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The Role of Innate Immune IL-10 Receptor Signaling in Controlling Intestinal Immune Responses

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Introduction

Interleukin-10 (IL-10) is a key anti-inflammatory cytokine that is produced predominantly by leukocytes including T cells, B cells, monocytes, macrophages and dendritic cells, as well as by some epithelial cells IL-10 acts on both innate and adaptive immune cells and has a broad range of immunomodulatory activities that suppress proliferation, cytokine secretion, and costimulatory molecule expression of pro-inflammatory immune cells. A critical role for IL-10 signaling in modulating intestinal mucosal homeostasis became evident with the description that IL-10-deficient mice develop spontaneous enterocolitis. This was subsequently strengthened by the observation that interleukin 10 receptor (IL10R)-deficient mice also develop spontaneous colitis.

In humans, IL10 and IL10R play critical roles in controlling immune responses in the intestinal mucosa. Single-nucleotide polymorphisms (SNPs) in IL10 have been linked to inflammatory bowel disease (IBD) risk in genome-wide association studies. In addition, patients with deleterious mutations in either IL10 or its receptor develop severe IBD, typically presenting within the first months of life.

IL-10 mediates its anti-inflammatory effects through IL-10R-dependent signals emanating from the cell surface. The IL-10R is a hetero-tetramer that consists of two subunits of IL-10Ra and two subunits of IL-10Rb. Whereas the IL-10Ra subunit is unique to IL-10 signaling, the IL-10Rb subunit is shared by other cytokine receptors, including IL-22, IL-26, and interferon-λ. IL-10 downstream signaling through the IL-10R inhibits the induction of pro-inflammatory cytokines by blocking NF-kB-dependent signals.

Although the development of IBD is well established in mice and in humans with IL-10R deficiency, the precise mechanisms of IL-10R-dependent control of immune tolerance and intestinal mucosal homeostasis are not well defined. In mice, intact IL-10R signaling is important in T regulatory (Treg) cells for their suppressive function including prevention of colitis, and in T effector cells for preventing exaggerated T helper 17 cell responses in mucosal compartments. A role for innate immune IL-10R signaling in the regulation of intestinal immune tolerance has not been explored. Several groups have demonstrated that IL-10 sensing by innate immune cells is required for suppression of pro-inflammatory cytokines secretion. Moreover, IL-10R-deficient dendritic cells secrete high quantities of pro-inflammatory cytokines after LPS stimulation. We hypothesized that innate immune IL-10R signaling is required for maintenance of intestinal immune tolerance and prevention of IBD.

Here we demonstrate that IL-10R signaling in innate immune cells is critical for regulating mucosal homeostasis and prevention of colitis. Loss of IL-10R-dependent signaling rendered wild-type CD4+ T cells colitogenic and was associated with markedly aberrant Treg cells generation and function. Importantly, we show that IL-10R-dependent signals modulated the differentiation and function of bone-marrow-derived macrophages and intestinal macrophages into either pro-inflammatory macrophages or functionally competent anti-

inflammatory macrophages. Similarly, monocyte-derived macrophages from very early onset IBD patients harboring loss of function mutations in IL10RA and IL10RB genes also exhibited impaired differentiation and function of pro- and anti-inflammatory macrophages. These results define a unique and non-redundant role for IL-10R signaling in innate immune cell control of intestinal mucosal homeostasis.

The work presented here, as part of the in requirements for the Degree of Master of Medical Sciences in the Master's Program in Clinical and Translational Investigation of Harvard University was published in *Immunity* in May 2014. The data has also been reported in several national and international conferences.

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Interleukin-10 Receptor Signaling in Innate Immune Cells Regulates Mucosal Immune Tolerance and Anti-Inflammatory Macrophage Function

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SUMMARY

Intact interleukin-10 receptor (IL-10R) signaling on effector and T regulatory (Treg) cells are each independently required to maintain immune tolerance. Here we show that IL-10 sensing by innate immune cells, independent of its effects on T cells, was critical for regulating mucosal homeostasis. Following wild-type (WT) CD4$^+$ T cell transfer, *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice developed severe colitis in association with profound defects in generation and function of Treg cells. Moreover, loss of IL-10R signaling impaired the generation and function of anti-inflammatory intestinal and bone-marrow-derived macrophages and their ability to secrete IL-10. Importantly, transfer of WT but not *Il10rb*$^{-/-}$ anti-inflammatory macrophages ameliorated colitis induction by WT CD4$^+$ T cells in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice. Similar alterations in the generation and function of anti-inflammatory macrophages were observed in IL-10R-deficient patients with very early onset inflammatory bowel disease. Collectively, our studies define innate immune IL-10R signaling as a key factor regulating mucosal immune homeostasis in mice and humans.

INTRODUCTION

Interleukin-10 (IL-10) is a key immunosuppressive cytokine that is produced by a wide range of leukocytes, as well as nonhematopoietic cells (Shouval et al., 2014). Polymorphisms in the *IL10* locus confer risk for ulcerative colitis and Crohn's disease (Franke et al., 2008; Franke et al., 2010), and mice and humans deficient in either IL-10 or IL-10 receptor (IL-10R) exhibit severe intestinal inflammation and marked proinflammatory cytokines

secretion (Begue et al., 2011; Glocker et al., 2010; Glocker et al., 2009; Kotlarz et al., 2012; Kühn et al., 1993; Moran et al., 2013; Spencer et al., 1998). Thus, IL-10 has a central role in regulation of intestinal mucosal homeostasis and prevention of inflammatory bowel disease (IBD).

IL-10 mediates its anti-inflammatory effects through IL-10R-dependent signals emanating from the cell surface. The IL-10R is a heterotetramer that consists of two subunits of IL-10Rα and two subunits of IL-10Rβ (Moore et al., 2001). Whereas the IL-10Rα subunit is unique to IL-10 signaling, the IL-10Rβ subunit is shared by other cytokine receptors, including IL-22, IL-26, and interferon-λ (IFN-λ) (Moore et al., 2001). IL-10 downstream signaling through the IL-10R inhibits the induction of proinflammatory cytokines by blocking NF-κB-dependent signals (Saraiva and O'Garra, 2010).

Although the development of IBD is well established in mice and in humans with IL-10R deficiency, the precise mechanisms of IL-10R-dependent control of immune tolerance and intestinal mucosal homeostasis are not well defined. In mice, intact IL-10R signaling is important in T regulatory (Treg) cells for their suppressive function including prevention of colitis, and in T effector cells for preventing exaggerated T helper 17 (Th17) cell responses in mucosal compartments (Chaudhry et al., 2011; Huber et al., 2011; Kamanaka et al., 2011; Murai et al., 2009). While innate immune cell production of IL-10 is critical for maintaining mucosal homeostasis (Liu et al., 2011; Murai et al., 2009), a role for innate immune IL-10R signaling in the regulation of intestinal immune tolerance has not been explored. Several groups have demonstrated that IL-10 sensing by innate immune cells is required for suppression of proinflammatory cytokines secretion (Gu et al., 2008; Pils et al., 2010). Moreover, IL-10R-deficient dendritic cells (DCs) secrete high quantities of proinflammatory cytokines after LPS stimulation (Girard-Madoux et al., 2012). We hypothesized that innate immune IL-10R signaling is required for maintenance of intestinal immune tolerance and prevention of IBD.

Here we demonstrate that IL-10R signaling in innate immune cells was critical for regulating mucosal homeostasis and prevention of colitis. Loss of IL-10R-dependent signaling rendered wild-type (WT) CD4$^+$ T cells colitogenic and was associated with markedly aberrant Treg cells generation and function. Importantly, we show that IL-10R-dependent signals modulated the differentiation and function of bone-marrow-derived macrophages (BMDM) and intestinal macrophages into either proinflammatory macrophages or functionally competent anti-inflammatory macrophages. Similarly, monocyte-derived macrophages from very early onset IBD patients harboring loss of function mutations in *IL10RA* and *IL10RB* also exhibited impaired differentiation and function of pro- and anti-inflammatory macrophages. These results define a unique and nonredundant role for IL-10R signaling in innate immune cell control of intestinal mucosal homeostasis.

RESULTS

IL-10 Regulates Intestinal Inflammation Independent of T Cell-Specific IL-10R Signaling

We have recently reported that aberrant interactions between innate immune cells devoid of the cytoskeletal regulator Wiskott-Aldrich syndrome protein (WASP) and WT CD4$^+$ T cells lead to colitis development (Nguyen et al., 2012a). In this model, *Was*$^{-/-}$*Rag2*$^{-/-}$ mice develop severe intestinal inflammation following WT CD4$^+$ T cell transfer, characterized by reduced production of IL-10; colitis development can be prevented by exogenous administration of IL-10Ig. To elucidate whether IL-10 acts on innate or adaptive immune cells in this model, we transferred *Il10rb*$^{-/-}$ CD4$^+$ T cells into *Was*$^{-/-}$*Rag2*$^{-/-}$ mice, which resulted in severe colitis in less than 2 weeks. We then assessed the effects of exogenous IL-10 in preventing disease, and as depicted in Figure S1 available online, colitis was readily abrogated by exogenous IL-10Ig administration, indicating that IL-10 can prevent intestinal inflammation independent of its function on either regulatory or effector CD4$^+$ T cells. These data are consistent with aberrant function of IL-10R signaling in innate immune cells in the setting of WASP-deficiency.

Colitis Development in *Il10rb*$^{-/-}$ Mice Requires an Adaptive Immune System

To assess directly the role of IL-10R-dependent signals in innate immune cells in the control of mucosal homeostasis, we first analyzed *Il10rb*$^{-/-}$ mice. Consistent with prior observations (Spencer et al., 1998), *Il10rb*$^{-/-}$ mice (on the 129SvEv background) developed spontaneous colitis starting around 3 months of age, characterized by extensive bowel wall thickening, lamina propria (LP) lymphoid cell infiltration, and presence of crypt abscesses, in association with increased IFN-γ$^+$- and IL-17A$^+$-producing CD4$^+$ T cells in the LP and mesenteric lymph node (MLN) (Figure S2). In order to assess whether lymphocytes are required for colitis development in *Il10rb*$^{-/-}$ mice we generated *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice, which lack mature B and T lymphocytes. Importantly, these mice are viable and do not develop clinical, endoscopic, or microscopic signs of colitis (data not shown). These data indicate that lymphocytes are essential for colitis development in *Il10rb*$^{-/-}$ mice.

Il10rb$^{-/-}$ Innate Immune Cells Render WT CD4$^+$ T Cells Colitogenic

We next hypothesized that colitis development in *Il10rb*$^{-/-}$ mice, although lymphocyte-dependent, is initiated by defects in the innate immune compartment. To assess whether *Il10rb*$^{-/-}$ deficient innate immune cells cause WT CD4$^+$ T cells to become colitogenic, we introduced unfractionated WT CD4$^+$ T cells by intraperitoneal (i.p.) injection into *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ recipient mice. *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice developed severe colitis following WT CD4$^+$ T cell transfer within 3–4 weeks (Figures 1A and 1B). Hematoxylin and eosin (H&E)-stained colonic sections demonstrated significant hyperplasia and immune cell infiltration of the LP, as well as occasional crypt abscesses (Figure 1C).

Because IL-10Rβ is also expressed on nonhematopoietic cells (Moore et al., 2001), we assessed whether loss of IL-10Rβ signaling in innate immune cells was sufficient to drive intestinal inflammation by generating bone-marrow (BM) chimeric animals. BM cells were isolated from either *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice and transferred into lethally irradiated *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ recipient mice, which after reconstitution received unfractionated WT CD4$^+$ T cells. Upon T cell transfer, *Rag2*$^{-/-}$ mice reconstituted with *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ BM developed colitis within several weeks (Figures 1D and 1E). In contrast, transfer of WT T cells into *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice reconstituted

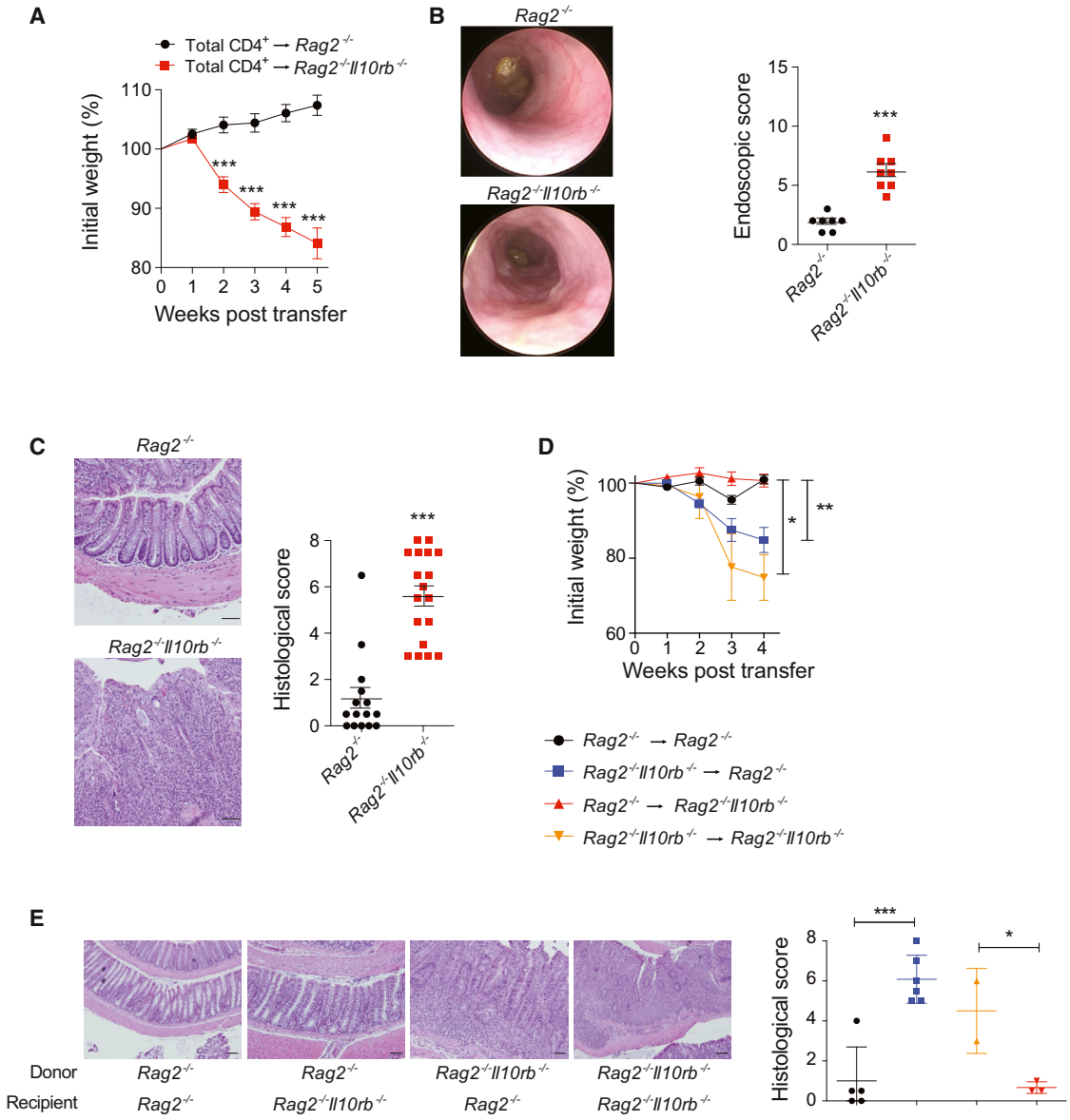

Figure 1. Transfer of WT CD4+ T Cells into *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ Mice Induces Severe Colitis

Rag2$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice were injected i.p. with 1 × 10^6 WT CD4+ T cells.

