Regulatory T-cell depletion in the gut caused by integrin β_7 deficiency exacerbates DSS colitis by evoking aberrant innate immunity

HL Zhang[1,4], YJ Zheng[1,4], YD Pan[1], C Xie[2], H Sun[1], YH Zhang[1], MY Yuan[1], BL Song[2,3] and JF Chen[1]

Integrin $\alpha_4\beta_7$ controls lymphocyte trafficking into the gut and has essential roles in inflammatory bowel disease (IBD). The $\alpha_4\beta_7$-blocking antibody vedolizumab is approved for IBD treatment; however, high dose of vedolizumab aggravates colitis in a small percentage of patients. Herein, we show that integrin β_7 deficiency results in colonic regulatory T (Treg) cell depletion and exacerbates dextran sulfate sodium (DSS) colitis by evoking aberrant innate immunity. In DSS-treated β_7-deficient mice, the loss of colonic Treg cells induces excessive macrophage infiltration in the colon via upregulation of colonic epithelial intercellular adhesion molecule 1 and increases proinflammatory cytokine expression, thereby exacerbating DSS-induced colitis. Moreover, reconstitution of the colonic Treg cell population in β_7-deficient mice suppresses aberrant innate immune response in the colon and attenuates DSS colitis. Thus, integrin $\alpha_4\beta_7$ is essential for suppression of DSS colitis as it regulates the colonic Treg cell population and innate immunity.

INTRODUCTION

Inflammatory bowel disease (IBD) is an idiopathic intestinal disorder caused by dysregulated immune responses to intestinal microbiota.[1] The two major types of IBD are ulcerative colitis (UC) and Crohn's disease (CD). A hallmark of IBD is the rapid recruitment and prolonged persistence of leukocytes at the site of inflammation, which has a major role both in the induction and maintenance of the inflammatory state.[2] Aberrant infiltration of mononuclear phagocytes, neutrophils, and inflammatory T cells is observed in the colonic lamina propria of IBD patients, suggesting that both innate and adaptive immunity contribute to progression of IBD.[3,4]

In the dextran sulfate sodium (DSS)-induced murine model of colitis, which mimics the hallmarks of IBD in human patients, innate and adaptive immune cells are consecutively involved in disease progression.[5,6] Acute colitis is triggered by epithelial barrier disruption, allowing intestinal bacteria to penetrate the injured mucosa and leading to an initial, rapid increase of innate immune cell infiltration.[6] Macrophages are one of the most important innate immune cells that are differentiated from monocytes recruited from the circulation into the affected tissues at the onset of inflammation.[7–9] These macrophages often show a proinflammatory phenotype through secretion of a variety of inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein 1, during the early stages of an immune response.[10,11] If the inflammatory macrophage response is not quickly controlled, it becomes pathogenic and facilitates IBD progression.[12,13] Subsequently, the activated innate immune cells present peptide antigens to T cells in secondary lymphoid organs of the gut. At day 6 after DSS treatment, the adaptive immune responses are evoked and inflammatory T cells, including type 1 helper T (Th1) cells and IL-17-producing helper T (Th17) cells, are recruited to the pathogenic site, where they are important for combating bacterial infection in the gut;[14,15] however, aberrant activation of these cells can lead to aggravated colitis.[2,5]

The recruitment of leukocytes from the peripheral circulation to the gut mucosa has a critical role in IBD.[16] This process is facilitated by the interaction of integrins with their respective

[1]State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. [2]State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China and [3]Department of Biochemistry, College of Life Sciences, Wuhan University, Wuhan, China. Correspondence: JF Chen (jfchen@sibcb.ac.cn)
[4]These authors contribute equally to this work.

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endothelial and mucosal ligands.[16,17] Integrin $\alpha_4\beta_7$ is the most important adhesion molecule mediating lymphocyte trafficking to gut-associated lymphoid tissues via binding to its ligand, mucosal addressing cell adhesion molecule 1.[17] Blocking the function of integrin $\alpha_4\beta_7$ can suppress the migration of lymphocytes to the gut and consequently inhibits adaptive immune-mediated colitis.[18–20] Vedolizumab, a humanized monoclonal antibody (mAb) that specifically blocks $\alpha_4\beta_7$ function, has recently been approved for the treatment of adults with UC and CD. Clinical studies report that vedolizumab effectively maintains clinical remission in some IBD patients; however, aggravated colitis is observed in a small percentage of UC patients treated with high dose of vedolizumab.[21,22] In addition, clinical study on another β_7-blocking antibody etrolizumab have reported that UC patients who received 100 mg etrolizumab show significant higher remission rate than patients receiving 300 mg etrolizumab,[23] suggesting excessive inhibition of integrin β_7 function might have some adverse effect on the management of UC. Thus, we speculated that excessive inhibition of integrin $\alpha_4\beta_7$ function might exacerbate colitis under certain conditions through an unknown mechanism.

Herein, we demonstrate that integrin β_7-deficiency causes severe colonic regulatory T (Treg) cell insufficiencies in integrin β_7-null (*Itgb7*$^{-/-}$) C57BL/6 mice and exacerbates DSS colitis because of aberrant innate immunity in the colon. In DSS-treated *Itgb7*$^{-/-}$ mice, this decrease in colonic Treg cells induces excessive macrophage infiltration in the colon via upregulation of intercellular adhesion molecule 1 (ICAM-1) expression in colonic epithelial cells and increases colonic expression of proinflammatory cytokines, which together result in the exacerbated DSS-induced colitis. Reconstitution of the colonic Treg cell population in *Itgb7*$^{-/-}$ mice by Treg cell transfer effectively suppresses the excessive macrophage infiltration and proinflammatory cytokine expression in the colon, which result in the attenuation of colonic inflammation. Thus, integrin $\alpha_4\beta_7$ is essential for suppression of DSS colitis because of its regulation of the Treg cell population and innate immune response in the colon. Furthermore, excessive inhibition of $\alpha_4\beta_7$ function can exacerbate DSS-induced colitis by evoking aberrant innate immune responses in the gut, which may aggravate the colitis in some IBD patients.

RESULTS

Integrin β_7 deficiency exacerbates DSS-induced acute colitis

Integrin $\alpha_4\beta_7$ has been shown to be required for lymphocyte recruitment to the gut-associated lymphoid tissue and to play critical roles in gut immunity.[21,24] Thus, we first examined the influence of integrin β_7 deficiency on the gut using *Itgb7*$^{-/-}$ C57BL/6 mice. The small intestine and colon of *Itgb7*$^{-/-}$ mice exhibited basically normal architectures (**Supplementary Figure S1a** and **b** online); however, smaller Peyer's patches with decreased cellularity and rudimentary follicles were observed in *Itgb7*$^{-/-}$ mice compared with wild-type (WT) mice (**Supplementary Figure S1c**). In addition, the thickness of the glycocalyx on the apical portion of microvilli in small intestine and colon of *Itgb7*$^{-/-}$ mice was similar to that of WT mice (**Supplementary Figure S1d–f**).

