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

Dror S. Shouval,1,2,23 Amlan Biswas,1,2,23 Jeremy A. Goettel,1,2,23 Katelyn McCann,1,23 Evan Conaway,3 Naresh S. Redhu,1,2,23 Ivan D. Mascalfroni,4 Ziad Al Adham,5 Sydney Lavoie,1 Mouna Ibourk,1 Deanna D. Nguyen,6,7 Janneke N. Samsom,8,23 Johanna C. Escher,9,23 Raz Somech,10,12,23 Batia Weiss,11,12,23 Rita Beier,13,23 Laurie S. Conklin,14,23 Christen L. Ebens,15,23 Fernanda G.M.S. Santos,16,23 Alexandre R. Ferreira,16,23 Mary Sherlock,17,23 Atul K. Bhan,18,19 Werner Müller,20 J. Rodrigo Mora,6,7 Francisco J. Quintana,4 Christoph Klein,21,23 Aleixo M. Muise,5,23 Bruce H. Horwitz,2,3,23 and Scott B. Snapper1,7,22,23,*

1Division of Gastroenterology, Hepatology and Nutrition, Boston Children’s Hospital, Boston, MA 02115, USA
2Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA
3Department of Pathology
4Center of Neurological Diseases
5Brigham and Women’s Hospital, Boston, MA 02115, USA
6Division of Gastroenterology, Hepatology, and Nutrition, Department of Paediatrics, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada
7Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA 02114, USA
8Department of Medicine, Harvard Medical School, Boston, MA 02115, USA
9Laboratory of Pediatric Gastroenterology
10Department of Pediatrics
11Erasmus Medical Center-Sophia Children’s Hospital, 3000 CA Rotterdam, the Netherlands
12Pediatric Immunology Service
13Division of Pediatric Gastroenterology and Nutrition
14Edmond and Lily Safra Children’s Hospital, Sheba Medical Center, Tel Hashomer 52661, Israel
15Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
16Department of Pediatric Hematology and Oncology, Hannover Medical School, 30625 Hannover, Germany
17Department of Gastroenterology, Children’s National Medical Center, Washington, D.C. 20010, USA
18Division of Pediatric Hematology and Oncology, University of Michigan, Ann Arbor, MI 48109, USA
19Hospital das Clinicas, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais 30130-100, Brazil
20Division of Gastroenterology, McMaster Children’s Hospital, West Hamilton, Ontario L8N 3Z5, Canada
21Department of Pathology, Massachusetts General Hospital, Boston, MA 02114, USA
22Department of Pediatrics, Brigham and Women’s Hospital, Boston, MA 02115, USA
23Division of Pediatric Gastroenterology and Nutrition
24InterNational Early Onset Paediatric IBD Cohort Study (NEOPICS)

*Correspondence: ssnapper@hms.harvard.edu
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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.
IL-10 Signaling Regulates Macrophage Function

Immunity

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

... (Sugue et al., 2011; Groh 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 interleukin-10 (IFN-γ) (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 (Su 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-10lg. 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-10lg 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.

II10rb−/− 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...
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-\(\gamma\), IL-6, IL-12, and IL-1\(\beta\), 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-\(\gamma\)^{+} 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 3C 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-10R 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-10R deficiency in innate immune cells affecting the function of either effector and/or regulatory T cell populations. Following transfer of unfractoned 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 naïve cells (CD4⁺CD25⁺ CD45RBhi) 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⁺CD45RBhi) with WT T naïve 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-10R signaling on innate immune cells regulates the suppressive function of WT Treg cells. Upon transfer of WT CD4⁺ T naïve 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 unfractoned WT CD4⁺ T cells (Figure S4A and S4B). Moreover, transfer of sorted CD4⁺CD45RB⁺FOXP3⁺naïve 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⁺FOXP3⁺naïve 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-10R⁻/⁻ signaling impairs the generation and function of WT Treg cells in vivo.

IL-10R⁻/⁻-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-10R⁻/⁻-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 precolicit mice minimized identifying nonspecific effects that might be attributable to inflammation alone. LP cell analysis of precolicit 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⁺ 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 Pdcd112 (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-10R⁻/⁻ 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<sup>−−</sup> Innate Immune Cells Impair WT Treg Cells Suppression and Generation In Vivo

(A) Frequency of Treg cells in LP and MLN of Rag2<sup>−−</sup> and Rag2<sup>−−</sup>Il10rb<sup>−−</sup> mice that were transferred with unfractionated WT CD4<sup>+</sup> T cell transfer. Representative flow cytometry plots of FOXP3<sup>+</sup> cells among CD4<sup>+</sup> T cells are followed by cumulative data in LP and MLN.

(B) Mean % initial body weights ± SEM following transfer of WT T naive (CD4<sup>+</sup>CD25<sup>−</sup>CD45RB<sup>hi</sup>) cells alone or in combination with Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup>) at a 1:1 ratio.