(A) Mean % initial body weights ± SEM following transfer (n = 30 for each group).

(B) Representative endoscopic images and scores ± SEM of *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice at 5 weeks posttransfer.

(C) Representative H&E colonic section images (20X) and histological score ± SEM of *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice following transfer.

(D and E) BM chimeras were generated by transferring *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ BM cells into lethally irradiated *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ recipients, and after 7 weeks WT CD4+ T cells were transferred into these mice. Mean weights ± SEM following T cells transfer displayed in (D) and images of representative H&E stained colonic sections (20X) and mean histological colitis scores ± SEM are displayed in (E). Scale bar represents 200 μm. The data are representative of two or more independent experiments. Figures S1–S3 and S6 accompany.

with *Rag2*$^{-/-}$ BM did not lead to intestinal inflammation. Overall, these findings demonstrate that *Il10rb*$^{-/-}$ innate immune cells transmit a colitogenic signal to WT CD4+ T cells.

Exaggerated Proinflammatory Cytokine Responses in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ Mice following WT CD4+ T Cell Transfer

We next assessed the effects of innate immune IL-10R deficiency on cytokine expression by analyzing *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice following WT CD4+ T cell transfer. Prior to transfer, inflammatory cytokines were not elevated in the LP of either *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice (data not shown). Following WT CD4+ T cell transfer, the T helper 1 (Th1) cell-associated cytokines tumor necrosis factor (TNF), IFN-γ, IL-6, IL-12, and IL-1β, but not IL-17A, were elevated in colonic explants and tissue extracts from *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ compared to *Rag2*$^{-/-}$ recipient mice (Figures S3A and S3B). Comparable frequencies of IL-17A+ and IFN-γ+ CD4+ T cells were detected by flow cytometry in the LP of both *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice

following WT T cell transfer; however, the absolute numbers of $CD4^+$ IFN-γ^+ T cells were significantly increased in LP of *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ compared to *Rag2*$^{-/-}$ mice (Figures S3C and S3D). Enhanced Th1 cell activity was reported in mice with a conditional deletion in macrophages and granulocytes of STAT3, a transcription factor downstream of IL-10 (Takeda et al., 1999). Overall, our data also suggests that loss of IL-10R signaling on innate immune cells is associated with exaggerated proinflammatory cytokine responses.

Loss of Innate Immune IL-10Rβ Signaling Impairs the Function and Generation of WT Treg Cells In Vivo

We next hypothesized that colitis development in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice following T cell transfer results from IL-10Rβ deficiency in innate immune cells affecting the function of either effector and/or regulatory T cell populations. Following transfer of unfractionated WT $CD4^+$ T cells the frequency of $FOXP3^+$ Treg cells was significantly reduced in the LP and MLN of *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice versus *Rag2*$^{-/-}$ mice (Figure 2A). Transfer of WT T naive cells ($CD4^+CD25^-CD45RB^{hi}$) elicited colitis in both *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ recipient mice; however, *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ recipient mice lost significantly more weight compared with *Rag2*$^{-/-}$ control group (Figures 2B and 2C). We then assessed whether cotransfer of WT Treg cells ($CD4^+CD25^+CD45RB^{lo}$) with WT T naive cells at a ratio of 1:1 (standard ratio used in the T cell transfer model is 1:4) was protective against colitis development in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice. Despite the marked increase in the fraction of Treg cells, only *Rag2*$^{-/-}$ recipient mice, but not *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ recipients, were protected from colitis development (Figures 2B and 2C), suggesting that IL-10Rβ signaling on innate immune cells regulates the suppressive function of WT Treg cells. Upon transfer of WT $CD4^+$ T naive cells, the generation of inducible Treg cells was also severely impaired in the LP and MLN of *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ recipient mice (Figure 2D).

To facilitate tracking of specific cell populations, additional transfer experiments were performed utilizing *Rag1*$^{-/-}$*Il10rb*$^{-/-}$ recipient mice on the C57BL/6 background. Similar to *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice on the 129SvEv background, these mice rapidly lost weight following transfer of unfractionated WT $CD4^+$ T cells (Figure S4A and S4B). Moreover, transfer of sorted $CD4^+CD45RB^{hi}FOXP3^{neg}$ cells into *Rag1*$^{-/-}$*Il10rb*$^{-/-}$ mice led to severe colitis, and, similar to *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ recipient mice on the 129SvEv background, was accompanied by a marked reduction in the generation of inducible $FOXP3^+$ Treg cells in the LP (Figures S4C–S4E). To assess further Treg cell maintenance, $CD4^+CD45RB^{lo}FOXP3^{pos}$ T cells were transferred into either *Rag1*$^{-/-}$*Il10rb*$^{-/-}$ or *Rag1*$^{-/-}$ mice. Treg cells transfer did not, as expected, induce colitis in either *Rag1*$^{-/-}$*Il10rb*$^{-/-}$ or *Rag1*$^{-/-}$ mice (data not shown); in addition, the frequency of Treg cells isolated from the LP and MLN was comparable between both recipient groups (Figure S4F). Collectively, our data suggest that loss of innate immune IL-10Rβ signaling impairs the generation and function of WT Treg cells in vivo.

IL-10Rβ-Dependent Signals Regulate Intestinal Macrophage Differentiation

We next sought to investigate whether sensing of IL-10 by intestinal macrophages is important for controlling mucosal homeostasis. Nomenclature for intestinal macrophage subsets is evolving rapidly (Bain et al., 2013; Rivollier et al., 2012; Tamoutounour et al., 2012; Zigmond et al., 2012); for simplicity we have followed the nomenclature described by Tamoutounour et al., who showed that circulating monocytes migrate into the LP and undergo a multistep differentiation process that progresses through four stages of development, including the proinflammatory P2 stage and the anti-inflammatory P3 and P4 stages. Throughout this manuscript, we refer to the P3 and P4 LP macrophage subsets in mice as anti-inflammatory macrophages. To evaluate whether IL-10Rβ-dependent signals regulate this differentiation process we evaluated *Il10rb*$^{-/-}$ mice at 5 weeks of age that lacked any clinical (data not shown), endoscopic, or histologic signs of intestinal inflammation (Figure 3A). Initial evaluation by flow cytometry of precolitic mice minimized identifying nonspecific effects that might be attributable to inflammation alone. LP cell analysis of precolitic *Il10rb*$^{-/-}$ mice demonstrated a significant increase in proinflammatory macrophages and a concomitant decrease in anti-inflammatory macrophages (Figures 3B and 3C). Moreover, expression of *Retnla* (Fizz1), a classical marker of anti-inflammatory macrophages and also identified in $CX3CR1^{hi}$ intestinal (presumably P4) macrophages (Zigmond et al., 2012), was decreased in the anti-inflammatory macrophages population of *Il10rb*$^{-/-}$ mice compared to WT (Figure 3D). *Il10rb*$^{-/-}$ anti-inflammatory macrophages also expressed less *Il10* and *Pdcd1l2* (programmed cell death 1 ligand 2, PD-L2) (Figure 3D). Importantly, similar results, demonstrating a reduction of anti-inflammatory macrophages, were observed in the LP of colitic *Il10rb*$^{-/-}$ mice and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice following T cell transfer (Figure S5). Collectively, these results implicate a critical role for IL-10Rβ signaling in the differentiation of intestinal macrophages.

Exogenous IL-10 Fails to Prevent Colitis in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ following T Cell Transfer

Because *Il10rb*$^{-/-}$ anti-inflammatory intestinal macrophages produce less IL-10, we assessed whether reduced IL-10 concentrations might be responsible for colitis development in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ by treating recipient mice with exogenous IL-10 following WT $CD4^+$ T cell transfer. *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice that received IL-10Ig treatment exhibited weight loss and signs of intestinal inflammation, similar to isotype control treated mice (Figures 3E and 3F), suggesting that IL-10 deficiency is not solely responsible for the colitis development. Moreover, as the $CD4^+$ T cells in these experiments express an intact IL-10R, this indicates that IL-10R signaling on $CD4^+$ T cells is insufficient to prevent colitis development in this model and suggests a nonredundant role for innate immune IL-10R signaling in regulating mucosal homeostasis.

Il10rb$^{-/-}$ M1 BMDM Produce High Quantities of Proinflammatory Cytokines and Promote Proliferation of WT $CD4^+$ T Cells

We next assessed whether BMDM, like their intestinal counterparts, were also dependent on IL-10R signaling for their differentiation and function. Stimulation of BMDM in vitro with LPS and IFN-γ generates M1 proinflammatory macrophages, while varying combinations of IL-4, IL-13, transforming growth

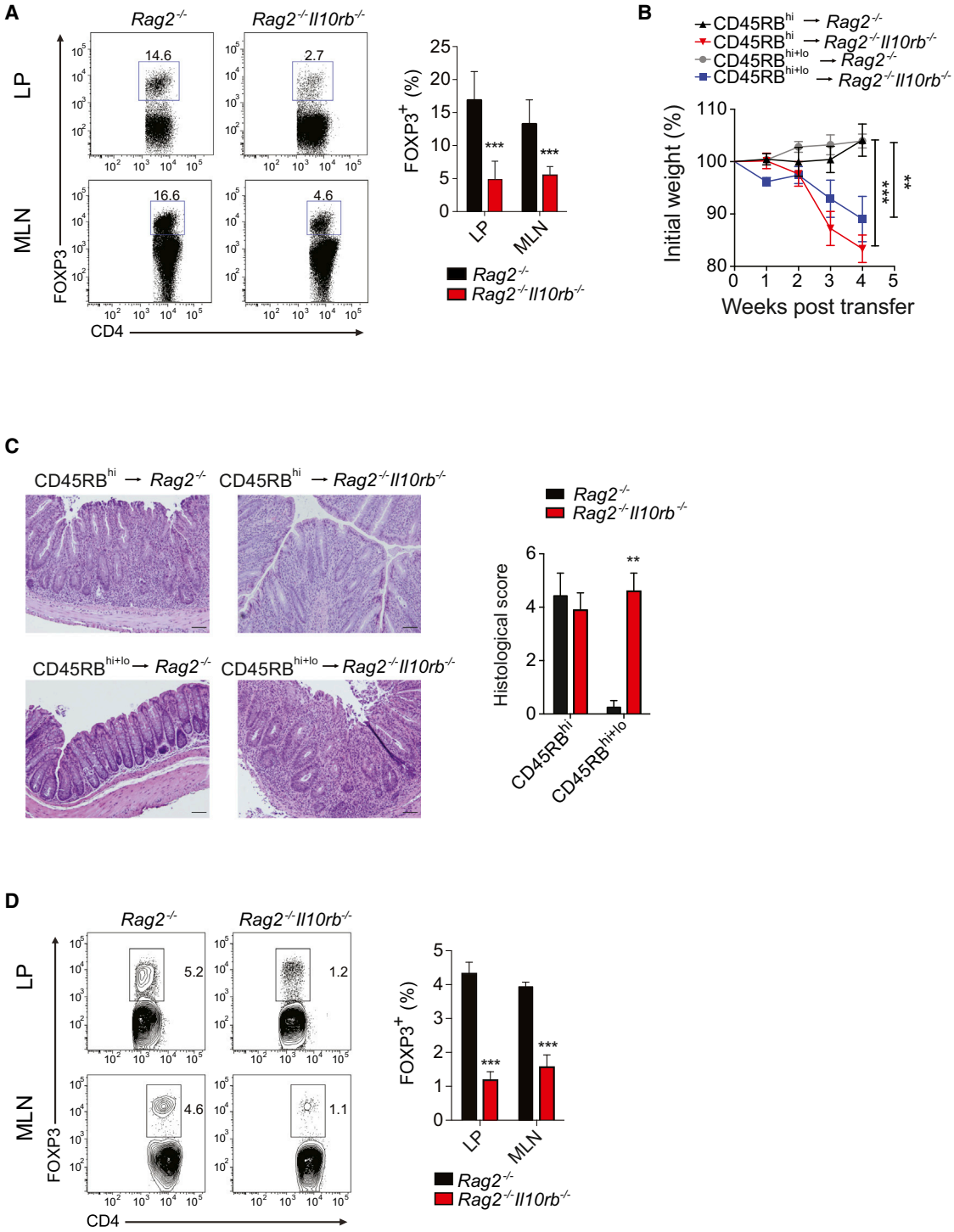

Figure 2. *Il10rb*$^{-/-}$ Innate Immune Cells Impair WT Treg Cells Suppression and Generation In Vivo

(A) Frequency of Treg cells in LP and MLN of *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice that were transferred with unfractionated WT CD4^{+} T cell transfer. Representative flow cytometry plots of FOXP3^{+} cells among CD4^{+} T cells are followed by cumulative data in LP and MLN.

(B) Mean % initial body weights ± SEM following transfer of WT T naive (CD4^{+}CD25^{-}CD45RB^{hi}) cells alone or in combination with Treg cells (CD4^{+}CD25^{+} CD45RB^{lo}) at a 1:1 ratio.

(C) Representative H&E images (20X) of colonic sections from *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice following transfer and mean histological colitis scores ± SEM. Scale bar represents 200 μm.

(D) Representative flow cytometry plots of the generation of inducible Treg cells in vivo assessed by FOXP3^{+} expression among CD4^{+} T cells in LP and MLN, 4 weeks after CD45RB^{hi} transfer, followed by cumulative data. Results are pooled from two independent experiments. Figures S4 and S6 accompany.