Next, we evaluated the role of integrin $\alpha_4\beta_7$ in IBD using the DSS-induced acute colitis model.[5] To avoid rapid death of *Itgb7*$^{-/-}$ mice caused by high doses of DSS, WT and *Itgb7*$^{-/-}$ mice were treated with 2% DSS for 5 days, followed by regular drinking water.[5,25] Compared with WT mice that showed good tolerance to 2% DSS, *Itgb7*$^{-/-}$ mice developed severe clinical symptoms of colitis at day 10 after the initial DSS treatment, including diarrhea and bloody stools (**Figure 1a**), reduced colon length (**Figure 1b**), massive infiltration of inflammatory cells, and thickened walls and disruption of mucosal structures in the colon (**Figure 1c**). In addition, *Itgb7*$^{-/-}$ mice showed rapid body weight loss after DSS treatment (**Figure 1d**) and exhibited a death rate of about 80% by day 13 (**Figure 1e**). By contrast, WT mice did not lose body weight significantly and all survived throughout the observation period. Thus, DSS induce much more severe colitis in *Itgb7*$^{-/-}$ mice than in WT mice, indicating that loss of integrin β_7 function exacerbates DSS-induced colitis.

Colonic macrophage infiltration and ICAM-1 expression are upregulated in *Itgb7*$^{-/-}$ mice upon DSS treatment

In both IBD patients and the DSS-induced colitis model, macrophages are considered a critical factor involved in disease progression.[4,25,26] Compared with the barely detectable macrophages in the colon of untreated WT and *Itgb7*$^{-/-}$ mice, WT mice receiving DSS treatment exhibited more macrophage aggregation in the colon at day 10, whereas *Itgb7*$^{-/-}$ mice displayed massive macrophage infiltration (**Figure 1f**). Along with the increased macrophage infiltration, colonic expression of proinflammatory cytokines (IL-6, TNF-α, and IL-1β) was significantly elevated in DSS-treated mice compared with untreated controls (**Figure 1g**). Notably, DSS treatment induced much higher levels of these proinflammatory cytokines in *Itgb7*$^{-/-}$ mice than in WT mice. These data indicate that loss of integrin β_7 function significantly enhances macrophage infiltration and proinflammatory cytokine expression in the colon of DSS-treated *Itgb7*$^{-/-}$ mice.

ICAM-1 has an important role in facilitating leukocyte infiltration in IBD[27] and is markedly upregulated on the epithelium of colonic biopsies from UC and CD patients.[28,29] Therefore, we examined ICAM-1 expression in colonic epithelial cells in mice at day 4 after the initial DSS treatment (**Figure 1h**). Before DSS treatment, no visible expression of ICAM-1 was observed in colonic epithelial cells in WT and *Itgb7*$^{-/-}$ mice. After DSS treatment for 4 days, WT mice exhibited no obvious change in ICAM-1 expression, whereas *Itgb7*$^{-/-}$ mice showed significantly elevated expression of ICAM-1 in colonic epithelial cells in accordance with massive macrophage infiltration in the invasive front area. Along with the increased ICAM-1 expression, the expression of TNF-α also increased in the colon in *Itgb7*$^{-/-}$ mice upon DSS treatment (**Supplementary Figure S2**), which has been shown to upregulate ICAM-1 expression in intestinal epithelial cells.[30] Thus, integrin β_7 deficiency results in upregulated ICAM-1

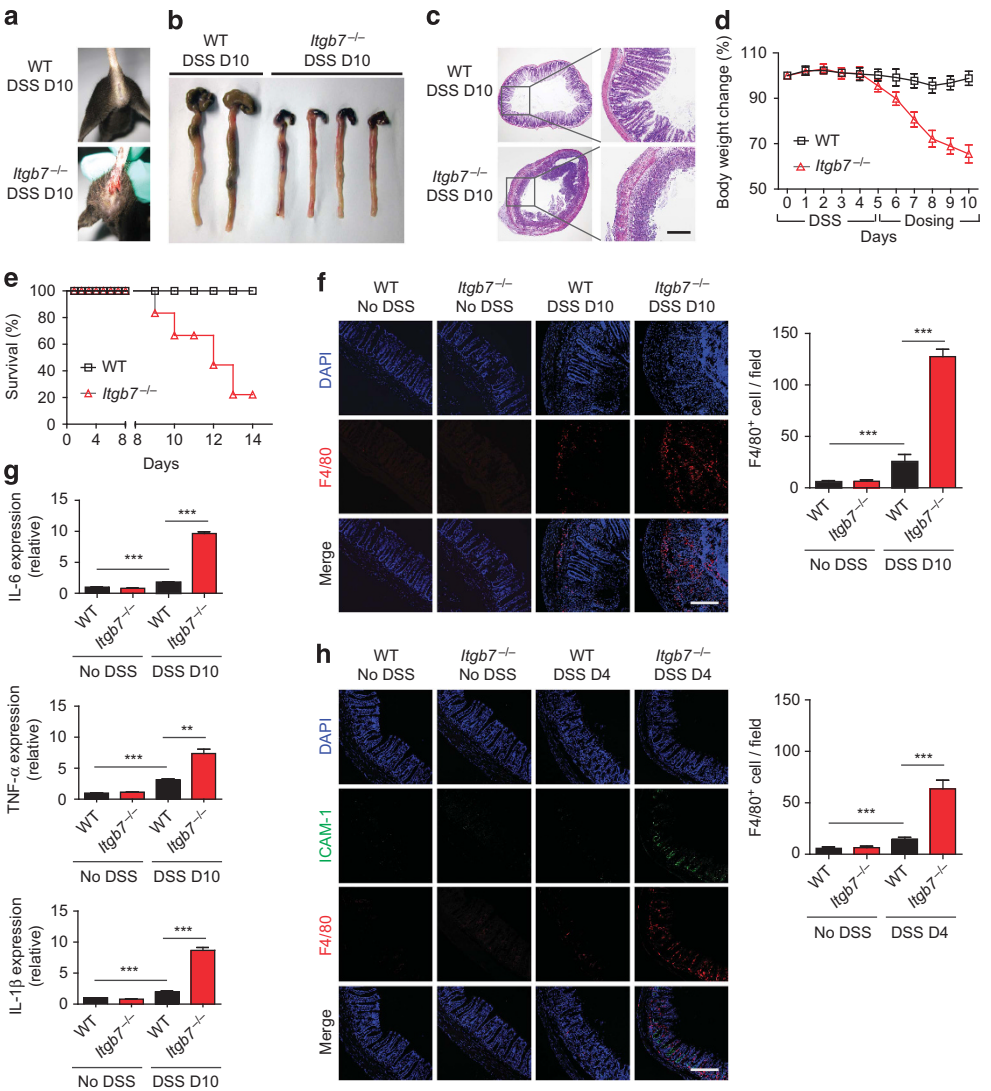

Figure 1 Integrin β_7 deficiency exacerbates DSS-induced acute colitis. 8-week-old mice were treated with 2% DSS for 5 days, followed by regular drinking water (dosing). (**a**–**c**) Macroscopic view of diarrhea and bloody stools (**a**), colon length (**b**), and hematoxylin and eosin staining of distal colon sections (**c**) at day 10 after DSS treatment (DSS D10). (**d**,**e**) Body weight change (**d**) and survival ratio (**e**) of mice ($n = 12$) after DSS treatment. Repeated measures two-way analysis of variance in **d**: WT vs. *Itgb7*$^{-/-}$ $F(10, 220) = 100.3$, $P < 0.001$. (**f**) Immunofluorescence staining of distal colon sections with anti-F4/80 (red) and 4,6-diamidino-2-phenylindole (DAPI; blue) before or after DSS treatment for 10 days. (**g**) Quantitative PCR analysis of IL-6, TNF-α, and IL-1β expression in distal colon tissue from individual groups of mice ($n = 12$). Results are normalized to GAPDH. (**h**) Immunofluorescence staining of distal colon sections with anti-ICAM-1 (green), anti-F4/80 (red), and DAPI (blue) before or after DSS treatment for 4 days. Scale bars, 100 μm (**c**,**f**,**h**). $^{**}P < 0.005$, $^{***}P < 0.001$ (Student's *t*-test in **f**–**h**). Data are representative of three independent experiments (mean ± s.d. in **d**, **f**–**h**).

expression in colonic epithelial cells during DSS colitis, which may enhance macrophage infiltration in the colon.

