of hemoglobin in peripheral blood from Itgb7<sup>+/+</sup>Il10<sup>-/-</sup> mice (n = 20) and Itgb7<sup>-/-</sup>Il10<sup>-/-</sup> mice (n = 15) at day 75 are shown. The concentration of hemoglobin in peripheral blood from Itgb7<sup>+/+</sup>Il10<sup>-/-</sup> mice was approximately 13–14 g/dL. Data represent means ± SEM. Two-tailed t-test. (F and G) Representative H&E staining of Swiss rolls of distal colon sections from Itgb7<sup>+/+</sup>Il10<sup>-/-</sup> mice (n = 20) and Itgb7<sup>-/-</sup>Il10<sup>-/-</sup> mice (n = 15) at day 75. (F) Scale bars: 500 μm. (G) Histology score was as described in the Material and Methods section. Data represent means ± SEM. Two-tailed t-test. (H) Survival ratio of Itgb7<sup>+/+</sup>Il10<sup>-/-</sup> mice (n = 15) and Itgb7<sup>-/-</sup>Il10<sup>-/-</sup> mice (n = 14) after piroxicam treatment (200 ppm) for 2 weeks. Data represent means ± SEM. Two-way analysis of variance with the Bonferroni posttest. *, P < .05; **, P < .01; ***, P < .001. WT, Itgb7<sup>+/+</sup>Il10<sup>-/-</sup> mice; Itgb7<sup>-/-</sup> Ilgb7<sup>-/-</sup>Il10<sup>-/-</sup> mice. (E and G) Data include surviving mice from panel A and additional mice that were not assessed for daily weights.
Figure 2. RNA expression of cytokines in **Itgb7**+/+/*Il10**-/− mice and **Itgb7**−/−/*Il10**−/− mice. Messenger RNA (mRNA) expression of IL-1β, tumor necrosis factor (TNF)-α, IL6, interferon (IFN)γ, and IL17A in distal colon tissue from **Itgb7**+/+/*Il10**−/− mice (n = 13) and **Itgb7**−/−/*Il10**−/− mice (n = 12). Results are normalized to glyceraldehyde-3-phosphate dehydrogenase. Data represent means ± SEM. Two-tailed t test. *P < .01, **P < .01, ***P < .001. WT, **Itgb7**+/+/*Il10**−/− mice; **Itgb7**−/−, **Itgb7**−/−/*Il10**−/− mice.

A

Colonic Lamina propria

B

Spleen

C

Colonic Lamina propria (piroxicam)

Figure 3. Loss of β7 expression impaired migration of Tregs to the colon in IL10-deficient mice. (A and B) Number and percentage of Tregs in CD4+ T cells in (A) colonic lamina propria and (B) spleen from **Itgb7**+/+/*Il10**−/− mice (n = 8) and **Itgb7**−/−/*Il10**−/− mice (n = 8). (C) Number and percentage of Tregs in CD4+ T cells in colonic lamina propria from **Itgb7**−/−/*Il10**−/− mice (n = 12) and **Itgb7**−/−/*Il10**−/− mice (n = 12) after piroxicam treatment (200 ppm) for 2 weeks. Data represent means ± SEM. Two-tailed t test. *P < .05, **P < .01, ***P < .001. WT, **Itgb7**+/+/*Il10**−/− mice; **Itgb7**−/−, **Itgb7**−/−/*Il10**−/− mice.
of Tregs in GALT; however, with the exception of αEβ7, β7-null Tregs that enter the colon express a typical repertoire of Treg-associated markers and transcription factors.

**Integrin β7–Deficient CD4+ T Cells Ameliorate Adoptive T-Cell–Transfer–Induced Colitis**

The foregoing results led us to ask whether the deleterious effects of loss of β7 function were mediated by effects on Tconv or Tregs. To investigate the role of β7 in Tconv cells and their capacity to induce colitis, we transferred CD4+CD25+CD45RBhigh T cells (Tconv cells) from wild-type (WT) mice or Itgb7−/− mice into Rag1−/− recipient mice. Rag1−/− mice injected with WT Tconv cells showed a dramatic loss in body weight starting at 20–30 days, and one third of the mice died by 90 days (Figure 7A and B). In contrast, Rag1−/− mice injected with Itgb7−/− Tconv cells showed a significantly milder and delayed weight loss compared with WT Tconv cells and most of the mice survived (Figure 7A and B).