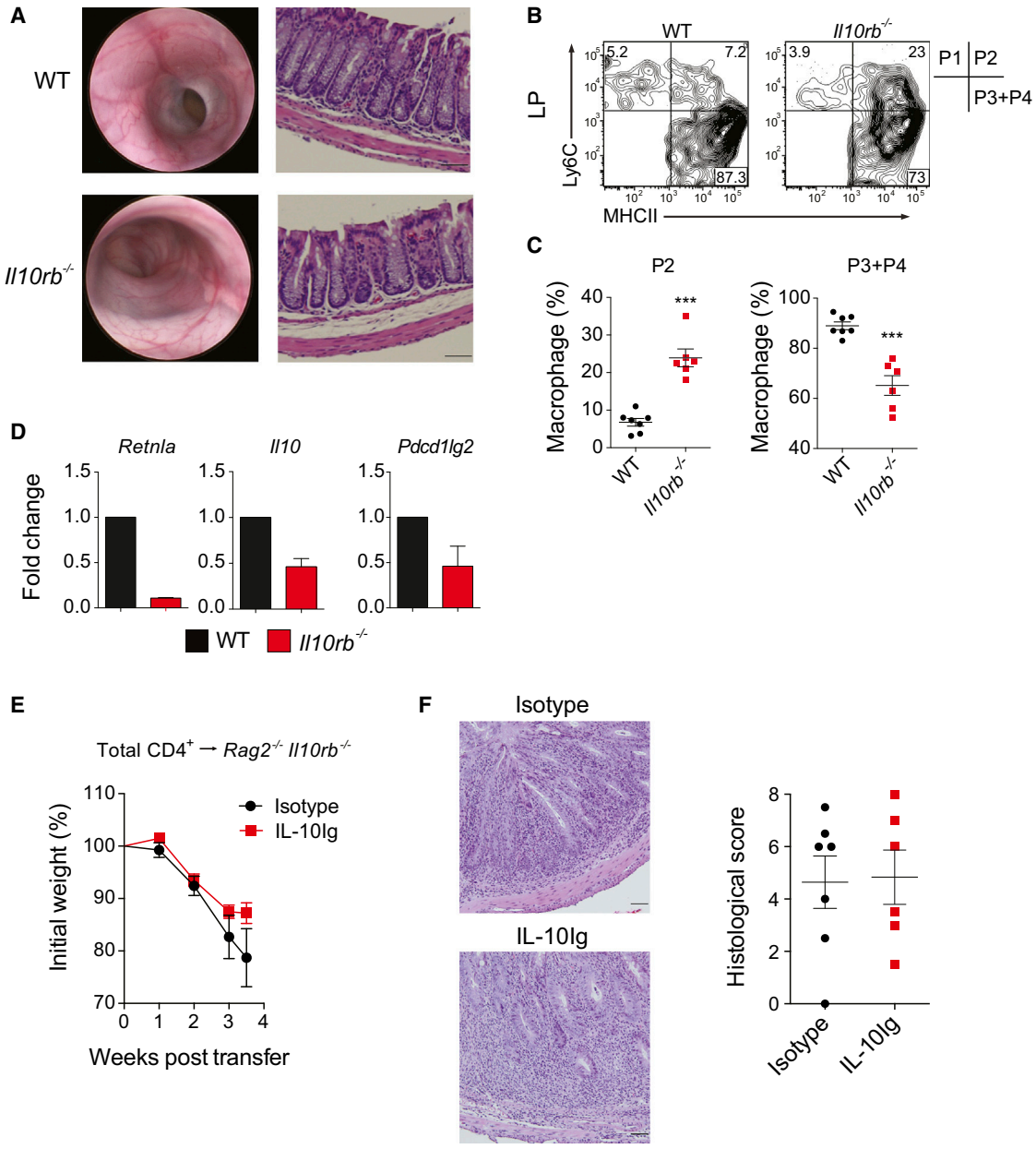

Figure 3. Reduction in Anti-Inflammatory Intestinal Macrophages in Precolitic *Il10rb*$^{-/-}$ Mice

(A) Endoscopic and histological colonic (20X) images of WT and *Il10rb*$^{-/-}$ mice at 5 weeks of age.

(B and C) Representative flow cytometry plots of macrophage subsets in LP of 5-week-old WT and *Il10rb*$^{-/-}$ mice, followed by quantification of the pro- and anti-inflammatory populations. Proinflammatory population was defined as Ly6C^{+}MHCII^{+} cells and anti-inflammatory as Ly6C^{-}MHCII^{+}.

(D) LP anti-inflammatory macrophages were sorted from WT (n = 20) and *Il10rb*$^{-/-}$ (n = 14) 5-week-old mice and qRT-PCR was performed to quantify expression of various anti-inflammatory transcripts. Results are representative of two independent experiments.

(E and F) *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ were injected with 1 μg of IL-10Ig or isotype one day prior to WT CD4^{+} T cell transfer, and then twice weekly. Mean weights ± SEM shown in (E) and representative H&E colonic section images (20X) and histological scores ± SEM of both groups shown in (F). Scale bar represents 200 μm. Results are pooled from two independent experiments. Figures S5 and S6 accompany.

factor-β (TGF-β) and IL-10 generate M2 tolerogenic macrophages (Martinez et al., 2008). Parsa and colleagues recently reported that stimulation of BMDM with IL-4, TGF-β, and IL-10 yields macrophages with increased tolerogenic properties that were defined as M2r macrophages (Parsa et al., 2012). These M2r macrophages highly express programmed death-ligand 1 (PD-L1) and PD-L2, secrete IL-10 and TGF-β, and, when transferred into NOD mice, prevent diabetes (Parsa et al., 2012).

We observed comparable expression of pro- and anti-inflammatory cytokines and costimulatory molecules between WT and *Il10rb*$^{-/-}$ unstimulated (M0) BMDM (data not shown). However,

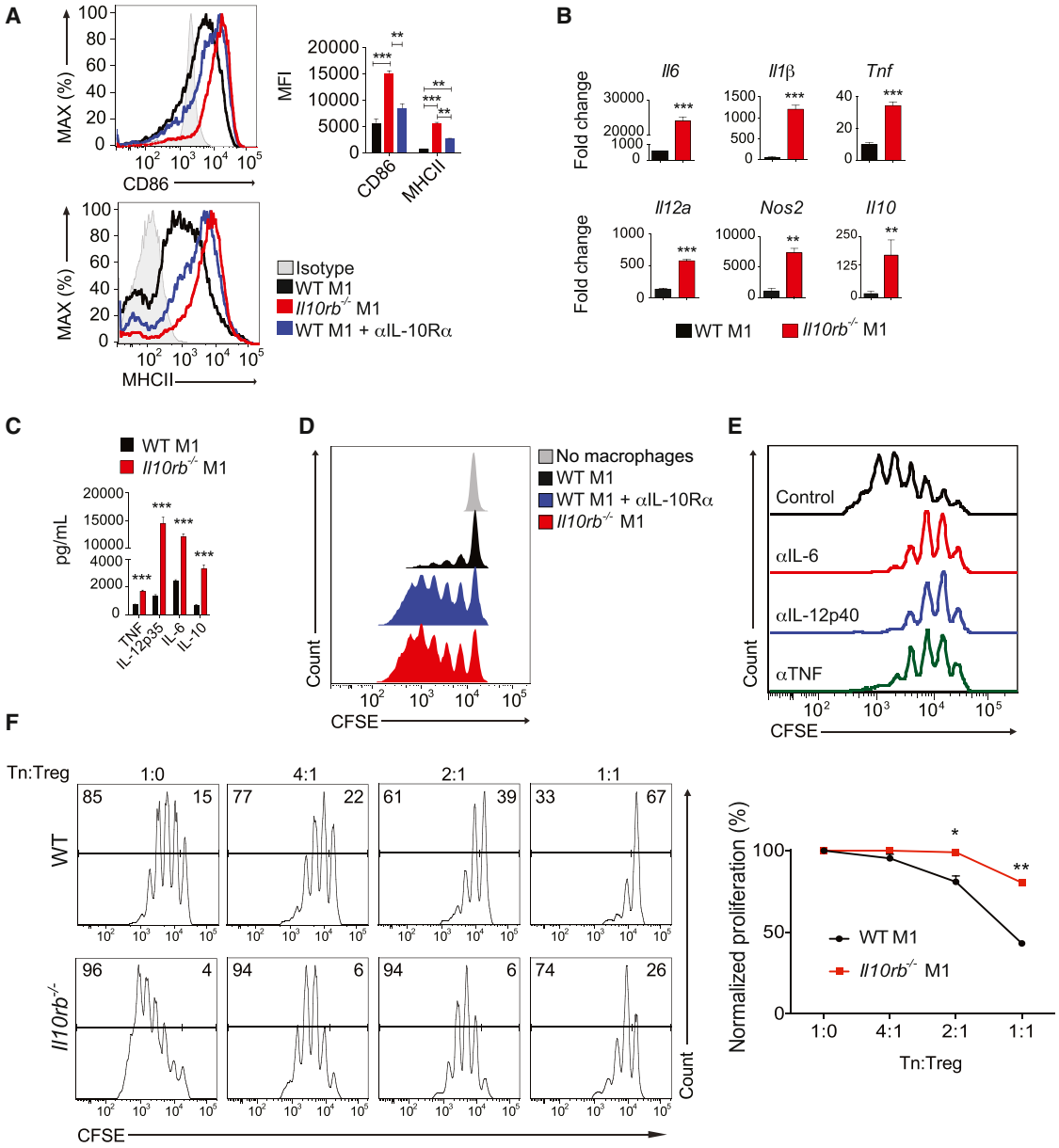

Figure 4. *Il10rb*$^{-/-}$ M1 BMDM Exhibit a Pronounced Proinflammatory Phenotype

(A) Mean fluorescence intensity (MFI) of MHCII and CD86 expression on M1 WT and *Il10rb*$^{-/-}$ BMDM or WT BMDM cultured with an IL-10Rα blocking antibody in M1 conditions.

(B) Cytokine mRNA expression determined by qRT-PCR of BMDM cultured for 24 hr in M1 conditions; fold change is relative to unstimulated (M0) WT BMDM.

(C) Cytokine concentrations determined by ELISA in supernatants of BMDM cultured for 48 hr under M1 conditions.

(D) Representative flow cytometry plots of CFSE-labeled WT CD4$^+$CD25$^-$ T naive cells cultured without macrophages or in the presence of WT M1 BMDM, *Il10rb*$^{-/-}$ M1 BMDM, or WT BMDM cultured with an IL-10Rα blocking antibody in M1 conditions.

(E) *Il10rb*$^{-/-}$ M1 BMDM were cultured with sorted T naive cells, in the presence of neutralizing antibodies to IL-6, IL-12p40, or TNF.

(F) WT T naive cells were cultured with WT or *Il10rb*$^{-/-}$ M1 BMDM in the presence of varying concentrations of WT Treg cells. Representative flow cytometry plots presented following cumulative data showing degree of proliferation normalized to conditions without Treg cells. All data are representative of two or more independent experiments. Figure S6 accompanies.

major histocompatibility complex class II (MHCII) glycoproteins, CD86 and proinflammatory mediators were highly expressed in *Il10rb*$^{-/-}$ BMDM cultured in M1 conditions, when compared to WT M1 BMDM (Figures 4A–4C). Similarly, culture of WT BMDM in M1 conditions with a blocking IL-10Rα antibody also led to a significant increase in expression of CD86 and MHCII (Figure 4A). *Il10rb*$^{-/-}$ M1 BMDM produced significantly more IL-10 (Figures 4B and 4C), suggesting that IL-10Rβ-dependent signaling in proinflammatory macrophages inhibits IL-10 production. In addition, in a coculture system with WT

CD4$^+$CD25$^-$ T naive cells and M1 BMDM serving as antigen-presenting cells, when compared to WT M1 BMDM, *Il10rb*$^{-/-}$ M1 BMDM or WT BMDM cultured with anti-IL-10Rα antibody under M1 conditions promoted increased proliferation of WT T naive cells (Figure 4D). Addition of neutralizing antibodies in this in vitro coculture system against IL-6, IL-12p40, or TNF decreased the degree of T cell proliferation generated by *Il10rb*$^{-/-}$ M1 BMDM (Figure 4E), suggesting that excessive T cell proliferation is not caused by an excess of a single proinflammatory cytokine. Moreover, *Il10rb*$^{-/-}$ M1 BMDM impaired the ability of WT Treg cells to suppress proliferation of T effector cells (Figure 4F). To rule out the possibility that defective signaling through cytokine receptors that also utilize IL-10Rβ (i.e., IL-22, IL-26, and IFN-λ) might contribute to the observed phenotypes of *Il10rb*$^{-/-}$ M1 BMDM, we performed additional experiments with BM obtained from *Il10ra*$^{-/-}$ mice that lack only defective IL-10R signaling. Like *Il10rb*$^{-/-}$ M1 BMDM, *Il10ra*$^{-/-}$ M1 BMDM when compared with WT M1 BMDM highly expressed CD86 and MHCII and, when cultured with WT CD4$^+$ CD25$^-$ T naive cells, promoted increased T cell proliferation (data not shown). Collectively, our data indicate that IL-10R signaling regulates the function of inflammatory macrophages, which in turn can modulate T cell responses.

IL-10R Signaling Promotes Tolerogenic Properties of Anti-Inflammatory BMDM

Under M2r conditions, *Il10rb*$^{-/-}$ BMDM expressed significantly less *Arg1* and *Retnla* (Figure 5A), which are classical markers of M2 anti-inflammatory macrophages (Martinez et al., 2008). Similarly, incubation of WT BMDM with IL-4 and TGF-β, but not including IL-10, also resulted in reduced *Arg1* and *Retnla* expression when compared to M2r conditions (Figure 5A), implying that IL-10 is required for the induction of the anti-inflammatory program in BMDM. Sensing of IL-10 by WT BMDM increased production of IL-10 (Figure 5A), indicating that IL-10R-dependent signals positively regulate IL-10 production by M2 macrophages. Baseline secretion of proinflammatory cytokines was low and comparable between WT and *Il10rb*$^{-/-}$ M2r BMDM (data not shown). However, restimulation with LPS of established *Il10rb*$^{-/-}$ M2r cells, or WT BMDM cultured with IL-4 and TGF-β, but not IL-10, led to a significant increase in the expression of proinflammatory cytokines compared to WT BMDM cultured under M2r conditions (Figure 5B). These data suggest that IL-10R signaling in macrophages is required to inhibit TLR4-dependent proinflammatory responses. In addition, *Il10rb*$^{-/-}$ M2r BMDM, compared to WT M2r BMDM, promoted less Treg cells generation when cocultured with WT CD4$^+$CD25$^-$ T naive cells (Figure 5C). This correlated with lower expression on *Il10rb*$^{-/-}$ M2r BMDM of PD-L1 and PD-L2 molecules known to promote Treg cells generation (Francisco et al., 2009; Zhang et al., 2006) (Figure 5D). Finally, we assessed whether transfer of WT M2r BMDM would inhibit the T cell transfer-induced colitis in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice. Administration of WT M2r BMDM i.p. 1 day prior to WT CD4$^+$ T cell transfer protected *Rag2*$^{-/-}$ *Il10rb*$^{-/-}$ mice from intestinal inflammation, whereas transfer of *Il10rb*$^{-/-}$ M2r BMDM was associated with rapid weight loss and increased mortality among transferred mice within 2–3 weeks (Figures 5E and 5F). Overall, our data suggests that loss of IL-10Rβ signaling impairs the generation and function of anti-inflammatory macrophages and that restoration of aberrant macrophage function can ameliorate colitis in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice.