Integrin β_7 deficiency results in decreased Treg cell recruitment to the gut

It is reported that Treg cells can negatively regulate ICAM-1 expression to suppress leukocyte infiltration.[31,32] Thus, it is tempting to speculate that integrin β_7 deficiency may induce Treg cell insufficiency in the colon, which enhances colonic ICAM-1 expression and macrophage infiltration. Immunofluorescence staining revealed significantly reduced numbers of Foxp3$^+$ Treg cells in the colon of *Itgb7*$^{-/-}$ mice compared with WT mice (**Figure 2a**). On day 4 after the initial DSS treatment WT mice showed a reduction in Treg cells in the colon, whereas colonic Treg cells were barely detected in *Itgb7*$^{-/-}$ mice. In support of this, analysis of the absolute number of colonic Treg cells in WT and *Itgb7*$^{-/-}$ mice by flow cytometry showed that *Itgb7*$^{-/-}$ mice had significantly fewer colonic Treg cells than WT mice both before and after DSS treatment (**Figure 2b**). Collectively, these data indicate that integrin β_7 deficiency results in severe colonic Treg cell depletion.

Induced Treg (iTreg) cells suppress ICAM-1 expression in colonic epithelial cells

To investigate the function of Treg cells in regulating colonic ICAM-1 expression, naive CD4$^+$ T cells were isolated from

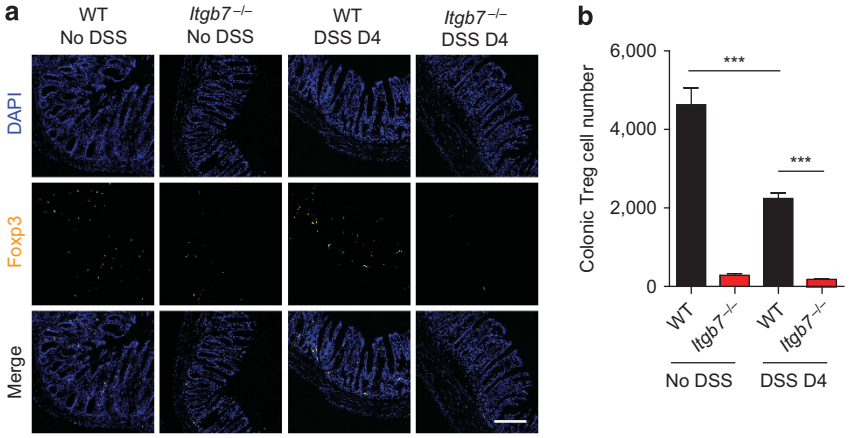

Figure 2 Impaired recruitment of Treg cells to the gut in integrin β_7-deficient mice. (**a**) Immunofluorescence staining of distal colon sections with anti-Foxp3 (yellow) and 4,6-diamidino-2-phenylindole (DAPI; blue) before or after DSS treatment for 4 days. Scale bar, 100 μm. (**b**) Flow cytometric analysis of Foxp3$^+$ Treg cell number in colonic tissues from individual groups of mice ($n = 9$). ***$P < 0.001$ (Student's *t*-test). Data are representative of three independent experiments (mean ± s.d. in **b**).

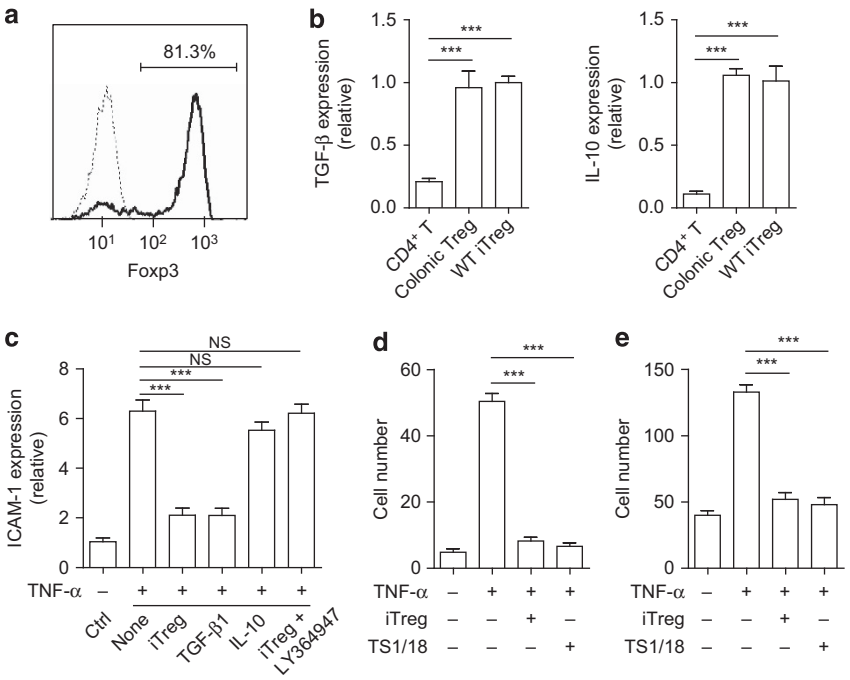

Figure 3 iTreg cells suppress macrophage adhesion to and across the colonic epithelial cell monolayer in a TGF-β-dependent manner. (**a**) Flow cytometric analysis of Foxp3 expression on iTreg cells, the numbers indicate the percentage of Foxp3$^+$ cells out of the total CD4$^+$ T-cell population. (**b**) Quantitative PCR analysis of TGF-β and IL-10 expression in WT CD4$^+$ T cells, colonic Treg cells, and iTreg cells. Results are normalized to GAPDH. (**c**) Quantitative PCR analysis of ICAM-1 expression in SW480 cells. SW480 cells were cocultured with iTreg cells, with TGF-β1, IL-10, or iTreg cells and LY364947. Subsequently, the iTreg cells were removed and the SW480 cells were further stimulated with TNF-α. Untreated SW480 cells were used as control. Results are normalized to GAPDH. (**d**,**e**) Analysis of THP-1 cell adhesion to the monolayer of SW480 cells at a wall shear stress of 1 dyn cm^{-2} (**d**) and THP-1 cell transmigration through the monolayer of SW480 cells (**e**). SW480 cells were pretreated with iTreg cells and TNF-α as in **c**. TS1/18 was used to block the function of integrin β_2. ***$P < 0.001$ (Student's *t*-test). Data are representatives of three independent experiments (mean ± s.d. in **b**–**e**).

