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IL-10 engages macrophages to shift Th17 cytokine dependency and pathogenicity during T cell-mediated colitis

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Abstract

Polymorphisms attenuating IL-10 signaling confer genetic risk for inflammatory bowel disease. Yet how IL-10 prevents mucosal autoinflammation is incompletely understood. We demonstrate using lineage-specific deletions of IL-10Rα that IL-10 acts primarily through macrophages to limit colitis. Colitis depends on IL-6 to support pathologic Th17 cell generation in wild type mice. However, specific ablation of macrophage IL-10Rα provokes excessive IL-1β production that overrides Th17 IL-6 dependence, amplifying the colonic Th17 response and disease severity. IL-10 not only inhibits pro-IL-1β production transcriptionally in macrophages, but suppresses caspase-1 activation and caspase-1 dependent maturation of pro-IL-1β to IL-1β. Therefore lineage-specific effects of IL-10 skew the cytokine dependency of Th17 development required for colitis pathogenesis. Coordinated interventions may be needed to fully suppress Th17-mediated immunopathology.

Keywords

IL-10; IL-1β; macrophage; Th17; Inflammasome

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are characterized by chronic relapsing intestinal inflammation and disorganized immune

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Disclosure/Competing Financial Interests

The authors declare no competing financial interests.

Author Contributions

B. L. designed and conducted research studies, analyzed data, prepared figures, and wrote the manuscript. P. G. and R. K. S. M. performed the Western analyses on IL-1b and caspase-1 with B. L. P. V. performed blinded histologic analyses and scoring of colon sections. T. D. K. provided guidance and advice on experimental design. T. L. G. coordinated the research efforts, designed the studies with B.L., assisted in data analysis, revised the manuscript and assisted in preparing figures.

responses in the gastrointestinal tract[1]. Although IBD pathogenesis is incompletely understood, mucosal T-helper type 17 (Th17) cells play a key pathologic role[2–4]. Th17 induction is supported by IL-1β, IL-6, IL-23 and TGF-β[5–7], cytokines that upregulate and stabilize retinoic acid receptor-related organ receptor (RORγT) and pro-inflammatory function[8].

The cytokine IL-10 preserves gastrointestinal homeostasis[9]. Mice deficient in IL-10 or IL-10Rβ develop spontaneous enterocolitis[10, 11]. Bi-allelic mutations in IL-10 lead to infantile enterocolitis, polymorphisms in IL-10 are associated with ulcerative colitis and Crohn's disease, and an association has been further reported between IL-10Rα SNPs and early onset UC[12–15].

Dynamic interactions between IL-10 and different IL-10 responsive immune cell lineages participate in IBD pathogenesis[16]. Mice with a deletion of IL-10 or its receptor solely in $Foxp3^+$ T cells can develop spontaneous colitis[17, 18]. The IL-10R-deficient $Foxp3^+$ T cells display decreased IL-10 secretion itself, potentially linking these phenotypes. Further, two recent studies have implicated the IL-10 response by myeloid populations to mucosal homeostasis and colitis susceptibility. Zigmond, et. al. used a CX3CR1 promoter-directed Cre to selectively delete IL-10Rα. These mice developed spontaneous colitis, and implicated IL-10 in the generation of anti-inflammatory $CX3CR1^+$ myeloid cells necessary for colonic homeostasis[19]. Shouval, et. al. demonstrated that IL-10Rβ$^{-/-}$ Rag2$^{-/-}$ mice are susceptible to colitis mediated by total CD4 T cell transfer. The IL-10Rβ deficiency impedes the generation and activity of anti-inflammatory macrophages, and impairs iTreg generation and Treg function[20]. IL-10-producing myeloid cells have also been shown to prevent the downregulation of Foxp3 in T cell transfer colitis, indicating a central role for IL-10 in the crosstalk between regulatory T cell and myeloid populations[21]. Implicating IL-10 in T cell effector function as well, T cell-specific blockade of IL-10 signaling using a dominant negative IL-10 receptor (IL-10RDN) leads to increased Th17 cells in an anti-CD3 Ab induced model of small intestinal inflammation[22].