Aberrant Generation and Function of Monocyte-Derived Macrophages from IL-10R-Deficient Patients

We next sought to investigate whether patients with null mutations in *IL-10R* genes also exhibit alterations in the generation and function of macrophage subsets. Through our interNational Early Onset Pediatric IBD Cohort Study (NEOPICS; www.neopics.org), we obtained blood samples from seven rare patients with loss of function mutations in *IL10RA* and *IL10RB* genes, all diagnosed with severe infantile IBD (Table S1). In humans, stimulation of CD14$^+$ blood monocytes with granulocyte macrophage-colony stimulating factor (GM-CSF) for 8 days generates M1 proinflammatory macrophages (Rey-Giraud et al., 2012), while M-CSF treatment for 7 days followed by 24 hr culture with IL-4 generates M2 macrophages (Hedl and Abraham, 2012). Similar to murine *Il10rb*$^{-/-}$ M1 BMDM, human IL-10R-deficient M1 macrophages highly expressed proinflammatory cytokines when compared to controls (Figure 6A), whereas *IL10* expression among patients was variable (Figure 6A). Human IL-10R-deficient M1 macrophages also expressed elevated concentrations of CD86 and HLA-DR (Figure 6B) and augmented proliferation of CD4$^+$CD25$^-$ T naive cells from allogeneic control subjects (Figure 6C), data that is consistent with the results observed in murine IL-10R deficient M1 BMDM.

The generation and function of M2 macrophages was also impaired in IL-10R-deficient patients, with lower expression of several human M2 markers, whereas *IL10* expression was variable (Figure 7A). In addition, expression of CD86 and HLA-DR was higher in IL-10R-deficient M2 macrophages (Figure 7B), and when re-stimulated with LPS, these cells secreted significantly more proinflammatory cytokines (Figure 7C), similar to findings in murine *Il10rb*$^{-/-}$ anti-inflammatory BMDM. *IL10* expression was significantly reduced in human *IL10R*-deficient M2 macrophages following secondary LPS stimulation (Figure 7C), suggesting that in human anti-inflammatory macrophages IL-10R signaling is required for IL-10 production after TLR-4 stimulation. Finally, as observed in mice, human IL-10R-deficient M2 macrophages expressed lower concentrations of *PDL2* (Figure 7D) and promoted less generation of Treg cells in vitro (Figure 7E). This human data and our murine data described above indicate that IL-10R signaling modulates the generation and function of pro- and anti-inflammatory macrophages across species. Collectively, based on our findings, we propose a model depicting the role of IL-10R signaling on macrophages in the regulation of intestinal immune homeostasis (Figure S6).

DISCUSSION

Numerous murine studies have established a role for IL-10 and downstream IL-10R signaling as major regulators of immune tolerance in mucosal compartments (Shouval et al., 2014). Recent studies in humans have identified causal loss-of-function mutations of *IL10* or either *IL10RA* or *IL10RB* in rare patients presenting with very early onset IBD and have identified hematopoietic cells broadly as the responsible cells mediating this

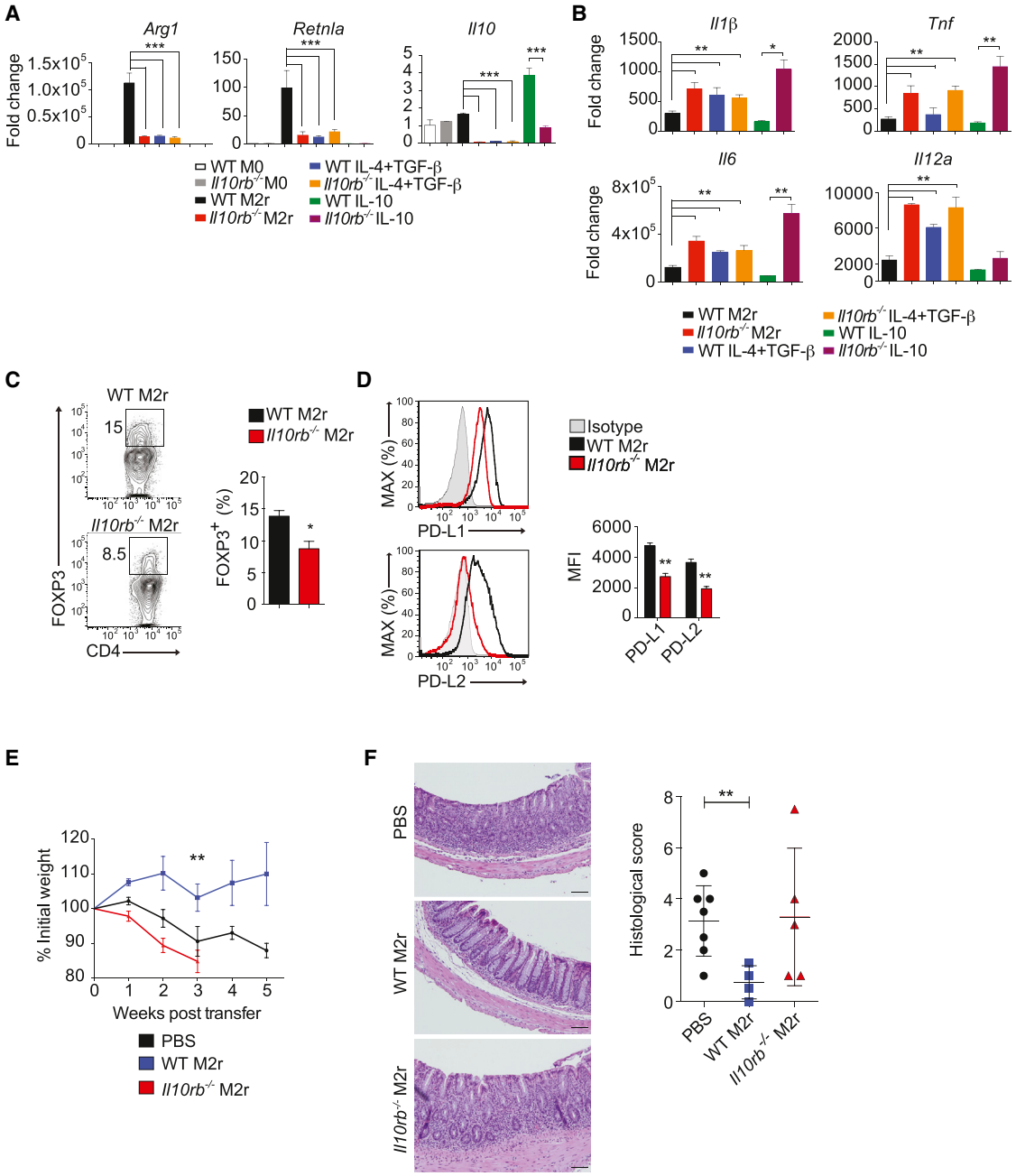

Figure 5. Loss of IL-10Rβ Signaling Impairs the Generation and Function of Anti-Inflammatory M2r BMDM

(A) qRT-PCR analysis of *Arg1*, *Retnla* (Fizz1), and *Il10* transcripts produced by WT or *Il10rb*$^{-/-}$ BMDM cultured for 24 hr under different conditions.

(B) Proinflammatory cytokines mRNA expression by WT and *Il10rb*$^{-/-}$ BMDM cultured in different conditions for 24 hr and then restimulated for 4 hr with LPS.

(C) Representative flow cytometry plots and cumulative data of in vitro generation of FOXP3^{+} Treg cells among CD4^{+} T cells in the presence of WT or *Il10rb*$^{-/-}$ M2r macrophages.

(D) Representative flow cyometry plots and cumulative MFI of PD-L1 and PD-L2 surface expression on WT and *Il10rb*$^{-/-}$ M2r BMDM.

(E) 1 ×10^{6} WT or *Il10rb*$^{-/-}$ M2r BMDM or PBS were injected i.p. into *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice one day prior to WT CD4^{+} T cell transfer. Figure depicts mean % initial body weights ± SEM following transfer.

(F) Representative H&E stained colonic sections (20X) followed by histological scores ± SEM for treated groups. Scale bar represents 200 μm. Results are pooled from two or more independent experiments. Figure S6 accompanies.

phenotype (Engelhardt et al., 2013; Glocker et al., 2010; Glocker et al., 2009; Kotlarz et al., 2012; Moran et al., 2013). More mechanistic studies exploring cell types dependent on IL-10R signaling have been limited to murine models and have concentrated largely on the regulation of mucosal T cell responses (Chaudhry et al., 2011; Huber et al., 2011; Kamanaka et al.,

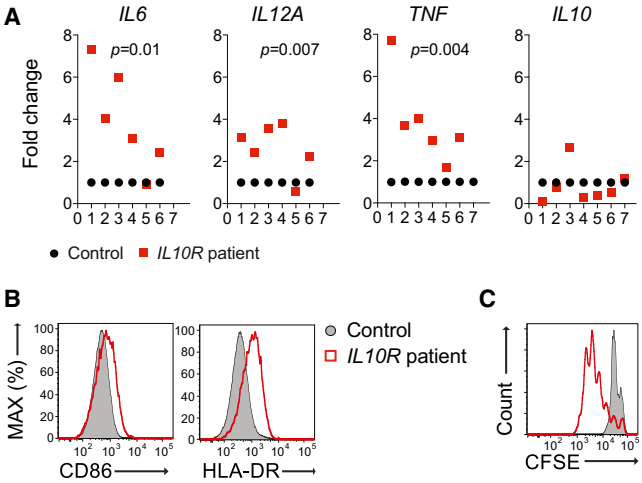

Figure 6. Increased Proinflammatory Cytokine Production and CD4$^+$ T Cell Proliferation by Human IL-10R-Deficient M1 Macrophages

(A) qRT-PCR analysis of proinflammatory cytokines among seven patients with loss-of-function mutations in *IL10R* genes versus healthy controls. Each red circle represents a unique patient, whereas each black rectangle represents an individual healthy control subject in the same experiment. Cytokine expression is normalized to corresponding healthy controls.

(B) Flow cytometry plots demonstrating high CD86 and HLA-DR expression on M1 macrophages from an IL-10R-deficient patient, compared to healthy control.

(C) Proliferation of CFSE-labeled CD4$^+$CD25$^-$ T naive cells obtained from an allogeneic healthy donor in the presence of IL-10R-deficient M1 macrophages from a patient compared to M1 macrophages obtained from a healthy control. Surface marker expression and proliferation data are representative of five patients. Table S1 and Figure S6 accompany.

2011; Murai et al., 2009). Although IL-10 is known to control anti-inflammatory responses in DCs and macrophages in peripheral compartments (Bhattacharyya et al., 2004; Fiorentino et al., 1991; Steinbrink et al., 1997), the role of IL-10R-dependent signals in the intestine has not been explored. Here, we demonstrated that loss of IL-10R signaling on innate cells impairs their crosstalk with T cells, leading to defective mucosal immune regulation and severe intestinal inflammation.

Our data show that IL-10R signaling coordinates the differentiation and function of pro- and anti-inflammatory macrophages in both intestinal and peripheral immune compartments. IL-10R-dependent signals suppress the generation of proinflammatory LP P2 macrophages, facilitate the generation of tolerogenic intestinal macrophages, and enhance their ability to secrete IL-10. IL-10R-dependent signals also suppress proinflammatory M1 macrophages derived from BM by inhibiting the secretion of proinflammatory cytokines and the ability of these cells to drive CD4$^+$ T naive cell proliferation. Moreover, the differentiation and function of anti-inflammatory BMDM also requires IL-10-dependent signals, because the expression of M2 markers and the ability of M2r macrophages to both suppress TLR-4-mediated proinflammatory cytokine secretion and to generate inducible Treg cells is reduced in IL-10R-deficient macrophages. Importantly, mirroring our findings in the murine system, we observed aberrant differentiation and function of pro- and anti-inflammatory macrophages in seven IL-10R-deficient patients who presented with infantile IBD, hence identifying IL-10R signaling as a critical modulator of the development and function of pathogenic and tolerogenic macrophages in mice and humans.

Amelioration of disease by the transfer of WT M2r BMDM, but not *Il10rb*$^{-/-}$ M2r BMDM, in mice lacking IL-10R in innate immune cells further suggests that IL-10R signaling on macrophages plays a key role in driving intestinal inflammation. Medina-Contreras and colleagues have reported that transfer of WT BMDM can ameliorate DSS-induced colitis in CX3CR1-deficient mice (Medina-Contreras et al., 2011). Similarly and consistent with our findings, Kayama and colleagues have recently reported that transfer of sorted intestinal CX3CR1^{hi} macrophages alleviates colitis in *Rag1*$^{-/-}$ mice transferred with CD45RB^{hi} cells (Kayama et al., 2012). However, transfer of CX3CR1^{hi} macrophages obtained from mice with conditional deletion of STAT3 in macrophages failed to rescue disease (Kayama et al., 2012). These findings are also consistent with recent data by Zigmond et al. showing that IL-10Rα deficiency in CX3CR1$^+$ macrophages results in spontaneous colitis (Zigmond et al., 2014).

Several aberrant macrophage-dependent immunoregulatory mechanisms resulting from IL-10R-deficiency might promote intestinal inflammation. Among anti-inflammatory cells, our data indicate that *Il10rb*$^{-/-}$ mice exhibit a decrease in generation of anti-inflammatory macrophage subsets and a decrease in *Il10* and *Pdcd1l2* expression, which, in turn, might result in decreased Treg cell generation observed in vitro and in vivo. Diminished generation and function of M2r BMDM in *Il10rb*$^{-/-}$ mice, with reduced PD-L1 and PD-L2 surface expression, IL-10 production, and Treg cell generation, further support the intestinal findings. Murai and colleagues have reported that IL-10 production by intestinal CD11b$^+$ innate immune cells, likely macrophages, is required for Treg cell maintenance (Murai et al., 2009). In addition, CX3CR1$^+$ macrophages promote the generation and expansion of Treg cells (Denning et al., 2007; Hadis et al., 2011). Our data from seven very early onset IBD patients harboring causal mutations of *IL10RA* and *IL10RB* show aberrant generation of M2 macrophages, diminished *IL10* expression, and decreased generation of inducible Treg cells, and hence further validate and add greater relevance to our findings in the murine system. Colitis development in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice cannot be attributed solely to diminished IL-10 production by IL-10Rβ-deficient innate immune cells because exogenous administration of IL-10Ig did not protect these mice from intestinal inflammation.