WT mice and differentiated into Foxp3$^+$ iTreg cells (**Figure 3a**),[33] which expressed TGF-β and IL-10 at levels similar to those of colonic Treg cells isolated from WT mice (**Figure 3b**). We then examined whether iTreg cells regulate TNF-α-induced ICAM-1 expression in a human colorectal epithelial cell line, SW480. Treatment with TNF-α significantly increased ICAM-1 expression in SW480 cells, which could be inhibited by coculture with iTreg cells (**Figure 3c**). In addition, TNF-α-induced ICAM-1 expression was inhibited by TGF-β but not by IL-10, and blocking TGF-β signaling with the LY364947 inhibitor[34] completely abolished the iTreg cell-dependent downregulation of ICAM-1 expression in TNF-α-

treated SW480 cells (**Figure 3c**). Thus, iTreg cells inhibit TNF-α-induced ICAM-1 expression in human colorectal epithelial cells in a TGF-β-dependent manner.

iTreg cells suppress adhesion and transmigration of monocytes on colonic epithelial cell monolayer

Colonic macrophages arise from monocytes recruited from the blood.[7,9] Next, we investigate the roles of iTreg cells in regulating adhesion and transmigration of monocyte cell line THP-1 on a monolayer of SW480 cells. Compared with the adhesion of THP-1 cells to an untreated monolayer of SW480 cells at a wall shear stress of 1 dyn cm^{-2}, THP-1 cell adhesion to the TNF-α-treated SW480 cells was significantly increased (**Figure 3d**). In addition, the transmigration of THP-1 cells across the monolayer of SW480 cells was also significantly enhanced upon treatment of SW480 cells with TNF-α (**Figure 3e**). The TNF-α-induced increase of THP-1 cell adhesion and transmigration were almost completely blocked by pretreating THP-1 cells with the β_2-blocking antibody TS1/18, suggesting that this effect is dependent on the ICAM-1–β_2 integrin interaction (**Figure 3d** and **e**). Importantly, coculture of iTreg cells with the SW480 cell monolayer abolished the TNF-α-induced increase in THP-1 cell adhesion and transmigration (**Figure 3d** and **e**), which is consistent with the result that iTreg cells inhibited TNF-α-induced ICAM-1 expression in SW480 cells (**Figure 3c**).

Collectively, these data suggest that decrease of colonic Treg cell population caused by integrin β_7 deficiency results in the upregulated expression of ICAM-1 on the colonic epithelia, facilitating monocyte recruitment via enhanced adhesion between monocyte β_2 integrins and colonic epithelial ICAM-1.

iTreg cells suppress the proinflammatory response of macrophages to lipopolysaccharide (LPS)

Next, we examined the ability of iTreg cell-treated macrophages to respond to LPS. Macrophages were isolated from the peritoneal cavity of WT mice as previously described (**Supplementary Figure S3a**).[35] Following coculture with iTreg cells, macrophages were significantly suppressed in their capacity to produce proinflammatory cytokines (IL-6, TNF-α, and IL-1β) compared with macrophages cultured alone (**Figure 4**). Blocking IL-10 function by silencing the IL-10 receptor (**Supplementary Figure S3b**) or inhibiting TGF-β function with LY364947 only partially restored LPS-induced proinflammatory cytokine expression in iTreg cell-treated macrophages. In addition, simultaneous inhibition of both IL-10 and TGF-β fully rescued cytokine expression in iTreg cell-treated macrophages, suggesting Treg cells suppress the proinflammatory response of macrophages to LPS via both IL-10 and TGF-β pathways.

Taken together, integrin β_7 deficiency-induced colonic Treg cell insufficiency results in enhanced ICAM-1 expression and macrophage infiltration in the colon and promotes the proinflammatory response of macrophages to LPS, contributing to the aggravated DSS colitis in *Itgb7*$^{-/-}$ mice.

Reconstitution of colonic Treg cells suppresses aberrant macrophage infiltration and cytokine expression in the gut of DSS-treated *Itgb7*$^{-/-}$ mice

Using adoptive Treg cell transfer approach,[36] we next examined the function of Treg cells in regulating macrophage infiltration and proinflammatory cytokine expression in the gut of *Itgb7*$^{-/-}$ mice upon DSS treatment. As WT iTreg cells, β_7-deficient iTreg cells expressed TGF-β and IL-10 at levels similar to those of colonic Treg cells isolated from WT mice (**Supplementary Figure S4a** and **b**). The *Itgb7*$^{-/-}$ recipient mice were adoptively transferred with 1×10^6 WT or β_7-deficient iTreg cells. After 7 days, all mice were challenged with 2% DSS for 4 days, then the colons were removed for subsequent analysis. Transfer of WT iTreg cells significantly increased the number of Treg cells in the colon of *Itgb7*$^{-/-}$ mice to a level comparable to that of WT mice (**Figure 5a** and **b**). By contrast, transfer of β_7-deficient iTreg cells did not increase the colonic Treg cell population. Consistently, transfer of WT iTreg cells rather than β_7-deficient iTreg cells restored the expression of TGF-β and IL-10 (**Figure 5c**) and decreased ICAM-1 expression, macrophage infiltration, and the expression of IL-6, TNF-α, and IL-1β in the colon in *Itgb7*$^{-/-}$ mice to the levels similar to those of WT mice (**Figure 5d** and **e**). Thus, reconstitution of the colonic

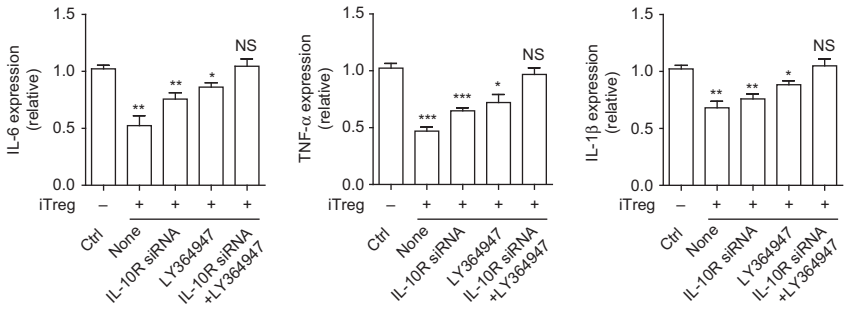

Figure 4 iTreg cells suppress the proinflammatory response of macrophages to LPS in a TGF-β and IL-10-dependent manner. Quantitative PCR analysis of IL-6, TNF-α, and IL-1β expression in LPS-treated macrophages. Macrophages were cocultured with iTreg cells along with IL-10R knockdown, inhibition of TGF-β with LY364947 or both. Subsequently, the iTreg cells were removed and the macrophages were further stimulated with LPS. Untreated macrophages were used as control. Results are normalized to GAPDH. $^*P<0.01$, $^{**}P<0.005$, and $^{***}P<0.001$ (Student's *t*-test). Data are representatives of three independent experiments (mean ± s.d).