(C) Representative H&E images (20X) of colonic sections from Rag2<sup>−−</sup> and Rag2<sup>−−</sup>Il10rb<sup>−−</sup> 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<sup>+</sup> expression among CD4<sup>+</sup> T cells in LP and MLN, 4 weeks after CD45RB<sup>hi</sup> transfer, followed by cumulative data. Results are pooled from two independent experiments. Figures S4 and S6 accompany.
factor-β (TGF-β) and IL-10 generate M2 tolerogenic macrophages (Martínez 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 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.
major histocompatibility complex class II (MHCII) glycoproteins, CD86 and proinflammatory mediators were highly expressed in Il10rb−/− M1 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-10Rx 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-10Rx-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, II10rb−/− 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 II10rb−/− M1 BMDM (Figure 4E), suggesting that excessive T cell proliferation is not caused by an excess of a single proinflammatory cytokine. Moreover, II10rb−/− 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 II10rb−/− M1 BMDM, we performed additional experiments with BM obtained from II10ra−/− mice that lack only defective IL-10R signaling. Like II10rb−/− M1 BMDM, II10ra−/− 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, II10rb−/− 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 II10rb−/− M2r BMDM (data not shown). However, restimulation with LPS of established II10rb−/− 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, II10rb−/− 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 II10rb−/− 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−/−II10rb−/− mice. Administration of WT M2r BMDM i.p. 1 day prior to WT CD4+ T cell transfer protected Rag2−/−II10rb−/− mice from intestinal inflammation, whereas transfer of II10rb−/− 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−/−II10rb−/− 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 II10rb−/− 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 II10rb−/− 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 IL10R 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
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., 2011). More studies have been performed on cell types dependent on IL-10R signaling.

Figure 5. Loss of IL-10R\beta 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\beta^{-/-} BMDM cultured for 24 hr under different conditions.

(B) Proinflammatory cytokines mRNA expression by WT and Il10rb\beta^{-/-} 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\beta^{-/-} M2r macrophages.

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

(E) 1 x 10\(^6\) WT or Il10rb\beta^{-/-} M2r BMDM or PBS were injected i.p. into Rag2\(^{-/-}\) Il10rb\beta^{-/-} 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 \(\mu\)m. Results are pooled from two or more independent experiments. Figure S6 accompanies.
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 naïve 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 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−/− mice (Medina-Contreras et al., 2011). Similarly and consistent with our findings, Kayama and colleagues have recently reported that transfer of sorted intestinal CX3CR1hi macrophages alleviates colitis in Rag1−/− mice transferred with CD45RBhi cells (Kayama et al., 2012). However, transfer of CX3CR1hi 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 Pdcd112 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-10R-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 Lz4m-cre- or Ilgax-cre-mediated deletion of Il10rc−/− 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-10Rz differentiate into a proinflammatory MHCIIhi macrophage subset (Nguyen et al., 2012b).
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-γ 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-10Rβ in specific innate immune cells are warranted. Indeed, the study by Zigmond et al., employing Cx3cr1-cre-mediated targeting of IL-10Rβ, suggests that defective IL-10Rβ-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 Collitis in Transfer Experiments**

In unfractonated 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 × 10⁶ 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 FACS Aria II SORP (BD Biosciences). T naive cells were defined as CD4+CD25−CD45RB+ and Treg cells as CD4+CD25+CD45RB−. Post-sort purity was typically >98%. Age-matched Rag2−/− mice or Rag2−/−Il10rb−/− mice were injected i.p. with 1–2 × 10⁷ WT T naive cells with or without Treg cells at a 1:1 ratio. Similarly, CD4+CD45RB+FOXP3+ or CD4CD45RB−FOXP3− 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 for 2 h followed by 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 sub- sets 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% CO₂. 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 M2 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 × 10⁵ CFSE-labeled 1 × 10⁶ WT CD4+ CD25+ T naive cells with 2 μg/ml soluble CD3 and either 2.5 × 10⁵ WT or Il10rb−/− M1 BMDM, for 4 days. Proliferation was determined by percent of CFSE dilution. For T reg generation experiments, 1 × 10⁵ WT CD4+CD25− sorted T naive cells were cultured with 2 μg/ml soluble CD3 (e Bioscience), 2 ng/ml human TGF-β1 (Peprotech) and either 2.5 × 10⁵ WT or Il10rb−/− M2r BMDM, for 5 days. Similar experiments were performed with human M1 or M2 macrophages from IL10R-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 spanning each exon. Purified PCR products were sequenced with the ABI 3730 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 manufacturer 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 TCT GGC CT) and full-length IL10RB (For: TGC TGT TGG AGG AAC 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.

**ACKNOWLEDGMENTS**

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REFERENCES


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

**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-10lg 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 \( \text{Rag2}^{-/-} \text{Il10rb}^{-/-} \) colons following T cell transfer. (A) Cytokine concentrations determined by ELISA in supernatants of colonic explants culture of \( \text{Rag2}^{-/-} \) and \( \text{Rag2}^{-/-} \text{Il10rb}^{-/-} \) mice. (B) Cytokine mRNA expression measured by qRT-PCR on colonic tissue of \( \text{Rag2}^{-/-} \) and \( \text{Rag2}^{-/-} \text{Il10rb}^{-/-} \) recipients of WT CD4\(^+\) T cells. Fold change is relative to \( \text{Rag2}^{-/-} \) recipient mice transferred with CD4\(^+\) T cells. (C) Representative FACS plot followed by cumulative frequencies of IFN-\( \gamma \)\(^+\) and IL-17A\(^+\) cells among CD4\(^+\) T cells in the LP of \( \text{Rag2}^{-/-} \) and \( \text{Rag2}^{-/-} \text{Il10rb}^{-/-} \) mice following CD4\(^+\) T cell transfer. (D) Absolute numbers (per \(1 \times 10^6\) LP cells) of T effector cell subsets in the LP of \( \text{Rag2}^{-/-} \) and \( \text{Rag2}^{-/-} \text{Il10rb}^{-/-} \) 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 \(\mu m\). (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\(^{hi}\)FOXP3\(^{neg}\) 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.
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<th>Current age (months)</th>
<th>Gender</th>
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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 2×10⁶ *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 mm² 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 -80⁰C until analysis. In other experiments, 1×10⁶ 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

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