Elevated proinflammatory cytokine production and augmentation of CD4$^+$ T cells proliferation in vitro in culture with *Il10rb*$^{-/-}$ M1 BMDM support the hypothesis that loss of IL-10R signaling might, independent of its role on anti-inflammatory macrophage function, lead to exaggerated intestinal inflammation. Our work is consistent with studies employing *LyzM-cre*- or *Itgax-cre*-mediated deletion of IL-10Rα predominantly in macrophages or DCs, respectively, that were associated with elevated LPS-induced proinflammatory cytokines and effector T cell responses in the skin (Girard-Madoux et al., 2012; Pils et al., 2010). Moreover, recent studies have demonstrated that peritoneal monocytes lacking IL-10Rα differentiate into a proinflammatory MHCII^{hi} macrophage subset (Nguyen et al., 2012b).

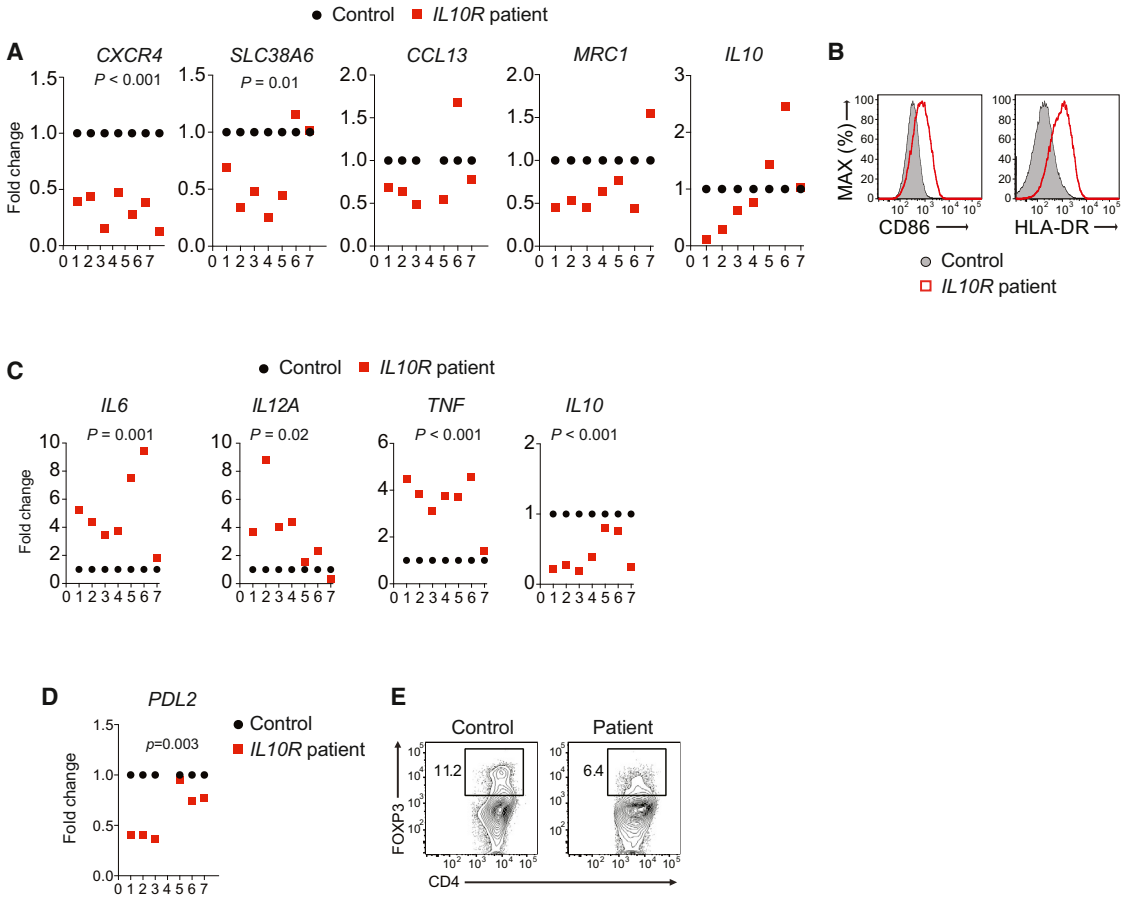

Figure 7. Impaired Generation and Function of Anti-Inflammatory Macrophages in Patients with Loss of IL-10R Signaling

(A) qRT-PCR analysis of M2 markers expressed in IL-4 stimulated monocyte-derived macrophages from IL-10R deficient patients versus healthy subjects.

(B) Surface expression of CD86 and HLA-DR by M2 macrophages generated from an IL-10R-deficient patient compared to healthy control.

(C) qRT-PCR analysis of various cytokines expressed by M2 macrophages following restimulation with LPS.

(D) *PDL2* expression by M2 macrophages detected by qRT-PCR.

(E) Flow cytometry plot illustrating in vitro Treg cells generation from $CD4^+$ T naive cells in the presence of M2 macrophages from an IL-10R-deficieint patient compared to healthy control. Treg cell generation and flow cytometry data are representative of two patients. Table S1 and Figure S6 accompany.

Finally, IL-10-mediated signaling is known to suppress IL-1β secretion (Guarda et al., 2011), and in turn, IL-1β-dependent signals drive effector T cell responses and colitis development (Coccia et al., 2012).

One limitation of *Il10rb*$^{-/-}$ mice as a model for studying the IL-10 pathway is that signaling by IL-22, IL-26, and IFN-λ also utilizes the IL-10Rβ chain as a coreceptor. Nonetheless, we speculate that the contribution of these later cytokines to colitis development in *Il10rb*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ is minimal, since they are almost exclusively expressed on nonhematopoietic cells (Lasfar et al., 2011; Sabat, 2010). Moreover, in vitro experiments utilizing *Il10ra*$^{-/-}$ M1 BMDM or administration of neutralizing IL-10Rα antibodies mimicked the phenotype observed in *Il10rb*$^{-/-}$ BMDM studies. Finally, to date, the clinical presentation of patients with mutations in either the *IL10RA* or *IL10RB* genes appear indistinguishable (Shouval et al., 2014), and in vitro studies with macrophages from *IL10RA*- and *IL10RB*-deficient patients appear similar. Nonetheless, a role for IL-10Rβ signals downstream of other cytokines cannot be excluded; because cytokines such as IL-22 are known to contribute to mucosal homeostasis (Zenewicz et al., 2013), more specific approaches targeting IL-10Rα in specific innate immune cells are warranted. Indeed, the study by Zigmond et al., employing *Cx3cr1-cre*-mediated targeting of IL-10Rα, suggests that defective IL-10Rα-signaling largely limited to this anti-inflammatory macrophage subset results in spontaneous colitis (Zigmond et al., 2014).

In conclusion, our data define a critical role for IL-10R signaling in innate immune populations in maintaining mucosal immune tolerance and preventing IBD. Our murine studies indicate that IL-10R-dependent signals suppress proinflammatory macrophage function as well as enhance tolerogenic macrophages properties, both in peripheral compartments and in the intestine. Data from several very early onset IBD patients harboring mutations in *IL10R* genes also strengthen these findings and define IL-10R as a key regulator of macrophages differentiation and function in humans as well. Targeted therapies delivering IL-10 to innate immune cells or modulating IL-10R-dependent signals

in these cells might provide a future direction of drug development for carefully selected IBD patients.

EXPERIMENTAL PROCEDURES

Mice

WT, *Il10rb*$^{-/-}$ (obtained from Genentech), *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Il10rb*$^{-/-}$, and *Was*$^{-/-}$*Rag2*$^{-/-}$ mice, all on 129 SvEv background, as well as WT, *Il10ra*$^{-/-}$, *Il10rb*$^{-/-}$ (courtesy of Thaddeus Stappenbeck, Washington University), *Rag1*$^{-/-}$, *Rag1*$^{-/-}$*Il10rb*$^{-/-}$, and FOXP3-GFP on the C57BL/6 background were maintained in specific pathogen-free animal facility at Boston Children's Hospital. Experiments were conducted after approval from the Animal Resources at Children's Hospital and according to regulations of the Institutional Animal Care and Use Committees (IACUC).

Induction of Colitis in Transfer Experiments

In unfractionated CD4$^+$ T cell transfer experiments, cells from peripheral lymph nodes, MLNs and spleens from WT mice were enriched for CD4$^+$ cells with a negative selection kit (Miltenyi Biotec). The purity of CD4$^+$ cells was >95%. *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ or *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Il10rb*$^{-/-}$ mice were adoptively transferred with 1 × 10^6 WT CD4$^+$ T cells by i.p. injection. In some experiments, *Il10rb*$^{-/-}$ CD4$^+$ T cells were isolated and transferred to *Was*$^{-/-}$*Rag2*$^{-/-}$ mice. For T naive and Treg cells adoptive transfer experiments, WT CD4$^+$ cells were enriched by negative selection as described above and further sorted by BD FACSAria II SORP (BD Biosciences). T naive cells were defined as CD4$^+$CD25$^-$CD45RB^{hi} and Treg cells as CD4$^+$CD25$^+$CD45RB^{lo}. Post-sort purity was typically >98%. Age-matched *Rag2*$^{-/-}$ mice or *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice were injected i.p. with 1–2 × 10^5 WT T naive cells with or without Treg cells at a 1:1 ratio. Similarly, CD4$^+$CD45RB^{hi}FOXP3^{neg} or CD4$^+$CD45RB^{lo}FOXP3^{pos} cells were obtained from FOXP3-GFP reporter mice and used for adoptive transfer experiments into *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Il10rb*$^{-/-}$ mice.

Isolation of LP Cells

Colons underwent epithelial layer stripping with agitation in 10 mM EDTA at 37°C twice before digestion in collagenase VIII. Following that specimens were enriched with a 40% and 90% Percoll (GE Healthcare) gradient to remove epithelial cells. In some experiments, LP macrophages were sorted. Gating strategy was based on Bain et al. who showed that distinct macrophages subsets can be isolated without using CX3CR1-GFP reporter mice (Bain et al., 2013). We performed some modifications to this method: following initial gating on live CD45$^+$ cells, we gated on CD11b$^+$CD64$^+$CD103$^-$ cells, then based on SSC and FSC (Bain et al., 2013), and finally on Ly6C and MHCII.

Generation of BMDM

BM was flushed from femur and tibia bones and cultured with DMEM, 20% FBS, penicillin 100 IU/ml, streptomycin 100 μg/ml and 30% L cell-conditioned medium, at 37°C in 5% CO_2. Media was supplemented every 2–3 days. Following 6–7 days, nonadherent cells were aspirated and adherent macrophages were removed by washing plate with ice-cold PBS and scraping. For generation of M1 macrophages, BMDM were stimulated for 24 hr with 100 ng/mL of LPS (Sigma-Aldrich) and 20 ng/mL IFN-γ (Peprotech). To generate M2r macrophages, BMDM were cultured for 24 hr with 20 ng/mL IL-4, 20 ng/mL human TGF-β1, and 20 ng/mL IL-10 (all from peprotech). In some experiments, WT BMDM were cultured with 10 μg/mL of anti-IL-10Rα blocking antibody (BioLegend) in M1 conditions.

Generation of Human Monocyte-Derived Macrophages

Blood was collected in EDTA tubes from patients with loss-of-function *IL10R* mutations and control subjects (either a healthy parent or an unrelated healthy donor) in accordance with the local Institutional Review Board and the Declaration of Helsinki. Blood samples were shipped at room temperature overnight to our laboratory at Boston Children's Hospital and upon arrival PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare) gradient, according to manufacturer's instructions. Monocytes were sorted with CD14 positive selection kit (Miltenyi Biotec) and cultured in RPMI 1640 supplemented with 20% FCS and antibiotics. To generate M1 macrophages, we supplemented media with 100 ng/mL GM-CSF for 8 days (Rey-Giraud et al., 2012) and for M2 macrophages media with 50 ng/mL of M-CSF for 7 days and an additional day with 20 ng/mL of IL-4 (Hedl and Abraham, 2012).

Quantitative RT-PCR

RNA was extracted from whole colons or from cells with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA was reverse transcribed from 1 μg total RNA with iScript Select cDNA Synthesis Kit (Bio-Rad). Analyses of transcripts were performed with iQ SYBR Green on a CFX96 Real-Time System (Bio-Rad). Cytokine transcripts were normalized against hypoxanthine-guanine phosphoribosyltransferase (HPRT), and normalized fold change was calculated with the ΔΔCt method against mean control ΔCt (*Rag2*$^{-/-}$ for *Rag2*$^{-/-}$*Il10rb*$^{-/-}$; WT for *Il10rb*$^{-/-}$ in BMDM experiments or macrophages from a healthy paired subject in experiments with monocytes derived macrophages from *IL10R*-deficient patients). For human M1 and M2 macrophages generation experiments, genes associated with each lineage were chosen as reported by Martinez et al. (Martinez et al., 2006).

In Vitro CD4$^+$ T Naive Proliferation and Treg Generation

To assess proliferation, we cultured 5 μM CFSE-labeled 1 × 10^5 WT CD4$^+$CD25$^-$ T naive cells with 2 μg/mL soluble αCD3 and either 2.5 × 10^4 WT or *Il10rb*$^{-/-}$ M1 BMDM, for 4 days. Proliferation was determined by percent of CFSE dilution. For Treg cells generation assays, 1 × 10^5 WT CD4$^+$CD25$^-$ sorted T naive cells were cultured with 2 μg/mL soluble αCD3 (eBioscience), 2 ng/mL human TGF-β1 (Peprotech) and either 2.5 × 10^4 WT or *Il10rb*$^{-/-}$ M2r BMDM, for 5 days. Similar experiments were performed with human M1 or M2 macrophages from IL-10R-deficient patients versus healthy controls. In these experiments CD4$^+$ T naive cells were isolated from an unrelated healthy subject. In some proliferation experiments, blocking antibodies against IL-6, IL-12p40, and TNF (BioLegend, 10 μg/mL) were added to the culture on day 0 and day 2.

Sequencing of *IL10R* Genes

Patients 1–3 were sequenced as reported elsewhere, while sequencing of patients 4–7 was performed at Muise laboratory at The Hospital for Sick Children, Toronto. Genomic DNA was purified from whole blood with the Puregene Blood Kit (QIAGEN). *IL10RA* and *IL10RB* were amplified with intronic primers flanking each exon. Purified PCR products were sequenced with the ABI3730 DNA analyzer (Applied Biosystems). *IL10RA* variant is numbered according to GeneBank accession number NM_001588. *IL10RB* variant is numbered according to GeneBank accession number NM_00628. Numbering of amino acid residues in *IL10RA* and *IL10RB* refers to their position in the immature protein that includes the signal peptide.