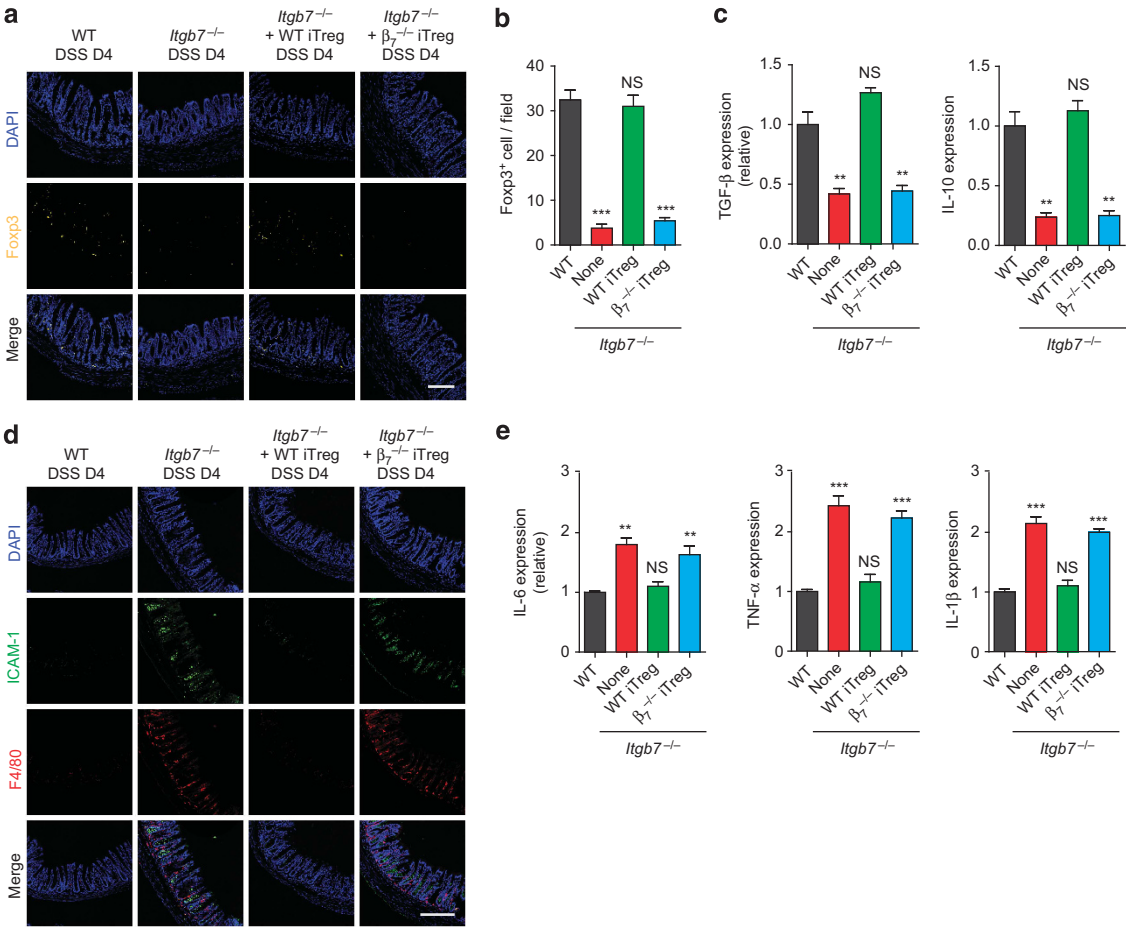

Figure 5 Reconstitution of colonic Treg cells suppresses aberrant macrophage infiltration and inflammatory cytokine expression in DSS-treated integrin β_7-deficient mice. (**a**) Immunofluorescence staining of colonic sections with anti-Foxp3 (yellow) and 4,6-diamidino-2-phenylindole (DAPI; blue) from WT mice, *Itgb7*$^{-/-}$ mice, *Itgb7*$^{-/-}$ mice transferred with WT iTreg cells (*Itgb7*$^{-/-}$ + WT iTreg) and *Itgb7*$^{-/-}$ mice transferred with β_7-deficient iTreg cells (*Itgb7*$^{-/-}$ + $\beta_7^{-/-}$ iTreg) at day 4 after DSS treatment. (**b**) Quantification of Foxp3$^+$ cell number in **a**. (**c**) Quantitative PCR (qPCR) analysis of TGF-β and IL-10 expression in distal colonic tissue from individual groups of mice as in **a** ($n = 9$). Results are normalized to GAPDH. (**d**) Immunofluorescence staining of colonic sections with anti-ICAM-1 (green), anti-F4/80 (red), and DAPI (blue) at day 4 after DSS treatment. Scale bars, 100 μm. (**e**) qPCR analysis of IL-6, TNF-α, and IL-1β expression in distal colonic tissues from individual groups of mice as in **a** ($n = 9$). Results are normalized to GAPDH. $^{**}P < 0.005$, $^{***}P < 0.001$ (Student's *t*-test). Data are representative of three independent experiments (mean ± s.d. in **b**,**c**,**e**).

Treg cell population restores TGF-β and IL-10 expression and suppresses colonic epithelial ICAM-1 expression in DSS-treated *Itgb7*$^{-/-}$ mice, resulting in decreased macrophage infiltration and proinflammatory cytokine production in the colon.

Reconstitution of colonic Treg cells relieves DSS-induced colitis in *Itgb7*$^{-/-}$ mice

Next, we evaluated the effect of iTreg cell transfer on DSS colitis progression in *Itgb7*$^{-/-}$ mice. WT mice, *Itgb7*$^{-/-}$ mice, and WT or β_7-deficient iTreg cell-transferred *Itgb7*$^{-/-}$ mice were treated with 2% DSS for 5 days, followed by regular drinking water. Compared with the rapid body weight loss of DSS-treated *Itgb7*$^{-/-}$ mice, WT mice and WT iTreg cell-transferred *Itgb7*$^{-/-}$ mice showed similar mild body weight changes throughout the observation period (**Figure 6a**). Transfer of β_7-deficient iTreg cell did not alleviate the body weight loss of DSS-treated *Itgb7*$^{-/-}$ mice. In addition, WT iTreg cell-transferred *Itgb7*$^{-/-}$ mice exhibited a 100% survival rate at day 12 compared with around 40% survival rate of *Itgb7*$^{-/-}$ mice or β_7-deficient iTreg cell-transferred *Itgb7*$^{-/-}$ mice (**Figure 6b**). Furthermore, WT iTreg cell-transferred *Itgb7*$^{-/-}$ mice developed more mild clinical symptoms of colitis, including normal colon length (**Figure 6c**), lower colitis activity score (**Figure 6d**), fewer inflammatory infiltrates, and moderate disruption of mucosal structures (**Figure 6e**). Collectively, these results indicate that integrin β_7 deficiency-induced exacerbation of DSS colitis can be effectively relieved by reconstitution of the colonic Treg cell population.