We next tested the role of integrin β7 on CD4+ T-cell migration using the competitive homing assay. β7-deficient CD4+ T cells were impaired for homing to MLNs and Peyr's patch (PP), but not PLNs and spleen (SP) (Figure 7C), which is consistent with previous studies. Thus, β7-deficient CD4+ T cells manifest a defective migration to the colon, which reduces adoptive T-cell–transfer–induced colitis.

**Integrin β7–Deficient Tregs Are Defective in the Capacity to Prevent Colitis**

We examined the effect of β7 deficiency on the ability of Tregs to prevent intestinal inflammation in the adoptive T-cell transfer model. Transfer of CD4+CD25+CD45RBhigh T cells (CD4+ Tconv) into Rag1−/− mice led to a severe colitis by 6–12 weeks after cell transfer. Loss of body weight started after 20 days (Figure 8A), and half of the mice had died by 90 days (Figure 8B). As expected, co-injection of Itgb7+/+ Tregs with Tconv prevented colitis (Figure 8A and B), whereas injection of Itgb7−/− Tregs with Tconv did not prevent disease. At 90 days, the disease markers in the mice receiving co-administered β7-deficient Tregs was similar to that of those receiving only Tconv (Figure 8C and D). Moreover, expression of proinflammatory cytokines in the colon was not significantly different in mice receiving Itgb7−/− Tregs compared with Tconv alone, whereas those that had received Itgb7+/+ Tregs showed only minimal cytokine expression (Figure 8E). These results show that β7 deficiency impairs the capacity of Tregs to prevent adoptive T-cell–transfer–induced colitis, whereas it impairs the capacity of Tconv to cause colitis.

**Defective Suppression of Colitis in β7-Deficient Tregs Is Ascribable to Defective Homing**

To evaluate the possibility that integrin β7-deficient Tregs lacked suppressive capacity we examined their ability to inhibit T-cell proliferation. Green fluorescent protein positive (GFP+) Tregs were sorted from Itgb7+/+Foxp3GFP or Itgb7−/−Foxp3GFP mice and their capacity to suppress proliferation of CD4+CD25+ responder cells was compared in a dye dilution assay. Carboxyfluorescein succinimidyl ester (CFSE)-labeled responder cells stimulated with immobilized anti-CD3 and anti-CD28 in the presence of IL2 for 4 days at 37°C proliferated vigorously as indicated by the dilution of fluorescence (Figure 9A). The addition of a 1:1 ratio of β7-deficient or replete Tregs markedly reduced proliferation in these cultures (Figure 9A). The proliferation index as a function of Treg/responder ratio was not significantly different between cultures with Treg lacking β7 and those sufficient for β7 (Figure 9B). Furthermore, β7-deficient Tregs showed similar expression of the antiinflammatory cytokines IL10 and transforming growth factor (TGF)-β as wild-type Tregs (Figure 9C). Thus, β7-deficient Tregs are functionally intact with regard to their suppressive functions.
The failure of \textit{Itgb7}^-/- Tregs to efficiently suppress colitis despite normal intrinsic suppressive function suggested that defective homing of \textit{Itgb7}^-/- Tregs may explain their reduced capacity to block colon inflammation. To test this idea, we used a competitive homing assay with GFP^+ Tregs isolated from \textit{Itgb7}^+/+ Foxp3^{GFP} or \textit{Itgb7}^-/- Foxp3^{GFP} mice, labeled them with a 1 \mu mol/L and 10 \mu mol/L concentration of eFluor670 proliferation dye, respectively, and co-injected equal numbers (1 \times 10^7) of differentially labeled cells into recipient mice. Lymphoid organs were harvested 3 hours after injection, and cells were isolated and analyzed by flow cytometry. Similar to the known effects of deficiency on Tconv homing (Figure 7C), \beta7-deficient Tregs homing to PP was reduced by approximately 90% compared with \beta7-sufficient cells, whereas homing to MLN was reduced modestly (~50%). In contrast, homing of Tregs to PLN and SP were similar in \textit{Itgb7}^-/- and \textit{Itgb7}^+/+ Tregs (Figure 9D).