In some cases, RNA was isolated from whole blood by the PAXgene Blood RNA kit (QIAGEN) according to the manufacture instructions. cDNA was synthesized with SuperScript III Reverse Transcriptase (Life Technologies). Primers for full-length *IL10RA* (For: TCA GTC CCA GCC CAA GGG TA; Rev: TGC AGG TCC AAG TTC TTC AGC TCT) and full-length *IL10RB* (For: TCG TGT GCT TGG AGG AAG CC; Rev: TAA GTC CAG GGT CTG GGA GTT CTA) were designed and synthesized at The Centre for Applied Genomics, Toronto. PCR was performed according to standard protocol and sequenced by ABI 3730 DNA analyzer (Applied Biosystems).

Statistical Analysis

Differences between groups were determined by unpaired two-tailed t test with GraphPad. Significance was defined if p value was less than 0.05 as following: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.03.011.

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REFERENCES

Bain, C.C., Scott, C.L., Uronen-Hansson, H., Gudjonsson, S., Jansson, O., Grip, O., Guilliams, M., Malissen, B., Agace, W.W., and Mowat, A.M. (2013). Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. Mucosal Immunol. *6*, 498–510.

Begue, B., Verdier, J., Rieux-Laucat, F., Goulet, O., Morali, A., Canioni, D., Hugot, J.P., Daussy, C., Verkarre, V., Pigneur, B., et al. (2011). Defective IL10 signaling defining a subgroup of patients with inflammatory bowel disease. Am. J. Gastroenterol. *106*, 1544–1555.

Bhattacharyya, S., Sen, P., Wallet, M., Long, B., Baldwin, A.S., Jr., and Tisch, R. (2004). Immunoregulation of dendritic cells by IL-10 is mediated through suppression of the PI3K/Akt pathway and of IkappaB kinase activity. Blood *104*, 1100–1109.

Chaudhry, A., Samstein, R.M., Treuting, P., Liang, Y., Pils, M.C., Heinrich, J.M., Jack, R.S., Wunderlich, F.T., Brüning, J.C., Müller, W., and Rudensky, A.Y. (2011). Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity *34*, 566–578.

Coccia, M., Harrison, O.J., Schiering, C., Asquith, M.J., Becher, B., Powrie, F., and Maloy, K.J. (2012). IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. J. Exp. Med. *209*, 1595–1609.

Denning, T.L., Wang, Y.C., Patel, S.R., Williams, I.R., and Pulendran, B. (2007). Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat. Immunol. *8*, 1086–1094.

Engelhardt, K.R., Shah, N., Faizura-Yeop, I., Kocacik Uygun, D.F., Frede, N., Muise, A.M., Shteyer, E., Filiz, S., Chee, R., Elawad, M., et al. (2013). Clinical outcome in IL-10- and IL-10 receptor-deficient patients with or without hematopoietic stem cell transplantation. J. Allergy Clin. Immunol. *131*, 825–830.

Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M., and O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. J. Immunol. *147*, 3815–3822.

Francisco, L.M., Salinas, V.H., Brown, K.E., Vanguri, V.K., Freeman, G.J., Kuchroo, V.K., and Sharpe, A.H. (2009). PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J. Exp. Med. *206*, 3015–3029.

Franke, A., Balschun, T., Karlsen, T.H., Sventoraityte, J., Nikolaus, S., Mayr, G., Domingues, F.S., Albrecht, M., Nothnagel, M., Ellinghaus, D., et al.; IBSEN study group (2008). Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. Nat. Genet. *40*, 1319–1323.

Franke, A., McGovern, D.P., Barrett, J.C., Wang, K., Radford-Smith, G.L., Ahmad, T., Lees, C.W., Balschun, T., Lee, J., Roberts, R., et al. (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat. Genet. *42*, 1118–1125.

Girard-Madoux, M.J., Kel, J.M., Reizis, B., and Clausen, B.E. (2012). IL-10 controls dendritic cell-induced T-cell reactivation in the skin to limit contact hypersensitivity. The Journal of allergy and clinical immunology *129*, 143–150 e141-110.

Glocker, E.O., Kotlarz, D., Boztug, K., Gertz, E.M., Schäffer, A.A., Noyan, F., Perro, M., Diestelhorst, J., Allroth, A., Murugan, D., et al. (2009). Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N. Engl. J. Med. *361*, 2033–2045.

Glocker, E.O., Frede, N., Perro, M., Sebire, N., Elawad, M., Shah, N., and Grimbacher, B. (2010). Infant colitis—it's in the genes. Lancet *376*, 1272.

Gu, Y., Yang, J., Ouyang, X., Liu, W., Li, H., Yang, J., Bromberg, J., Chen, S.H., Mayer, L., Unkeless, J.C., and Xiong, H. (2008). Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. Eur. J. Immunol. *38*, 1807–1813.

Guarda, G., Braun, M., Staehli, F., Tardivel, A., Mattmann, C., Förster, I., Farlik, M., Decker, T., Du Pasquier, R.A., Romero, P., and Tschopp, J. (2011). Type I interferon inhibits interleukin-1 production and inflammasome activation. Immunity *34*, 213–223.

Hadis, U., Wahl, B., Schulz, O., Hardtke-Wolenski, M., Schippers, A., Wagner, N., Müller, W., Sparwasser, T., Förster, R., and Pabst, O. (2011). Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. Immunity *34*, 237–246.

Hedl, M., and Abraham, C. (2012). Nod2-induced autocrine interleukin-1 alters signaling by ERK and p38 to differentially regulate secretion of inflammatory cytokines. Gastroenterology *143*, 1530–1543.

Huber, S., Gagliani, N., Esplugues, E., O'Connor, W., Jr., Huber, F.J., Chaudhry, A., Kamanaka, M., Kobayashi, Y., Booth, C.J., Rudensky, A.Y., et al. (2011). Th17 cells express interleukin-10 receptor and are controlled by Foxp3^{-} and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. Immunity *34*, 554–565.

Kamanaka, M., Huber, S., Zenewicz, L.A., Gagliani, N., Rathinam, C., O'Connor, W., Jr., Wan, Y.Y., Nakae, S., Iwakura, Y., Hao, L., and Flavell, R.A. (2011). Memory/effector (CD45RB(lo)) CD4 T cells are controlled directly by IL-10 and cause IL-22-dependent intestinal pathology. J. Exp. Med. *208*, 1027–1040.

Kayama, H., Ueda, Y., Sawa, Y., Jeon, S.G., Ma, J.S., Okumura, R., Kubo, A., Ishii, M., Okazaki, T., Murakami, M., et al. (2012). Intestinal CX3C chemokine receptor 1(high) (CX3CR1(high)) myeloid cells prevent T-cell-dependent colitis. Proc. Natl. Acad. Sci. USA *109*, 5010–5015.

Kotlarz, D., Beier, R., Murugan, D., Diestelhorst, J., Jensen, O., Boztug, K., Pfeifer, D., Kreipe, H., Pfister, E.D., Baumann, U., et al. (2012). Loss of interleukin-10 signaling and infantile inflammatory bowel disease: implications for diagnosis and therapy. Gastroenterology *143*, 347–355.

Kühn, R., Löhler, J., Rennick, D., Rajewsky, K., and Müller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell *75*, 263–274.

Lasfar, A., Abushahba, W., Balan, M., and Cohen-Solal, K.A. (2011). Interferon lambda: a new sword in cancer immunotherapy. Clin. Dev. Immunol. *2011*, 349575.

Liu, B., Tonkonogy, S.L., and Sartor, R.B. (2011). Antigen-presenting cell production of IL-10 inhibits T-helper 1 and 17 cell responses and suppresses colitis in mice. Gastroenterology *141*, 653–662, 662 e651–654.

Martinez, F.O., Gordon, S., Locati, M., and Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J. Immunol. *177*, 7303–7311.

Martinez, F.O., Sica, A., Mantovani, A., and Locati, M. (2008). Macrophage activation and polarization. Frontiers in bioscience: a journal and virtual library *13*, 453–461.

Medina-Contreras, O., Geem, D., Laur, O., Williams, I.R., Lira, S.A., Nusrat, A., Parkos, C.A., and Denning, T.L. (2011). CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice. J. Clin. Invest. *121*, 4787–4795.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. *19*, 683–765.

Moran, C.J., Walters, T.D., Guo, C.H., Kugathasan, S., Klein, C., Turner, D., Wolters, V.M., Bandsma, R.H., Mouzaki, M., Zachos, M., et al. (2013). IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. Inflamm. Bowel Dis. *19*, 115–123.

Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C.L., Cheroutre, H., and Kronenberg, M. (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat. Immunol. *10*, 1178–1184.

Nguyen, D.D., Wurbel, M.A., Goettel, J.A., Eston, M.A., Ahmed, O.S., Marin, R., Boden, E.K., Villablanca, E.J., Paidassi, H., Ahuja, V., et al. (2012a). Wiskott-Aldrich syndrome protein deficiency in innate immune cells leads to mucosal immune dysregulation and colitis in mice. Gastroenterology *143*, 719–729 e711–712.

Nguyen, H.H., Tran, B.T., Muller, W., and Jack, R.S. (2012b). IL-10 acts as a developmental switch guiding monocyte differentiation to macrophages during a murine peritoneal infection. J. Immunol. *189*, 3112–3120.

Parsa, R., Andresen, P., Gillett, A., Mia, S., Zhang, X.M., Mayans, S., Holmberg, D., and Harris, R.A. (2012). Adoptive transfer of immunomodulatory M2 macrophages prevents type 1 diabetes in NOD mice. Diabetes *61*, 2881–2892.

Pils, M.C., Pisano, F., Fasnacht, N., Heinrich, J.M., Groebe, L., Schippers, A., Rozell, B., Jack, R.S., and Müller, W. (2010). Monocytes/macrophages and/or neutrophils are the target of IL-10 in the LPS endotoxemia model. Eur. J. Immunol. *40*, 443–448.

Rey-Giraud, F., Hafner, M., and Ries, C.H. (2012). In vitro generation of monocyte-derived macrophages under serum-free conditions improves their tumor promoting functions. PLoS ONE *7*, e42656.

Rivollier, A., He, J., Kole, A., Valatas, V., and Kelsall, B.L. (2012). Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. J. Exp. Med. *209*, 139–155.

Sabat, R. (2010). IL-10 family of cytokines. Cytokine Growth Factor Rev. *21*, 315–324.

Saraiva, M., and O'Garra, A. (2010). The regulation of IL-10 production by immune cells. Nat. Rev. Immunol. *10*, 170–181.

Shouval, D.S., Ouahed, J., Biswas, A., Goettel, J.A., Horwitz, B.H., Klein, C., Muise, A.M., and Snapper, S.B. (2014). Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. Adv. Immunol. *122*, 177–210.

Spencer, S.D., Di Marco, F., Hooley, J., Pitts-Meek, S., Bauer, M., Ryan, A.M., Sordat, B., Gibbs, V.C., and Aguet, M. (1998). The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. J. Exp. Med. *187*, 571–578.

Steinbrink, K., Wölfl, M., Jonuleit, H., Knop, J., and Enk, A.H. (1997). Induction of tolerance by IL-10-treated dendritic cells. J. Immunol. *159*, 4772–4780.

Takeda, K., Clausen, B.E., Kaisho, T., Tsujimura, T., Terada, N., Förster, I., and Akira, S. (1999). Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity *10*, 39–49.

Tamoutounour, S., Henri, S., Lelouard, H., de Bovis, B., de Haar, C., van der Woude, C.J., Woltman, A.M., Reyal, Y., Bonnet, D., Sichien, D., et al. (2012). CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. Eur. J. Immunol. *42*, 3150–3166.

Zenewicz, L.A., Yin, X., Wang, G., Elinav, E., Hao, L., Zhao, L., and Flavell, R.A. (2013). IL-22 deficiency alters colonic microbiota to be transmissible and colitogenic. J. Immunol. *190*, 5306–5312.

Zhang, Y., Chung, Y., Bishop, C., Daugherty, B., Chute, H., Holst, P., Kurahara, C., Lott, F., Sun, N., Welcher, A.A., and Dong, C. (2006). Regulation of T cell activation and tolerance by PDL2. Proc. Natl. Acad. Sci. USA *103*, 11695–11700.

Zigmond, E., Varol, C., Farache, J., Elmaliah, E., Satpathy, A.T., Friedlander, G., Mack, M., Shpigel, N., Boneca, I.G., Murphy, K.M., et al. (2012). Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. Immunity *37*, 1076–1090.

Zigmond, E., Bernshtein, B., Friedlander, G., Walker, C.R., Yona, S., Kim, K.-W., Brenner, O., Krauthgamer, R., Varol, C., Müller, W., and Jung, S. (2014). Macrophage-restricted Interleukin-10 receptor-, but not IL-10 deficiency causes severe spontaneous colitis. Immunity *40*. Published online May 1, 2014. http://dx.doi.org/10.1016/j.immuni.2014.03.012.

Immunity, Volume *40*

Supplemental Information

Interleukin-10 Receptor Signaling in Innate Immune Cells Regulates Mucosal Immune Tolerance and Anti-Inflammatory Macrophage Function

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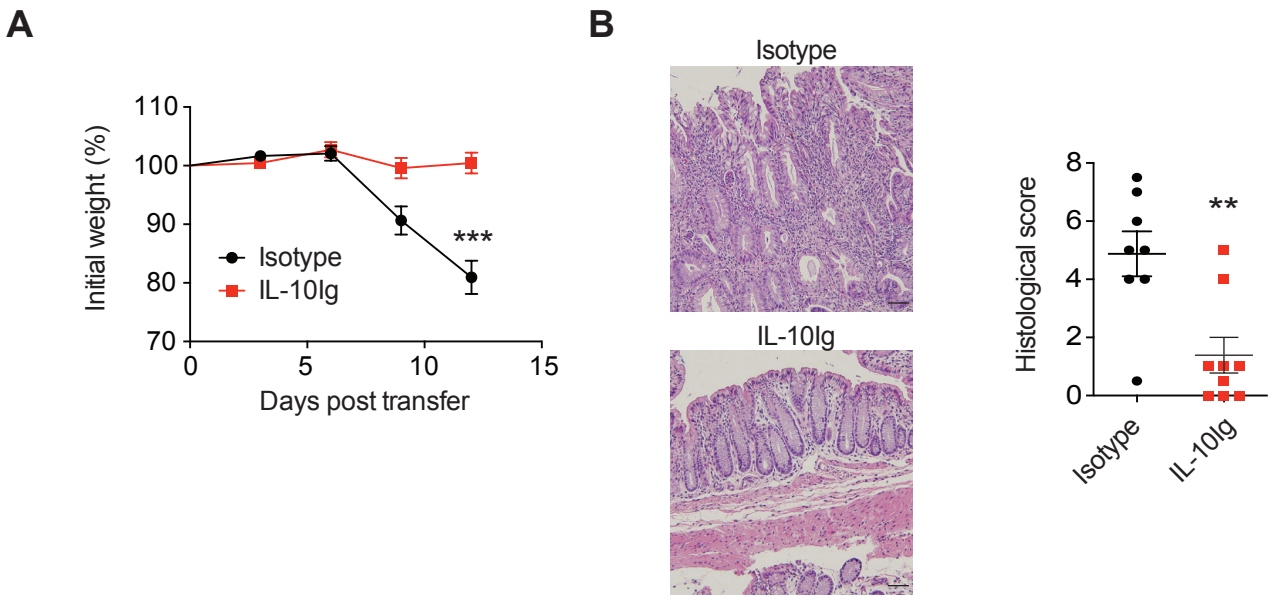

Figure S1, related to Figure 1. **Exogenous IL-10 prevents colitis in *Was*$^{-/-}$*Rag2*$^{-/-}$ mice independent of IL-10R signaling in CD4^{+} T cells**. *Was*$^{-/-}$*Rag2*$^{-/-}$ mice were injected *i.p.* with 1x10^{6} unfractionated *Il10rb*$^{-/-}$ CD4^{+} T cells and treated with 1 μg of IL-10Ig or isotype control twice weekly, starting one day prior to T cell transfer. (A) Mean % initial body weights displayed ± SEM. (B) Representative H&E stained sections (20X) of colonic tissue and mean histological scores ± SEM. Scale bar = 200 μm. Data pooled from 3 independent experiments.