Inhibition of ICAM-1 function relieves DSS-induced colitis in *Itgb7*$^{-/-}$ mice

To evaluate the contribution of ICAM-1 to the aggravated DSS colitis in *Itgb7*$^{-/-}$ mice, we examined the effect of blocking ICAM-1 function on DSS-induced colitis in *Itgb7*$^{-/-}$ mice. *Itgb7*$^{-/-}$ mice were intracolonically administrated with

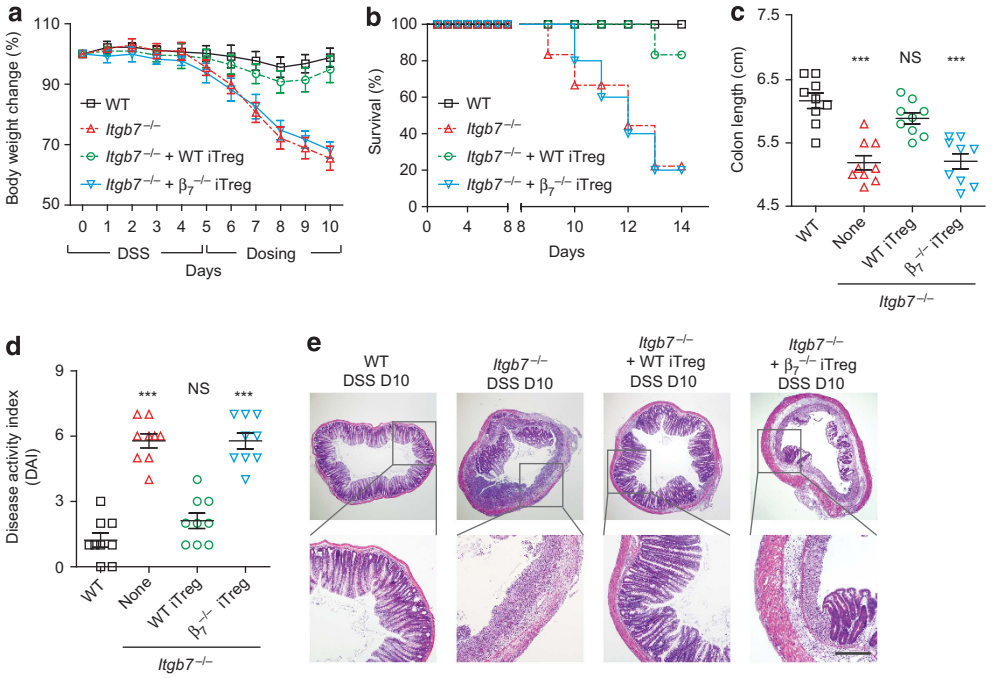

Figure 6 Reconstitution of colonic Treg cells relieves DSS-induced colitis in integrin β_7-deficient mice. 8-week-old WT mice, *Itgb7*$^{-/-}$ mice, *Itgb7*$^{-/-}$ mice transferred with WT iTreg cells (*Itgb7*$^{-/-}$ + WT iTreg) and *Itgb7*$^{-/-}$ mice transferred with β_7-deficient iTreg cells (*Itgb7*$^{-/-}$ + $\beta_7^{-/-}$ iTreg) were treated with 2% DSS for 5 days, followed by regular drinking water. (**a**,**b**) Body weight change (**a**) and survival ratio (**b**) were obtained from individual groups of mice ($n = 12$) Repeated measures two-way analysis of variance in **a**: WT vs. *Itgb7*$^{-/-}$ $F(10, 220) = 100.3$, $P < 0.001$; WT vs. WT iTreg cell-transferred *Itgb7*$^{-/-}$ (*Itgb7*$^{-/-}$ + WT iTreg) $F(10, 220) = 0.9636$, $P = 0.4761$; WT vs. $\beta_7^{-/-}$ iTreg cell-transferred *Itgb7*$^{-/-}$ (*Itgb7*$^{-/-}$ + $\beta_7^{-/-}$ iTreg) $F(10, 220) = 161.4$, $P < 0.001$. (**c**,**d**) Mean colon length (**c**) and disease activity score (**d**) were determined at day 10 after DSS treatment. (**e**) Hematoxylin and eosin staining of distal colon sections at day 10 after DSS treatment. Scale bar, 100 μm. *$P < 0.01$, **$P < 0.005$, ***$P < 0.001$ (Student's *t*-test in **c**,**d**). Data are representative of three independent experiments (mean ± s.d. in **a**,**c**,**d**).

ICAM-1-blocking antibody 3E2, then were treated with 2% DSS for 5 days, followed by regular drinking water. The results showed that blocking ICAM-1 function could efficiently block macrophage infiltration in the colon (**Figure 7a** and **b**) and significantly alleviate the body weight loss of DSS-treated *Itgb7*$^{-/-}$ mice (**Figure 7c**), indicating colonic Treg depletion-induced ICAM-1 expression has a major role in exacerbation of DSS colitis in *Itgb7*$^{-/-}$ mice.

DISCUSSION

Using a DSS-induced murine model of colitis, we demonstrate that loss of integrin β_7 function causes colonic Treg cell depletion and exacerbates DSS colitis mainly by evoking aberrant innate immunity in *Itgb7*$^{-/-}$ mice. Reconstitution of colonic Treg cells in *Itgb7*$^{-/-}$ mice suppresses aberrant innate immune responses in the colon, thereby alleviating DSS colitis.

During the early stages of an immune response following infection or injury, monocytes are recruited from the circulation into the affected tissues and differentiate into macrophages.[7,9] The macrophages then promote inflammatory response through secretion of inflammatory cytokines.[10,11] Here we show that integrin β_7-mediated Treg cells recruitment to gut-associated lymphoid tissue is essential to prevent aberrant macrophage infiltration and suppress inflammation in DSS-induced acute colitis. Interestingly, in contrast to the proinflammatory role of colonic macrophage in DSS-induced acute colitis, it has been reported that integrin β_7 is required to

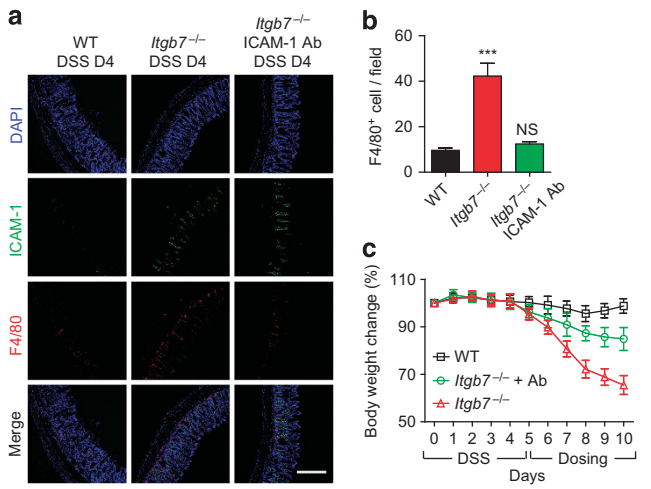

Figure 7 Inhibition of ICAM-1 function relieves DSS-induced colitis in *Itgb7*$^{-/-}$ mice. 8-week-old WT mice, *Itgb7*$^{-/-}$ mice, and *Itgb7*$^{-/-}$ mice received antibody against ICAM-1 intraconically in 0.2 ml PBS, at a dose of 1 mg kg^{-1} on each days 0, 2, and 4 were treated with 2% DSS for 5 days, followed by regular drinking water. (**a**) Immunofluorescence staining of distal colon sections with anti-ICAM-1 (green), anti-F4/80 (red), and 4,6-diamidino-2-phenylindole (DAPI; blue) at day 4 after DSS treatment. Scale bars, 100 μm. (**b**) Quantification of the F4/80$^+$ macrophage number in **a**. (**c**) Body weight change were obtained from individual groups of mice ($n = 12$). **$P < 0.005$, ***$P < 0.001$ (Student's *t*-test in **b**). Repeated measures two-way analysis of variance in **c**: WT vs. *Itgb7*$^{-/-}$ $F(10, 220) = 100.3$, $P < 0.001$; WT vs. blocking antibody-treated *Itgb7*$^{-/-}$ (*Itgb7*$^{-/-}$ + Ab) $F(10, 220) = 12.87$, $P < 0.001$. Data are representative of three independent experiments (mean ± s.d. in **b**,**c**).