The absolute cell numbers of both WT or \textit{Itgb7}^-/- Tregs in the different organs also were checked (Figure 10). Thus, there is a profound reduction in Tregs in the GALT of \textit{Itgb7}^-/- mice that is attributable, at least in part, to defective homing of these cells, and this reduction can account for the exacerbation of colitis on an \textit{Il10}^-/- background.

\section*{\beta7 Deficiency Does Not Exacerbate Acute Dextran Sulfate Sodium–Induced Colitis}

Increased colitis in \textit{Itgb7}^-/-\textit{Il10}^-/- mice seemed paradoxical in light of the therapeutic efficacy of \alpha4\beta7 blockade in IBD patients. We reasoned that the combination of reduced functionality of Tregs lacking IL10 combined with a reduction of Tregs in the GALT of \beta7-deficient mice might synergistically reduce Treg function in the gut sufficient to exacerbate colitis. To test this idea, we examined the effect
of β7 deficiency in a colitis model in which Treg function was normal. We administered dextran sulfate sodium (DSS) for 7 days and then analyzed the mice for an additional 7 days, times at which inflammation is dominated largely by the innate immune response to bacterial products that breach the intestinal barrier as a consequence of mucosal injury.31,35 Subsequently, an adaptive immune component can contribute to further chronic inflammation,36 thus potentially accounting for the finding by Zhang et al that β7 deficiency exacerbated a later stage of DSS colitis.30 Both Itgb7+/+ and Itgb7/− mice showed similar rapid body weight loss during the initial phase of DSS treatment. Once DSS administration was halted, Itgb7/− mice regained weight slightly but significantly more rapidly than Itgb7+/+ mice (Figure 11A). Itgb7−/− mice showed less mucosal immune cell infiltration and crypt destruction (Figure 11B), as shown by lower histologic scores (Figure 11C), and further supported by less anemia (Figure 6D). Thus, in sharp contrast to the IL10-deficient model, β7 deficiency did not exacerbate inflammation in this acute DSS model of colitis.

**Antibody Blockade of α4β7–MAdCAM-1 Interaction Aggravates Spontaneous Colitis Induced by IL10 Deficiency**

The foregoing studies showed that genetic inactivation of integrin β7 exacerbated colitis in Itgb7−/−/Il10−/− mice and ascribed the exacerbation to a combination of reduced Treg function and homing to GALT. These results raise 2 important issues: (1) β7 can combine with αE or α4; can loss of only α4β7 function exacerbate colitis in IL10-null mice? (2) Itgb7−/− mice lack β7 function throughout development: will loss of α4β7 function in an adult IL10-deficient mouse exacerbate colitis? MAdCAM-1, which is expressed preferentially on gut and GALT-associated endothelial cells, plays a vital role in α4β7-mediated leukocyte trafficking to the GALT and is a primary ligand of integrin α4β7. Because we did not have access to a function blocking antimurine α4β7, Il10−/− mice

![Figure 6. Loss of β7 expression on migration of Tregs to the MLN in IL10-deficient mice. The number of Tregs in MLN and PLN from Itgb7−/−/Il10−/− mice (n = 6) and Itgb7−/−/Il10−/− mice (n = 6) Data represent means ± SEM. One-way analysis of variance with the Bonferroni posttest. ***P < .001.](image)

![Figure 7. β7-deficient CD4+ T cells ameliorate adoptive T-cell-transfer–induced colitis. (A and B) CD4+CD25+CD45RBhigh Tconv cells (1 × 10⁶) from Itgb7−/− or Itgb7−/− mice were injected into Rag1−/− mice. Changes in (A) body weight and (B) survival ratio are shown. Changes in body weight are shown as a percentage of the original weight. The number of mice in each group is indicated. Data represent means ± SEM. Two-way analysis of variance with the Bonferroni posttest. (C) In vivo competitive homing of CD4+ T cells to lymphoid tissues. CD4+ T cells were isolated from either Itgb7−/− or Itgb7−/− mice, differentially labeled, and mixed before injection into C57BL/6 mice. CD4+ T cells homing to different lymphoid organs were analyzed by flow cytometry 3 hours after injection. The ratio of Itgb7−/− CD4+ T cells to Itgb7−/− CD4+ T cells (Itgb7−/−/WT) from different lymphoid organs is shown (n = 14). Data represent means ± SEM. One-way analysis of variance with the Bonferroni posttest. **P < .01. WT Tconv, Tconv cells from Itgb7−/−/− mice; Itgb7−/− Tconv, Tconv cells from Itgb7−/− mice.)
Discussion