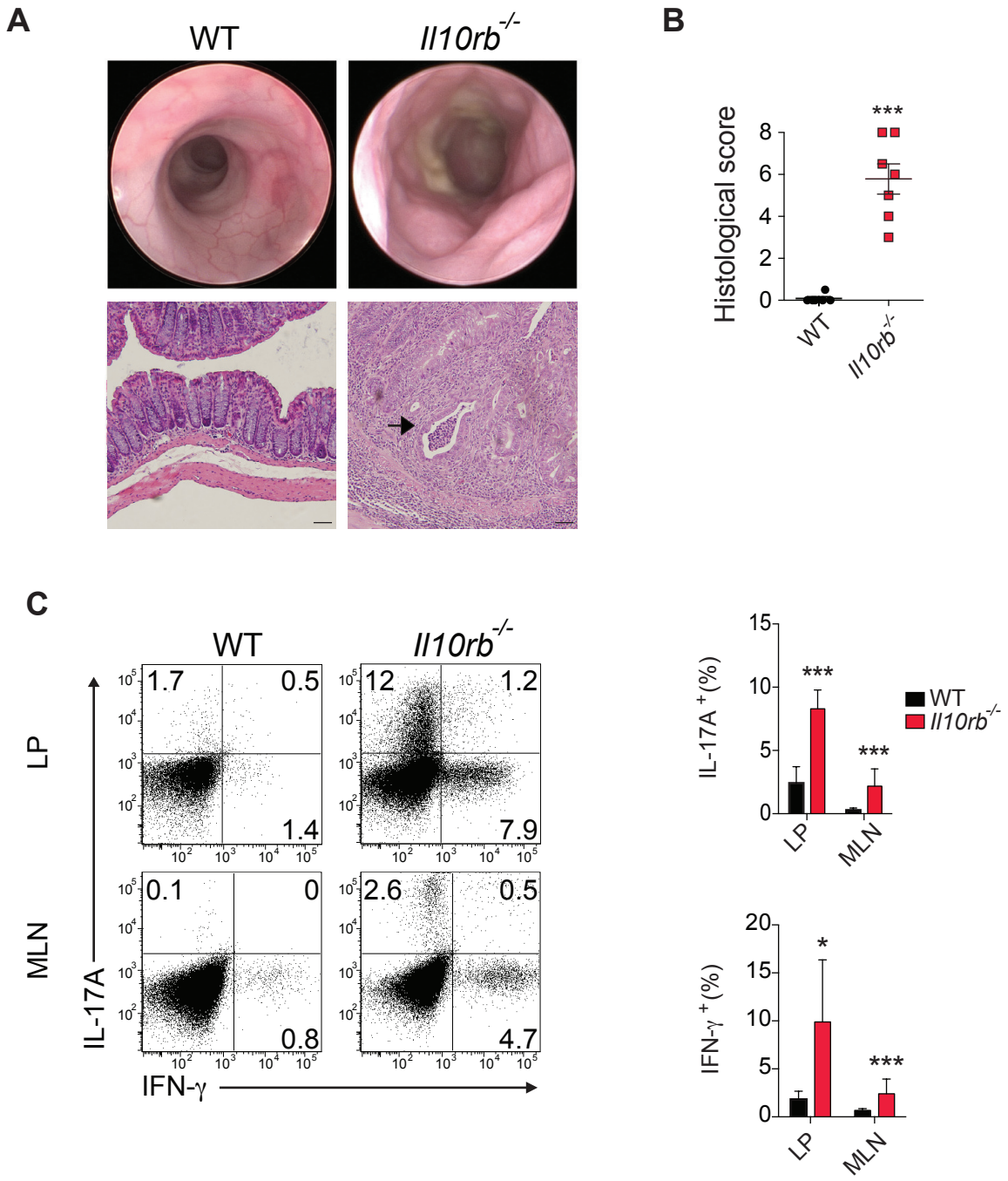

Figure S2, related to Figure 1. ***Il10rb*$^{-/-}$ mice develop spontaneous colitis associated with an increase in IFN-γ^{+}- and IL-17A^{+}-producing CD4^{+} T cells**. (A) Representative endoscopic images and H&E stained colonic sections (20X) of 6-month-old WT and *Il10rb*$^{-/-}$ mice. Arrow pointing to a crypt abscess frequently observed in *Il10rb*$^{-/-}$ mice. Scale bar = 200 μm. (B) Mean histological scores of colonic tissue ± SEM of WT and *Il10rb*$^{-/-}$ mice at 4-6 months of age. (C) Representative FACS plots and cumulative data ± SEM of IFN-γ^{+} and IL-17A^{+} cells among CD4^{+} T cells in the LP and MLN. Results were pooled from 3 independent experiments.

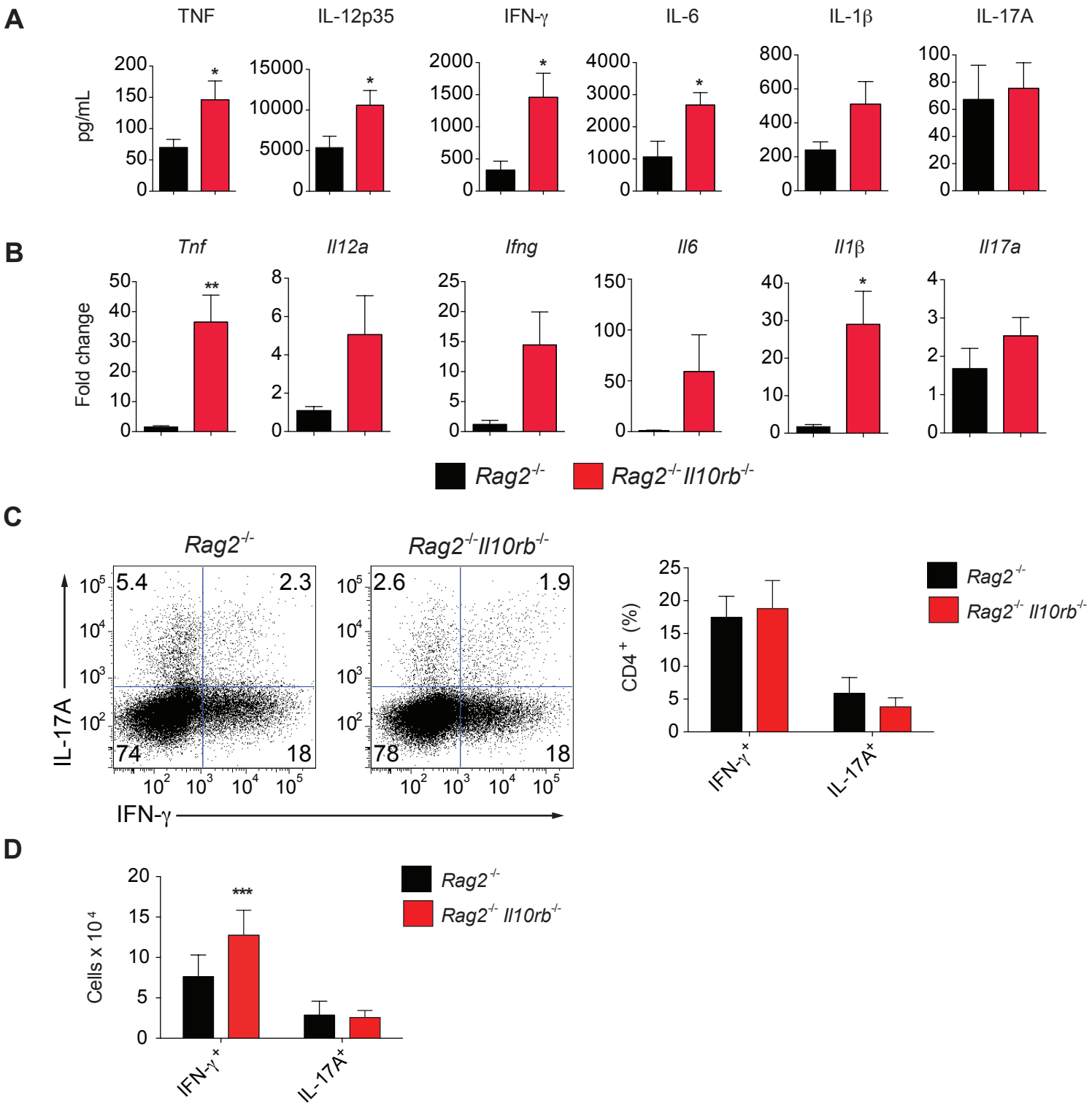

Figure S3, related to Figure 1. Exaggerated pro-inflammatory response in *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ colons following T cell transfer. (A) Cytokine concentrations determined by ELISA in supernatants of colonic explants culture of *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice. (B) Cytokine mRNA expression measured by qRT-PCR on colonic tissue of *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ recipients of WT CD4^{+} T cells. Fold change is relative to *Rag2*$^{-/-}$ recipient mice transferred with CD4^{+} T cells. n=5 or more for both experiments. (C) Representative FACS plot followed by cumulative frequencies of IFN-γ^{+} and IL-17A^{+} cells among CD4^{+} T cells in the LP of *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ *Il10rb*$^{-/-}$ mice following CD4^{+} T cell transfer. (D) Absolute numbers (per 1x10^{6} LP cells) of T effector cell subsets in the LP of *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice following CD4^{+} T cell transfer. Results pooled from 2 independent experiments.

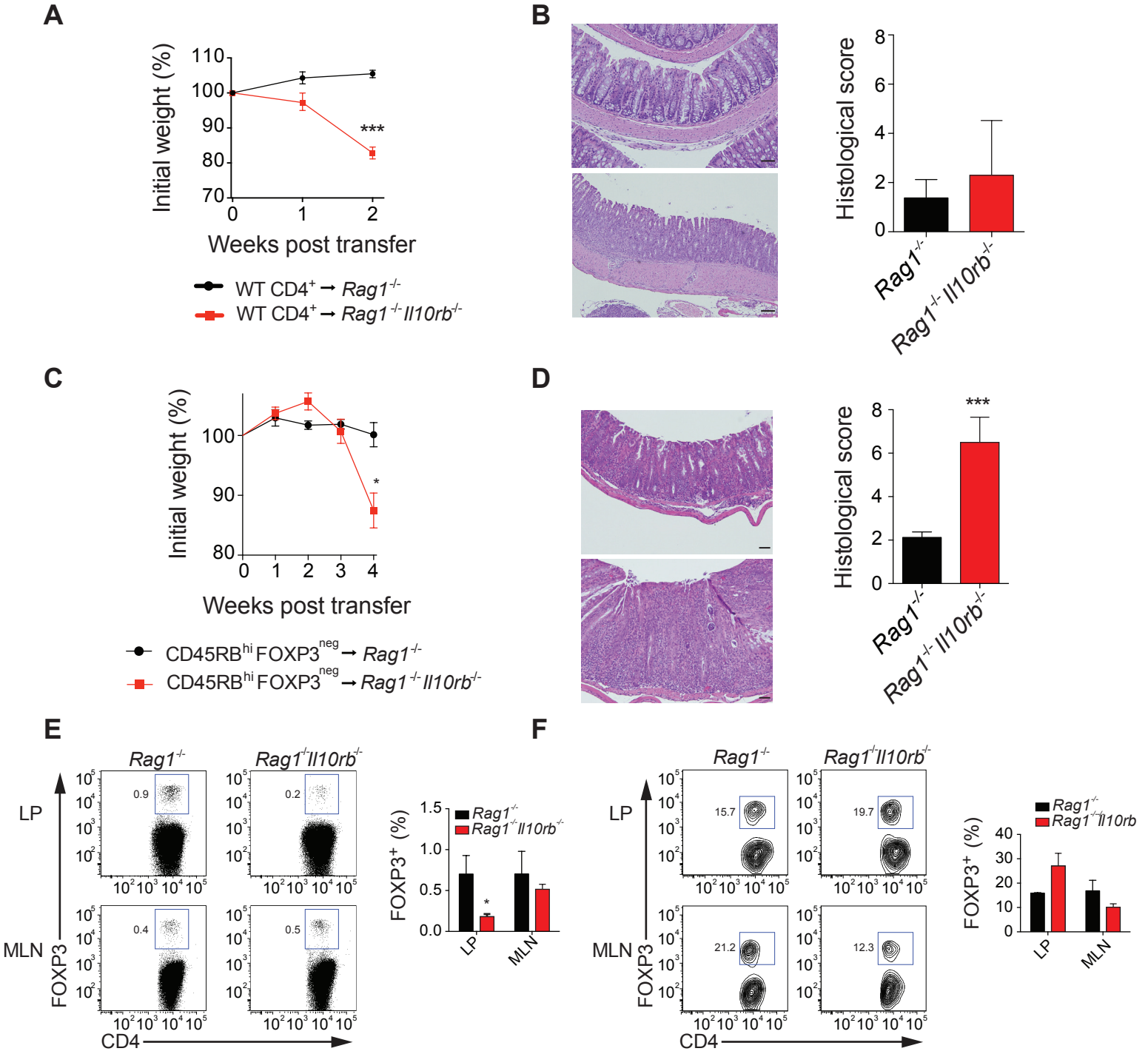

Figure S4, related to Figure 2: Impaired Treg cells generation in *Rag1$^{-/-}$Il10rb$^{-/-}$* mice following CD4^{+}CD45RB^{hi}FOXP3^{neg} T cell transfer. (A) Weight curves of *Rag1$^{-/-}$* and *Rag1$^{-/-}$ Il10rb$^{-/-}$* mice on the C57BL/6 background injected *i.p.* with 1x10^{6} WT CD4^{+} T cells, followed by (B) representative H&E section images (20X) and mean histological colitis scores ± SEM. (C) Weight curves of *Rag1$^{-/-}$* and *Rag1$^{-/-}$Il10rb$^{-/-}$* mice injected *i.p.* with 2x10^{5} CD4^{+}CD45RB^{hi}FOXP3^{neg} T cells, followed by (D) representative H&E section images (20X) and mean histological colitis scores ± SEM. Scale bar = 200 μm. (E) Generation of inducible Treg cells was assessed by determining the frequency of FOXP3^{+} cells among CD4^{+} T cells 4 weeks following transfer of CD4^{+}CD45RB^{hi}FOXP3^{neg} T cells into *Rag1$^{-/-}$* and *Rag1$^{-/-}$Il10rb$^{-/-}$* mice. Representative FACS plots shown with quantified data presented as the mean ± SEM. (F) Treg cells maintenance was determined by the frequency of FOXP3^{+} cells among CD4^{+} T cells 4 weeks following transfer of CD4^{+}CD45RB^{lo}FOXP3^{pos} cells. Representative FACS plots shown with quantified data presented as the mean ± SEM.