give rise to intestinal mononuclear phagocytes to prevent T cell-mediated chronic colitis.[37] Using T-cell transfer model of chronic colitis, Villablanca *et al.*[37] showed that T cell-mediated colitis was aggravated in *Itgb7*$^{-/-}$ *Rag2*$^{-/-}$ mice compared with *Rag2*$^{-/-}$ mice because of the impaired homing of mononuclear phagocytes to the gut, indicating a protective role of mononuclear phagocytes in chronic colitis. Taken together, mononuclear phagocytes might have distinct roles in inflammatory response in the gut under different conditions.

Denning *et al.* have reported that β_7-deficient Tregs can prevent CD4 CD45RB^{high} T cell-mediated colitis in *Rag2*$^{-/-}$ mice,[38] suggesting that localization of Treg cells in the gut is not necessary for the prevention of adaptive T cell-mediated immune responses in the colon. In contrast, here we demonstrate that adoptive transfer of β_7-deficient Treg cells into *Itgb7*$^{-/-}$ recipient mice fail to suppress aberrant innate immune responses in the colon induced by 2% DSS, suggesting that β_7 integrin-dependent Treg localization to the gut is critical for the suppression of innate immune responses during DSS colitis. Comparing the colitis models used in these studies, it is tempting to speculate that Treg cells could have distinct roles under different inflammatory conditions.

IL-17A has been shown to exert a protective role in DSS colitis.[39] Th17 trafficking to the gut could be inhibited by integrin β_7 deficiency, which might lower the IL-17A level in the colon and exacerbate DSS colitis. To investigate the effect of β_7 deficiency on colonic Th17 population and IL-17A expression, the absolute number of Th17 cells (**Supplementary Figure S5a**) and IL-17A expression in the colon, mesenteric lymph node, and spleen were determined (**Supplementary Figure S5b**). The results showed that the number of Th17 cell was decreased in the colon but not in the mesenteric lymph node and spleen in *Itgb7*$^{-/-}$ mice. Interestingly, IL-17A expression in colon tissue showed no significant decrease in *Itgb7*$^{-/-}$ mice compared with WT mice, which could be due to that IL-17A expressed by other IL-17-producing cells[40] might compromise the effect of decreased Th17 cells in the colon in *Itgb7*$^{-/-}$ mice. Thus, the impaired Th17 trafficking to the gut might not contribute to exacerbation of DSS colitis in *Itgb7*$^{-/-}$ mice because IL-17A level barely changed in the colon in *Itgb7*$^{-/-}$ mice.

The US Food and Drug Administration has approved the integrin $\alpha_4\beta_7$-blocking antibody vedolizumab for the treatment of adults with moderately to severely active UC or CD. By specifically blocking the interaction of integrin $\alpha_4\beta_7$ with mucosal addressing cell adhesion molecule 1, vedolizumab can efficiently suppresses the recruitment of inflammatory T and B cells to the gut, and consequently, inhibit adaptive immune-mediated colitis.[18,19] However, IBD is a result of the complicated interaction of innate and adaptive immune cells working in concert. Although excessive inhibition of integrin $\alpha_4\beta_7$ function by high dose of vedolizumab could excessively suppress $\alpha_4\beta_7$-depdent trafficking of immune cells into the gut, which might have potential adverse effects on the management of IBD. Our study shows one example that loss of β_7 function inhibits Treg recruitment to the colon and result in colonic Treg cell insufficiency, which may evoke aberrant innate immune responses and aggravate colitis in particular patients who have mucosal injury and microbiota-mediated infection in the gut. Thus, vedolizumab is ideal for the treatment of adaptive immune-induced IBD. High dose of vedolizumab should be avoided to prevent the complete loss of integrin β_7 function and the potential adverse effects on the management of IBD.

METHODS

Mice. *Itgb7*$^{-/-}$ C57BL/6 mice were from Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions. Age- and sex-matched *Itgb7*$^{-/-}$ mice and littermate controls were used at 8–10 weeks of age. All animal studies were approved by the Institutional Animal Care and Use Committee of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Antibodies and reagents. Human and mouse chemokines and cytokines were purchased from R&D Systems (Minneapolis, MN). The following mAbs were used in this study: phycoerythrin-conjugated anti-CD4 (GK1.5), anti-F4/80 (BM8); Alexa Fluor 647-conjugated anti-CD19 (1D3), anti-CD25 (PC61); purified anti-CD3 (145-2C11), anti-CD28 (37.51), anti-CD18 (TS1/18), anti-ICAM-1 (3E2) were all from BD Bioscience (San Jose, CA). Allophycocyanin-conjugated anti-Foxp3 (FJK-16s) was from eBioscience (San Diego, CA). FITC-conjugated anti-ICAM-1 (M-19) was from Santa Cruz (Santa Cruz, CA). The secondary antibody Cy3-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch (West Grove, PA). LPS, 4,6-diamidino-2-phenylindole, and LY364947 (TGF-β type I receptor kinase inhibitor) were from Sigma (St. Louis, MO).

DSS-induced acute colitis. Acute colitis was induced by the provision of 2% (wt/vol) DSS with molecular mass of 36–50 kDa (MP Biomedicals, Irvine, CA) in drinking water for a total of 5 days (days 0–5), followed by regulatory drinking water (days 6–15). Mice were assessed daily for body weight, diarrhea, and bloody stool. The disease activity index and histological damage were assessed by trained individuals blinded to the treatment groups, as reported previously.[41] On day 10, mice were killed and colons removed and analyzed. For interim analyses, experiments were done as described above but terminated on day 4. Colon tissues were used for histological and quantitative PCR analysis.

Histology and immunofluorescence microscopy. Formalin-fixed, paraffin-embedded distal colon sections of 4-μm thickness were mounted on glass slides and followed by hematoxylin and eosin staining or periodic acid–schiff staining. For immunostaining analysis, distal colons were collected at individual days after the initial DSS treatment. Cryostat sections were made permeable with cold acetone and blocked with 1% (wt/vol) BSA. Samples were incubated with fluorochrome-conjugated anti-F4/80 (5 μg ml^{-1}), anti-ICAM-1 (10 μg ml^{-1}), or anti-Foxp3 (5 μg ml^{-1}), and counterstaining of nuclei was with 4,6-diamidino-2-phenylindole (1 μg ml^{-1}). Images were acquired with a Leica TCS SP8 confocal microscope (Leica, Mannheim, Germany).