Leukocyte homing to GALT has complex roles in the pathogenesis of IBD.15,40–42 Antibodies against β7 integrins, which inhibit leukocyte trafficking to the gut and GALT, are not uniformly efficacious at ameliorating IBD.15 Here, we report that mice lacking IL10, a major immunosuppressive cytokine of Tregs, unexpectedly developed increased intestinal inflammation when β7 was genetically inactivated or α4β7-mediated GALT homing was blocked by antibodies. These effects of loss of β7 function were associated with reduced Tregs in the lamina propria owing to impaired Treg homing to the gut. In an adoptive transfer model of intestinal inflammation, we found that β7-deficient Tregs are impaired in their capacity to colonize GALT and to oppose intestinal inflammation despite possessing normal intrinsic suppressive functions. We propose that the combination of reduced Treg function in IL10-null mice and reduced Treg homing owing to loss of β7 function, in combination, decrease net Treg suppressive activity in the gut sufficiently to counteract the protective effect of blocking β7 on homing of conventional T cells. Thus, Treg numbers or functions could affect the efficacy of β7 blockade in IBD (Figure 13).
Surprisingly, loss of $\beta 7$ function in mice lacking IL10 caused increased intestinal inflammation. Genetic $\beta 7$ deficiency aggravated spontaneous colitis in IL10-deficient mice as judged by dramatic body weight loss accompanied by severe diarrhea and rectal bleeding. These symptoms were associated with histologic evidence of increased tissue damage and inflammation, worsened anemia, and increased colonic expression of proinflammatory cytokines. Importantly, because we were obliged to breed $\text{Il10}^{-/-}$ mice through crosses of $\text{Il10}^{+/+}$ mice, it was not feasible to generate sufficient numbers of littermate $\text{Ilbg7}^{-/-}\text{Il10}^{+/+}$ and $\text{Ilbg7}^{+/+}\text{Il10}^{-/-}$ controls. Because $\beta 7$ deficiency can impact the intestinal flora, even though $\text{Ilbg7}^{-/-}$ null and WT mice were on a C57 BL/6 background and were cohoused, it is possible that some of the effects of $\beta 7$ deficiency could be the result of an altered microbiome. That said, $\text{Il10}^{+/+}$ mice experienced a similar worsening of colitis after antibody blockade of $\alpha 4 \beta 7$-MadCAM-1 interaction in comparison with cohoused IgG-treated littermates. Furthermore, in the adoptive transfer model of colitis, we documented that $\beta 7$-null Tregs were defective in suppressing intestinal inflammation compared with WT Tregs when administered to cohoused littermate mice. These results sharply contrast with the beneficial effects of $\alpha 4 \beta 7$-blocking antibody in patients with active UC or CD. Earlier studies in mice also reported that inhibiting $\beta 7$-integrin function attenuated acute and chronic murine models of intestinal inflammation. Thus, loss of $\beta 7$ integrin function causes increased intestinal inflammation in IL10-deficient mice despite its often beneficial effects in other murine models and in human beings with IBD.

Vedolizumab blocks integrin $\alpha 4 \beta 7$ on both effector T cells and Tregs. Recent studies have reported that $\alpha 4 \beta 7$ is crucial for controlling homing of Tregs in patients with UC to the inflamed colon in vivo, and $\beta 7$ deficiency causes Treg depletion in the gut. Nevertheless, our data suggest that combined loss of Treg function and $\beta 7$-dependent Treg homing to gut and GALT together can counteract a protective effect of $\beta 7$ blockade of effector T-cell migration on intestinal inflammation. Integrin $\text{Itbg7}^{-/-}$ Tregs were defective in their capacity to prevent induction of colitis in immune-deficient mice reconstituted with Tconv. We note that Denning et al reported that $\text{Itbg7}^{-/-}$ Tregs could prevent colitis in their adoptive transfer model, a difference that might be a consequence of their use of a different...
**Figure 10.** Cell number of homed Tregs in different organs in the competitive homing assay. In vivo competitive homing of (A) CD4⁺ T cells or (B) Tregs to lymphoid tissues. (A) CD4⁺ T cells or (B) Tregs were isolated from either WT or Itgb7⁻/⁻ mice, differentially labeled and mixed before injection into C57BL/6 mice. The absolute cell numbers of WT or Itgb7⁻/⁻ (A) CD4⁺ T cells or (B) Tregs in different lymphoid organs was shown (n = 14). Data represent means ± SEM. One-way analysis of variance with the Bonferroni posttest. ***P < .001. WT, Itgb7⁺/⁺ mice; Itgb7⁻/⁻, Itgb7⁻/⁻ mice.