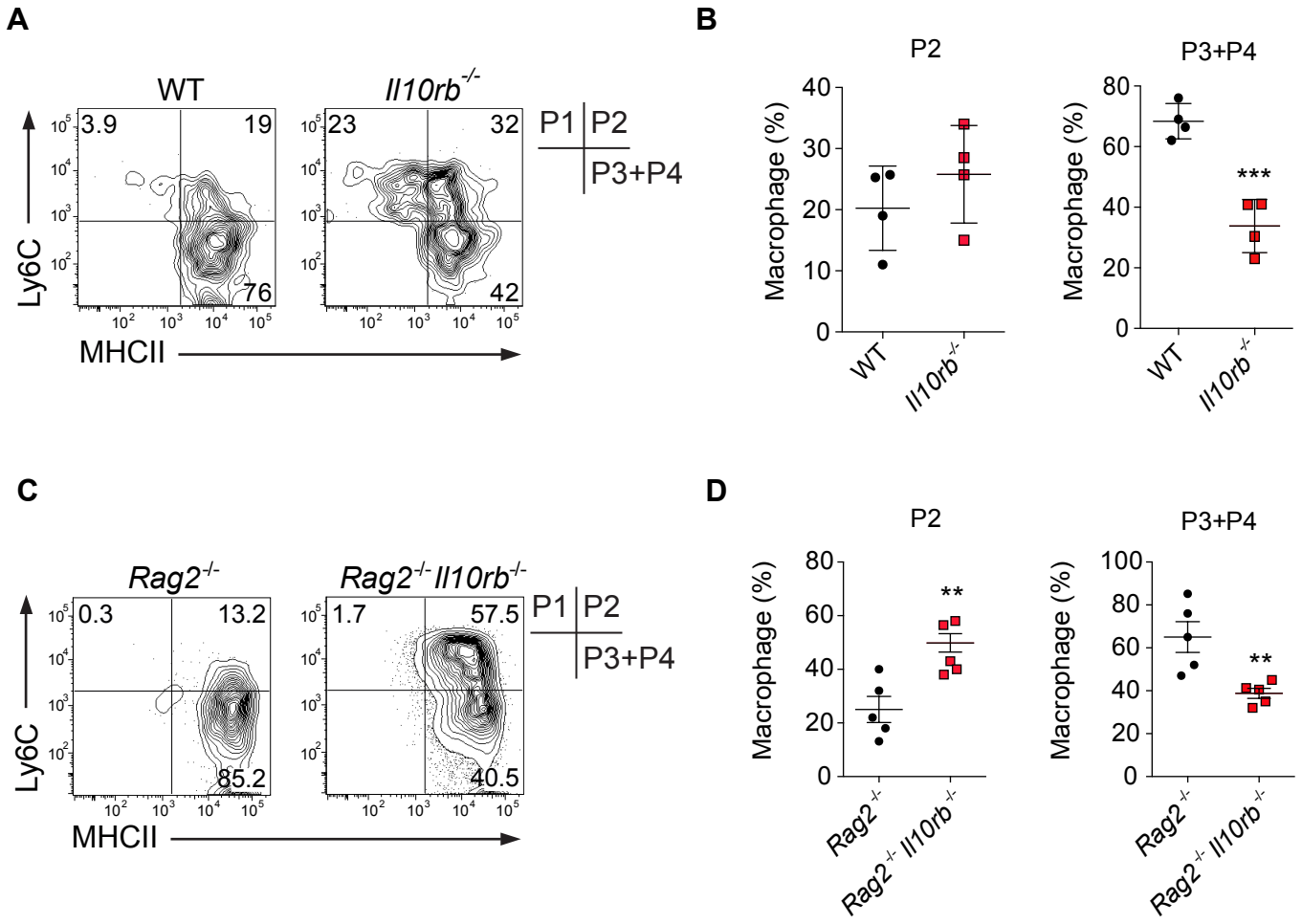

Figure S5, related to Figure 3. Reduction of intestinal anti-inflammatory macrophages in colitic *Il10rb*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice. Representative FACS plots of different macrophage subsets in the LP of (A) 4 months old WT and *Il10rb*$^{-/-}$ mice and (C) *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice 4 weeks following WT CD4^{+} T cell transfer. Comparison of the frequencies of pro- and anti-inflammatory macrophage subsets is shown in (B) for WT vs. *Il10rb*$^{-/-}$ mice and (D) for *Rag2*$^{-/-}$ vs. *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice following WT CD4^{+} T cell transfer. Results are pooled from two independent experiments.

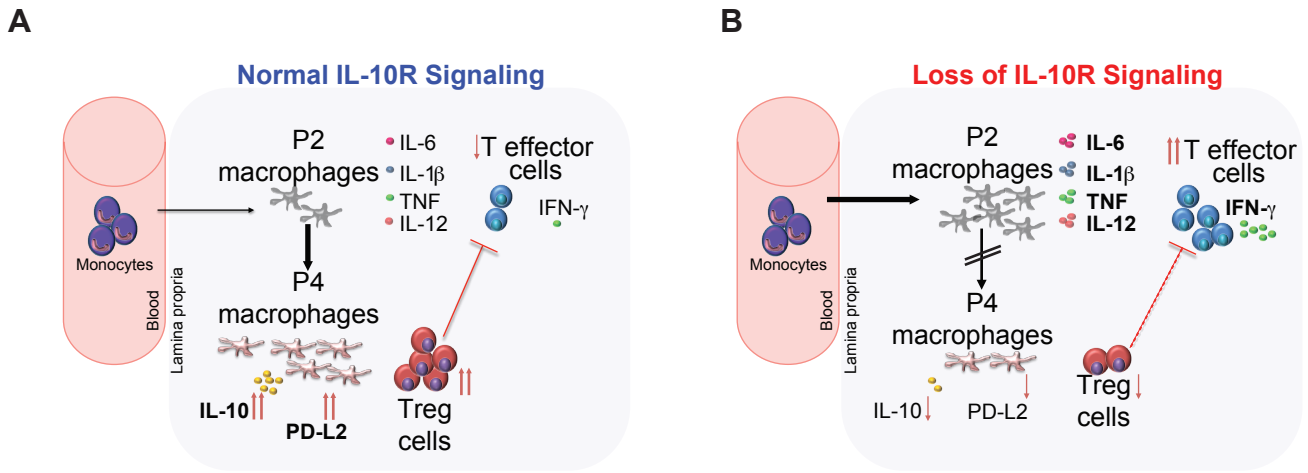

Figure S6, related to Figures 1-7: IL-10R responses in macrophages regulate mucosal homeostasis. Model showing how (A) intact and (B) aberrant IL-10R signaling on monocytes and macrophages influence differentiation of macrophages in the intestine, which in turn modulate T cell responses. In the presence of intact IL-10R signaling macrophages are polarized into tolerogenic P4 macrophages, which enhance the secretion of anti-inflammatory cytokines and promote the generation of inducible Treg cells. Defective IL-10R signaling on macrophages blunts the differentiation of circulating monocytes into tolerogenic P4 macrophages, with a concomitant increase in inflammatory P2 subset that secrete high levels of pro-inflammatory mediators and augment proliferation of T effector cells.

Patient	Age of Onset (months)	Current age (months)	Gender	*IL10R* mutation	Clinical features	Therapy
1*	3	48	Male	*IL10RB* c.*C52T (3' UTR)	Colitis, perianal abscesses, arthritis, folliculitis	Colectomy, diverting ileostomy, prednisone
2#	<1	130	Female	*IL10RA* c.585delT p.S196fs	Colitis, perianal abscesses, folliculitis, recurrent respiratory infections	Thalidomide, colchicine, immunoglobulins
3*	<2	154	Male	*IL10RB* c.477G>A p.W159X	Colitis, perianal and renal abscesses, folliculitis, arthritis	Colectomy, diverting ileostomy
4	<6	31	Male	*IL10RB* g.17030_22177 del5148	Colitis, perianal abscesses, folliculitis	Diverting ileostomy; awaiting SCT
5	<1	184	Female	*IL10RB* c.278A>C p.S58R/ c.717G>A p.W202X	Colitis, perianal abscesses, B cell lymphoma	Colectomy, diverting ileostomy, post chemotherapy; awaiting SCT
6	2	3	Female	*IL10RB* c.114G>A p.W3X	Colitis, recto-vaginal abscess	Diverting ileostomy; awaiting SCT
7	3	21	Male	*IL10RB* c.438-604del167 p.Y59_D110del (Δexon3)	Colitis, perianal abscesses	Steroids, azathioprine; awaiting SCT

Table S1, related to Figures 6-7. Demographic and clinical characteristics of patients with loss-of-function mutations in *IL10R* genes. SCT, stem cell transplantation. Patients 1-3 were reported elsewhere:* (Kotlarz et al., 2012); # (Pigneur et al., 2013)

Supplemental Experimental Procedures

Generation of BM chimeras

Rag2$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice were lethally irradiated with a single dose of 900 rads and several hours later mice were intravenously injected with either 2x10^{6} *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ total BM cells.

Colitis monitoring and scoring

During transfer experiments, mice were weighed weekly and monitored for signs of illness for up to 5 weeks. In some experiments a high-resolution colonoscopy (Karl Storz, Tuttlingen, Germany) was performed under anesthesia and findings were graded as previously described (Becker et al., 2006). H&E stained colon sections were scored in a blinded fashion by a pathologist as previously described (Nguyen et al., 2007). Briefly, scores ranged from 0–8 based on crypt hyperplasia (0-3), LP cell infiltration (0-3) and presence of crypt abscesses (0-2).

Exogenous IL-10 administration

Mice received 1 µg of IL-10Ig–containing serum or control Ig (IgG2a from Sigma-Aldrich) in 100 µL phosphate-buffered saline by *i.p.* injection, starting 1 day prior to WT CD4^{+} T cell transfer, with continued treatment twice weekly following cell transfer. Briefly, the serum of mice infected with an adenovirus encoding an IL-10IgG2a construct was used as the source of IL-10Ig (Tomczak et al., 2006). Detailed *in vivo* and *in vitro* characterization of the IL-10Ig product is presented elsewhere (Tomczak et al., 2006).

ELISA

Following WT CD4^{+} T cell transfer, the colons of *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il10rb*$^{-/-}$ mice were flushed, cut longitudinally and a 4 mm^{2} punch biopsy was obtained from distal colon and placed in culture with 400 µL RPMI supplemented with 10% FBS and antibiotics for 24 hours. Supernatants were obtained and kept in -80^{0}C until analysis. In other experiments, 1x10^{6} BMDM cells were cultured under M1 and M2r conditions in 1 mL, and supernatants were

collected. ELISA assays were performed using kits and antibodies obtained from BD Biosciences, Ebioscience, Biolegend, R&D systems and Thermo Scientific.

Flow cytometry

Cells were acquired using FACS Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo (Treestar, Ashlan, OR). For intra-cellular staining cells were stimulated for 4 hours with 500 ng/mL ionomycin and 50 ng/mL PMA in the presence of 1 μg/mL of Golgistop. Cell were then fixed, permeabilized (BD Bioscience) and stained for intracellular proteins. For FOXP3 staining fixation/permeabilization kit by Ebioscience was used.

Antibodies

The following were used for flow cytometry: CD45 (clone 30-f11; Biolegend) ;CD4 (clone GK1.5; biolegend), CD25 (clone PC61.5; Biolegend) CD45RB (clone C363-16A; Biolegend), Foxp3 (clone FJK-16s; eBioscience), CD11b (clone M1/70; biolegend), CD103 (clone 2E7, ebioscience); CD64 (clone X54-5/7.1; Biolegend) MHC II (clone M5/114.15.2; BioLegend), CD103 (clone 2E7; eBioscience), Ly6C (clone HK1.4, Biolegend), CD86 (clone GL-1; Biolegend), PD-L1 (clone 10F.9G2; Biolegend); PD-L2 (clone TY25; BD Bioscience);). IFNγ (clone XMG1.2; Biolegend) and IL-17A (clone TC11-18H10.1; Biolegend); FOXP3 (clone FJK-16s; Ebioscience).

Supplemental References

Becker, C., Fantini, M.C., and Neurath, M.F. (2006). High resolution colonoscopy in live mice. Nature protocols *1*, 2900-2904.
Kotlarz, D., Beier, R., Murugan, D., Diestelhorst, J., Jensen, O., Boztug, K., Pfeifer, D., Kreipe, H., Pfister, E.D., Baumann, U*., et al.* (2012). Loss of interleukin-10 signaling and infantile inflammatory bowel disease: implications for diagnosis and therapy. Gastroenterology *143*, 347-355.
Nguyen, D.D., Maillard, M.H., Cotta-de-Almeida, V., Mizoguchi, E., Klein, C., Fuss, I., Nagler, C., Mizoguchi, A., Bhan, A.K., and Snapper, S.B. (2007). Lymphocyte-dependent and Th2 cytokine-associated colitis in mice deficient in Wiskott-Aldrich syndrome protein. Gastroenterology *133*, 1188-1197.
Pigneur, B., Escher, J., Elawad, M., Lima, R., Buderus, S., Kierkus, J., Guariso, G., Canioni, D., Lambot, K., Talbotec, C*., et al.* (2013). Phenotypic characterization of very early-onset IBD due to mutations in the IL10, IL10 receptor alpha or beta gene: a survey of the Genius Working Group. Inflammatory bowel diseases *19*, 2820-2828.
Tomczak, M.F., Erdman, S.E., Davidson, A., Wang, Y.Y., Nambiar, P.R., Rogers, A.B., Rickman, B., Luchetti, D., Fox, J.G., and Horwitz, B.H. (2006). Inhibition of Helicobacter hepaticus-induced colitis by IL-10 requires the p50/p105 subunit of NF-kappa B. J Immunol *177*, 7332-7339.

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