***In vivo* antibody blocking assay**. *Itgb7*$^{-/-}$ mice were treated with DSS as previously described, and 1 mg kg^{-1} hamster anti-mouse ICAM-1 (3E2)-blocking antibody was intracolonically given to the mouse on each of days 0, 2, and 4.[42] The antibody was installed slowly through a polyethylene catheter carefully inserted until the tip was 3 cm proximal to the anus.

Colonic Treg cell isolation and flow cytometry. Mononuclear cells were isolated from colon as previously described.[43] The isolated cells were stained with anti-CD4 (3 μg ml^{-1}) and anti-CD25 (3 μg ml^{-1}) mAbs to sort CD4$^+$CD25$^+$ Treg cells by flow cytometry using a FACSAria II flow cytometer (BD Biosciences).[44] For intracellular

staining of Foxp3, cells were fixed and permeabilized using the mouse Treg cell staining kit (eBioscience) before staining with fluorochrome-conjugated anti-Foxp3 (3 μg ml^{-1}) mAb. Data were analyzed using WinMDI 2.9 software (The Scripps Research Institute, La Jolla, CA).

RNA isolation and real-time quantitative PCR. Total RNA was extracted from cells or mouse distal colon tissues with TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). For cDNA synthesis, RNA was reverse-transcribed with an M-MLV reverse transcriptase (Promega, Madison, WI). Then cDNA was amplified by real-time PCR (primers, **Supplementary Table 1**) with a SYBR Premix ExTaq kit (TaKaRa, Otsu, Japan) on an AbiPrism 7,500 sequence detector (Applied Biosystems, Foster City, CA). The expression of target genes was normalized to expression of the housekeeping gene GAPDH.

***In vitro* Treg cell induction and adoptive transfer**. iTreg cells were generated from naive CD4$^+$ T cells as described, with minor modifications.[33] Briefly, isolated naive CD4$^+$ T cells were resuspended in complete RPMI-1640 and plated in flat-bottom plates precoated with 3 μg ml^{-1} anti-CD3 (145-2C11) mAb, and then stimulated with 3 μg ml^{-1} anti-CD28 (37.51) mAb, 2 ng ml^{-1} IL-2, and 10 ng ml^{-1} TGF-β1. After stimulation for 72 h, Foxp3 expression was assessed by flow cytometry, and the expression of TGF-β and IL-10 was assessed by quantitative PCR. For adoptive cell transfer, 1×10^6 WT or $\beta_7^{-/-}$ iTreg cells were injected intravenously into *Itgb7*$^{-/-}$ mice, respectively. Recipients were acclimated for 7 days after injection and then DSS challenge was performed.[36,45]

Macrophage isolation, coculture, and stimulation. Peritoneal macrophage cells were harvested and cultured using a standard protocol.[35] Briefly, mice were injected peritoneally with 2.5 ml of 3% Brewer thioglycollate medium to harvest peritoneal macrophages. Subsequently, the cells were resuspended in RPMI-1640 medium supplemented with penicillin G/streptomycin (100 U ml^{-1}; 100 μg ml^{-1}) for further use.

Macrophages (2×10^6 per ml) or macrophages with knockdown of IL-10R were cocultured with WT iTreg cells (1×10^6 per ml) or with WT iTreg cells (1×10^6 per ml) and 5 μM LY364947 in RPMI-1640 medium (10% heat-inactivated fetal bovine serum, 2 mM glutamine) for 40 h. Subsequently, the iTreg cells were removed and the macrophages were further stimulated for 6 h with 50 ng ml^{-1} LPS and then collected for qRCR.

Small interfering RNA silencing of IL-10 receptor A (IL-10R) in macrophages. Murine IL-10R-specific small interfering RNA 5′-CCUGAGCAUCUUAGUCAUATT-3′ and the scramble control small interfering RNA 5′-UUCUCCGAACGUGUCACGUTT-3′ were purchased from GenePharma (Shanghai, China). Macrophages were transfected with small interfering RNA using Lipofectamine 2000 (Invitrogen). IL-10R silencing efficiency was assessed by quantitative PCR 48 h post transfection.

SW480 cell and iTreg cell coculture and stimulation. SW480 cells (2×10^6 per ml) were cultured in the following conditions: without iTreg cells, with iTreg cells (1×10^6 per ml), with 5 ng ml^{-1} TGF-β1, with 5 ng ml^{-1} IL-10, or with iTreg cells (1×10^6 per ml) + 5 μM LY364947 in RPMI-1640 medium (10% heat-inactivated fetal bovine serum, 2 mM glutamine) for 40 h. Subsequently, the iTreg cells were removed and the SW480 cells were further stimulated with 5 ng ml^{-1} TNF-α for 6 h and then collected for quantitative PCR, flow chamber, and Transwell assays.

Flow chamber assay. The untreated or iTreg cell-treated monolayers of SW480 cells that were left unstimulated or stimulated with 5 ng ml^{-1} TNF-α were used as the lower wall of the flow chamber. THP-1 cell was diluted to 1×10^6 cells per ml in serum-free RPMI-1640 medium and immediately perfused through the flow chamber in a constant flow of 1 dyn cm^{-2}. For β_2 integrin function blocking, THP-1 was pretreated with 10 μg ml^{-1} β_2 integrin-blocking antibody TS1/18 for 30 min at room temperature before being perfused into the flow chamber. All adhesive interactions between the flowing THP-1 and SW480 cell monolayer were determined by manually tracking the motions of individual cells for 1 min. The motion of each adherent cell was monitored following the initial adhesion point, and the cell adhesion event was defined as a cell that remained adherent for at least 5 s.

Transwell migration assay. Transwell migration was performed using Millicell inserts with 5 μm pore size (Millipore, Billerica, MA). Untreated or iTreg cell-treated monolayer of SW480 cells that were either left unstimulated or were stimulated with 5 ng ml^{-1} TNF-α were coated on the upper surface of inserts, and the lower chamber was filled with 1 ml RPMI-1640 medium with 10% fetal bovine serum. 2×10^5 THP-1 cells in 0.2 ml serum-free RPMI-1640 medium were added into the upper chamber. For β_2 integrin function blocking, THP-1 cells were pretreated with 10 μg ml^{-1} β_2 integrin- blocking antibody TS1/18 for 30 min at 37 °C before being added into the upper chamber. Cells were incubated for 6 h at 37 °C in 5% CO_2. Cells remaining on the upper surface of the inserts were scraped with a cotton swab, and cells migrating to the bottom surface were counted after fixation with 3.7% formaldehyde and staining with 4,6-diamidino-2-phenylindole.

Statistical analysis. Statistical analyses were performed with GraphPad PRISM software 5.0 (GraphPad Software, La Jolla, CA). Significances were determined by two-tailed Student's *t*-test or Repeated measures two-way analysis of variance as indicated. $^*P<0.01$, $^{**}P<0.005$ and $^{***}P<0.001$ were considered statistically significant in all figures.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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AUTHOR CONTRIBUTIONS

H.L.Z. and J.F.C. designed the experiments; H.L.Z., Y.J.Z., Y.D.P., C.X., H.S., Y.H.Z., and M.Y.Y. performed the experiments and analyzed data; H.L.Z. and J.F.C. interpreted results; J.F.C. and B.L.S. supervised the study; the manuscript was drafted by H.L.Z. and edited by J.F.C.

DISCLOSURE

The authors declare no conflict of interest.

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