**Figure 11.** β7 deficiency does not exacerbate DSS-induced acute colitis. Eight-week-old Itgb7⁺/⁺ and Itgb7⁻/⁻ mice were treated with 1.5% DSS for 7 days, followed by regular drinking water. (A) Changes in body weight are shown as a percentage of the original weight. Data represent means ± SEM. Two-way analysis of variance with the Bonferroni posttest. (B) Representative H&E staining of Swiss rolls of distal colon sections at day 14. Scale bars are labelled in the images. (C) Histology score was determined as described in the Materials and Methods section. (D) Hemoglobin concentration and hematocrit in peripheral blood from Itgb7⁺/⁺ and Itgb7⁻/⁻ mice at day 14 are shown. Data represent means ± SEM. Two-tailed t test. *P < .05, **P < .01. WT, Itgb7⁺/⁺; Itgb7⁻/⁻, Itgb7⁻/⁻ mice.
immunodeficient recipient mouse strain. Because Itgb7−/− Tregs manifested intact suppressor function in vitro, this defect likely was owing to their reduced ability to populate the gut and GALT, a reduction we can ascribe to reduced migration to the GALT as shown in competitive homing experiments. Furthermore, vedolizumab can block the homing of Tregs from UC patients to the inflamed gut. IL10 is a product of Tregs that is essential for the maintenance of intestinal homeostasis. It suppresses effector functions of T helper (Th)1/Th17 cells as well as natural killer cells and macrophages, thereby modulating both innate and adaptive immune responses and muting pathogenic Th17 responses to pathobionts. Tregs are a major source of IL10 to maintain homeostasis at the environmental interface of the intestine. Thus, reduced homing of Itgb7−/− Tregs to gut and GALT, combined with a lack of IL10, together will profoundly reduce Treg suppression of inflammation in the colon. In contrast, Itgb7−/− mice did not experience an exacerbation of the acute DSS model in which Treg function was not impaired. IBD can have a variety of underlying causes such as defects in the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome or defective Tregs. We show that reduced integrin β7-mediated homing in the setting of reduced loss of IL10 exacerbates intestinal inflammation. Thus, loss of IL10 expression or reduced Treg numbers or functions in IL10-deficient mice were injected intraperitoneally with anti-MAdCAM-1 blocking antibody MECA367 (100 μg/mouse) every 4 days. (A and B) Changes in (A) body weight and (B) survival ratio of murine IgG control- or MECA367-treated IL10-deficient mice. Changes in body weight are shown as a percentage of the original weight. Data represent means ± SEM. Two-way analysis of variance with the Bonferroni posttest. (C) Concentration of hemoglobin (HB) in peripheral blood at day 45 posttreatment is shown. Data represent means ± SEM. Two-tailed t test. (D and E) Representative H&E staining of distal colon sections from murine IgG control- or MECA367-treated IL10-deficient mice. (D) Scale bars: 250 μm. (E) Histology score was assessed as described in the Material and Methods section. Data represent means ± SEM. Two-tailed t test. *P < .01, **P < .01, ***P < .001. mIgG, Mouse Immunoglobulin G.

IL10 is a product of Tregs that is essential for the maintenance of intestinal homeostasis. It suppresses effector functions of T helper (Th)1/Th17 cells as well as natural killer cells and macrophages, thereby modulating both innate and adaptive immune responses and muting pathogenic Th17 responses to pathobionts. Tregs are a major source of IL10 to maintain homeostasis at the environmental interface of the intestine. Thus, reduced homing of Itgb7−/− Tregs to gut and GALT, combined with a lack of IL10, together will profoundly reduce Treg suppression of inflammation in the colon. In contrast, Itgb7−/− mice did not experience an exacerbation of the acute DSS model in which Treg function was not impaired. IBD can have a variety of underlying causes such as defects in the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome or defective Tregs. We show that reduced integrin β7-mediated homing in the setting of reduced loss of IL10 exacerbates intestinal inflammation. Thus, loss of IL10 expression or reduced Treg numbers or functions in IL10-deficient mice were injected intraperitoneally with anti-MAdCAM-1 blocking antibody MECA367 (100 μg/mouse) every 4 days. (A and B) Changes in (A) body weight and (B) survival ratio of murine IgG control- or MECA367-treated IL10-deficient mice. Changes in body weight are shown as a percentage of the original weight. Data represent means ± SEM. Two-way analysis of variance with the Bonferroni posttest. (C) Concentration of hemoglobin (HB) in peripheral blood at day 45 posttreatment is shown. Data represent means ± SEM. Two-tailed t test. (D and E) Representative H&E staining of distal colon sections from murine IgG control- or MECA367-treated IL10-deficient mice. (D) Scale bars: 250 μm. (E) Histology score was assessed as described in the Material and Methods section. Data represent means ± SEM. Two-tailed t test. *P < .01, **P < .01, ***P < .001. mIgG, Mouse Immunoglobulin G.

Figure 12. Blocking MAdCAM-1 interaction with α4β7 integrin worsened spontaneous colitis induced by IL10 deficiency. The IL10-deficient mice were injected intraperitoneally with anti-MAdCAM-1 blocking antibody MECA367 (100 μg/mouse) every 4 days. (A and B) Changes in (A) body weight and (B) survival ratio of murine IgG control- or MECA367-treated IL10-deficient mice. Changes in body weight are shown as a percentage of the original weight. Data represent means ± SEM. Two-way analysis of variance with the Bonferroni posttest. (C) Concentration of hemoglobin (HB) in peripheral blood at day 45 posttreatment is shown. Data represent means ± SEM. Two-tailed t test. (D and E) Representative H&E staining of distal colon sections from murine IgG control- or MECA367-treated IL10-deficient mice. (D) Scale bars: 250 μm. (E) Histology score was assessed as described in the Material and Methods section. Data represent means ± SEM. Two-tailed t test. *P < .01, **P < .01, ***P < .001. mIgG, Mouse Immunoglobulin G.

Figure 13. Schematic diagram of β7 function in gut inflammation. During gut inflammation, a disrupted protective mucus layer and epithelial barrier leads to increased uptake of luminal bacteria. Bacterial antigens result in the activation of immune cells such as T cells and macrophages, with the consequent release of proinflammatory cytokines, with further recruitment of leukocytes. β7 blockade suppresses effector T-cell migration to the gut, thereby ameliorating inflammation. However, it additionally reduced Treg homing to the gut, counteracting their anti-inflammatory activity.
patients with IBD may impact their response to therapeutic inhibition of β7 integrins.

Materials and Methods

Antibodies and Reagents

The following antibodies were from BioLegend (San Diego, CA): CD4 (GK1.5), β7 (FIB504), Foxp3 (MF-14), anti-CD3 (2C11), anti-CD28 (37.51), IL10 (JES5-16E3), TGF-β1 (TW7-16B4), and anti-MAdCAM-1 (MECA367). Secondary AlexaFluor-labeled antibodies were from Jackson ImmunoResearch (West Grove, PA). The Foxp3 transcription factor fixation/permeabilization kit was purchased from eBioscience. CFSE and eFluor®70 were purchased from Invitrogen (Carlsbad, CA) and BioLegend, respectively. Pirroxicam was from MilliporeSigma (Burlington, MA). Ionomycin, brefeldin A, and monensin were from BioLegend. The MojoSort mouse CD3 T-cell isolation kit and mouse CD4 T-cell isolation kit were from BioLegend. Liberase TL (research grade) and DNAse I were from Roche (Basel, Switzerland). Recombinant mouse MAdCAM-1–Fc was purified by protein A beads as previously described.39

Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California San Diego, and were conducted in accordance with federal regulations as well as institutional guidelines and regulations on animal studies. All mice were housed in specific pathogen-free conditions on corncob bedding and were fed ad libitum with a chow diet. C57BL/6 (CD45.1), C57BL/6 (CD45.2), Itgb7+/-, Il10+/-, Rag1+/- mice were from The Jackson Laboratory (Bar Harbor, ME). Foxp3GFP+/- mice have been described previously.26,50 All of the mice were on a C57BL/6 background. The phenotypes of mice spontaneously develop chronic IBD under pathogen-free conditions. The phenotypes of mice in the transfer model, 8- to 10-week-old mice were used (Tables 1 and 2).

Mouse Colitis Models

Il10+/- mice spontaneously develop 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 weeks. Because Il10+/- mice develop spontaneous colitis, which has negative consequences on their capacity to breed, we separately crossed the mice as Itgb7+/-/Il10+/- × Itgb7+/-/Il10+/- and Itgb7+/-/Il10+/- × Itgb7+/-/Il10+/- to generate Itgb7+/-/Il10+/- and Itgb7+/-/Il10+/-, respectively. Sex-matched Itgb7+/-/Il10+/- and Itgb7+/-/Il10+/- mice were cohoused starting at 3–4 weeks after weaning. For piroxicam treatment, mice were administered piroxicam (200 ppm in their diet everyday) for 2 weeks and killed 3 weeks after the end of piroxicam treatment.25 For the adoptive T-cell–transfer model, 8- to 10-week-old mice were used. CD4+CD25-CD45RBhigh conventional T cells (5 × 10⁵) from C57BL/6 mice were injected intraperitoneally into cohoused Rag1-/- mice in the presence or absence of 1 × 10⁶ CD4+CD25+CD45RBlow Tregs derived from the indicated mutant genotype littermate mice (0.2 mL phosphate-buffered saline each recipient). In acute colitis experiments, cohoused Itgb7+/- or Itgb7+/- littermates were administered 1.5% (wt/vol) DSS with a molecular mass of 36–50 kilodaltons (MP Biomedicals, Irvine, CA) in drinking water for a total of 7 days (days 0–7), followed by plain drinking water (days 8–14). Mice were assessed daily for body weight, diarrhea, and bloody stool. The disease activity index and histologic damage were assessed by trained individuals blinded to the treatment groups, as reported previously.31 For anti-MAdCAM-1 blocking antibody MEC367 treatment, the Il10+/- cohoused littermates were injected intraperitoneally with MEC367 or control IgG (100 µg/mouse) every 4 days. Mice were euthanized, and peripheral blood was collected to test hemoglobin levels. Colonos were isolated for histology and quantitative polymerase chain reaction (PCR) analysis.

Mouse body weight was measured every day 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 scoring a disease activity index. The disease activity index 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%), and 4 (>20%); (2) stool consistency: 0 (normal), 1 (soft), 2 (very soft), and 3 (diarrhea); (3) rectal bleeding: 0 (none), 1 (red), 2 (dark red), and 3 (gross bleeding); and (4) rectal prolapse: 0 (none), 1 (signs of prolapse), 2 (clear prolapse), and 3 (extensive prolapse). Mice were killed 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 Slide Scanner (Hamamatsu Nanozoomer 2.0 HT Slide Scanner, Hamamatsu City, Japan). Blinded histologic scoring was performed by 2 investigators based on the method described previously.22 Two different scoring schemes were used (Tables 1 and 2).

Flow Cytometry

Cells isolated from mouse tissues were washed and resuspended in Hank’s balanced salt solution containing 0.1% bovine serum albumin and 1 mmol/L Ca²⁺/Mg²⁺ and stained with conjugated antibody for 30 minutes at 4°C. Then cells were washed twice before flow cytometry analysis using an Accuri C6 Plus or FACSCalibur (BD Biosciences, San Diego, CA).
Sciences, Hatfield, PA) and permeabilized with the Foxp3 transcription factor fixation/permeabilization kit (eBio-science) before IL10, TGF-β, and Foxp3 staining.

### Treg Suppression Assays

CD4⁺ CD25⁻ T cells (responder cells) were isolated from spleens 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 Tregs. GFP⁺ Tregs were sorted with a FACSAria 2 (BD Biosciences) from Itgb7⁺/⁺ Foxp3GFP or Itgb7⁻/⁻ Foxp3GFP mice. Responder cells were labeled with CFSE and cocultured with Tregs (8:1, 4:1, 2:1, and 1:1 ratios) in the presence of 5 μg/mL immobilized anti-CD3 (2C11) and anti-CD28 (37.51) and IL2 for 4 days at 37°C. The proliferation index was calculated by FlowJo v10.

### In Vivo Competitive Lymphocyte Homing

The competitive homing assay used high- and low-dose cell tracker, as described. GFP⁺ Tregs were sorted with a FACSAria 2 (BD Biosciences) from Itgb7⁺/⁺ Foxp3GFP or Itgb7⁻/⁻ Foxp3GFP mice and labeled with 1 μmol/L and 10 μmol/L of eFluor670, resulting in readily discriminated cell populations (Figure 14). Equal numbers of differentially labeled Tregs (1 × 10⁶) were mixed and then injected intravenously into C57BL/6 recipient mice. Lymphoid organs were harvested 3 hours after injection and isolated cells were analyzed by flow cytometry. The ratio of Itgb7⁻/⁻ Tregs (10 μmol/L eFluor670) to Itgb7⁺/⁺ Tregs (1 μmol/L eFluor670) from different lymphoid organs are shown. For a competitive homing assay of β7-deficient CD4⁺ T cells, CD4⁺ T cells were isolated by the MojoSort mouse CD4 T-cell isolation kit from Itgb7⁺/⁺ or Itgb7⁻/⁻ mice and labeled with 1 μmol/L of CFSE and eFluor670, respectively.
Real-Time Quantitative PCR Analyses

Total RNA was isolated from colon using a tissue homogenizer (JXFSTPRP-24; ThunderSci, Shanghai, China) and TRIzol reagent according to the manufacturer’s protocol (Thermo Fisher Scientific, Waltham, MA). For gene expression analysis, single-stranded complementary DNA was produced from 10 μg colonic total RNA using SuperScript III First-Strand synthesis and oligo-dT (deoxythymine) primers according to the manufacturer’s protocol (Thermo Fisher Scientific). The kappa SybrFast quantitative PCR kit (Kapa Biosystems, Wilmington, MA) and thermal cycler (CFX96 Real-Time System; Bio-Rad Laboratories, Berkeley, CA) were used to determine the relative levels of the genes analyzed (primer sequences are shown in Table 3) according to the manufacturer’s protocol. The 2–delta delta CT (\(\Delta\Delta CT\)) method was used for analysis, and data were normalized to glyceraldehyde-3-phosphate dehydrogenase. Control values (WT mice or Rag1\(^{-/-}\) mice injected with phosphate-buffered saline) were set to 1 for comparisons.

Statistical Analysis

Statistical analysis was performed using PRISM software (version 6.00, GraphPad Software, San Diego, CA), and all data sets were checked for Gaussian normality distribution. Data analysis was performed using a 2-tailed t test, 1-way or 2-way analysis of variance, followed by the Bonferroni correction.

| Table 3. Primers for Quantitative PCR |
|--------------------------------------|
| **IL1β** | F | AGTGTTGGATCCCCAAGGAAATAC | CTCGCTATTCGCCTGTAGCTTTCT |
|          | R |                            |                          |
| **TNF-α** | F | AGTGAACAGGCCTGAGGCCT | GAGTTGACTTTCTCCTGTAGAT |
|          | R |                            |                          |
| **IL6** | F | CTCAAGAGGAGCAGGGGAGAGATTT | GAGTTGACTTTCTCCTGTAGAT |
|          | R |                            |                          |
| **IFNγ** | F | CTCTTCCTCATGGCTTCTTCTTCTT | TACTCTCAGCTCATCCGCTT |
|          | R |                            |                          |
| **IL17** | F | TCTCCACGGGCTGAGGGAGAC | CAGCTACGCTGCTGCTGCTGCT |
|          | R |                            |                          |
| **GAPDH** | F | CCAGTTGTTCTCCTGCGACTT | CTTGTTGCTGCTGCTGCTGCT |
|          | R |                            |                          |

F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; R, reverse; TNF, tumor necrosis factor.
posttest as indicated in the figure legends. The resulting $P$ values are indicated as follows: *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$. Plotted data are the means ± SEM of at least 3 independent experiments.

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Hao Sun and Mark H. Ginsberg conceived the project and designed the experiments; Hao Sun and Wun Kuk performed the experiments; Hao Sun analyzed the data; Jesús Rivera-Nieves, Miguel Alejandro Lopez-Ramirez, and Lars Eckmann contributed expert advice to the design of the experiments; and Hao Sun and Mark H. Ginsberg wrote the manuscript with contributions from all authors.

Conflicts of interest
The authors disclose no conflicts.

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