Despite the clear role of IL-10 in intestinal homeostasis, pharmacologic administration of IL-10 has not proven effective[23]. Further resolution of the core interactions, responsible cell types, and pertinent mechanisms underlying IL-10's activity will be necessary to develop effective interventions targeting this pathway. Here we use the transfer of naive $CD4^+CD45RB^{hi}$ T cells into lymphocyte-deficient strains to analyze how the lineage specific activities of IL-10 impact the pathologic T cell response.

Transferred naïve T cells are activated by microbial flora, provoking Th17-dependent colonic inflammation. Studies using IL-10RDN T cells have indicated that the T cell IL-10 response does not significantly restrain colitis development after $CD45RB^{hi}$ T cell transfer[24]. We verify this here using T cells conditionally deficient in the specific receptor for IL-10, IL-10Rα. However, we further show using additional targeted deletion models that IL-10 acts dominantly on macrophages (Mφs) to mediate its inhibitory effects, and define how this occurs through a shift in the cytokine dependency of pathologic effector T cells. We show that IL-10 acts on Mφs to potently suppress IL-1β production through several routes, including the inhibition pro-IL-1β production, caspase-1 activation, and IL-1β

maturation. This not only modulates colitis, but also transforms the pathologic Th17 response from one that is IL-6-independent and IL-1-dependent to one with an obligate requirement for IL-6. Our findings demonstrate a redundancy in operative Th17 induction pathways during colitis, the critical role of the Mφ response to IL-10 in controlling these pathways, and imply that coordinated therapies targeting redundant cytokines may be required to fully suppress disease.

Results

T cell IL-10 response does not impact colitis development

To evaluate lineage-specific effects of IL-10 in T cell transfer-mediated colitis, we first produced mice with a selective deletion of the specific IL-10 receptor, IL-10Rα, restricted to T cells (IL-10Rα^{Tdel})[25]. Colitis was then induced by transferring naive CD4^{+}CD25^{-}CD45RB^{hi} T cells from wild-type (WT) or IL-10Rα^{Tdel} mice into Rag1$^{-/-}$ recipients. Disease magnitude and quality were monitored through changes in body weight, colon histopathology, and colonic T cell and T cell subset (Th1, Th17, Foxp3^{+}) infiltration (Supplementary Fig. 1). No differences were identified between recipients of WT and IL-10Rα^{Tdel} T cells, indicating that IL-10 signaling into the transferred T cells does not modulate colitis severity in this T cell-dependent model.

Mϕ response to IL-10 restrains T cell-mediated colitis

We previously demonstrated that macrophage but not DC or T cell IL-10 signaling prominently alleviated inflammation in a distinct T cell-independent, toxin-induced model of acute colitis, DSS colitis[26]. Recent data have also implicated myeloid cells as regulators of disease severity in T cell transfer colitis[19–21]. To assess the impact of DC and Mφ-selective deletion of IL-10Rα (IL-10Rα^{DCdel}, IL-10Rα^{Mdel}) in T cell transfer colitis, we generated lineage-specific knock-outs on a Rag1$^{-/-}$ background and induced disease. Histologic and clinical disease did not differ between Rag1$^{-/-}$ and IL-10Rα^{DCdel}Rag1$^{-/-}$ recipients (Supplementary Fig. 2a, b). In contrast, disease was intensified in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice (Fig. 1a and Supplementary Fig. 2a, b). Weight loss was elevated and accelerated, with a mean±s.e.m. decrement at 8 wk of 19.1±1.1% vs 9.3±1.1% for Rag1$^{-/-}$ controls (Fig. 1a). IL-10Rα^{Mdel}Rag1$^{-/-}$ colons were shorter than Rag1$^{-/-}$ controls (8.2±0.3 vs 7.1±0.2 cm, Fig. 1b). Histopathology in IL-10Rα^{Mdel}Rag1$^{-/-}$ colons demonstrated increased inflammation, with more extensive cellular infiltrates, submucosal edema, and epithelial erosion (Fig. 1c). Total histologic score was 8.3±0.6 and 3.6±0.7 (scale 0–12) for IL-10Rα^{Mdel}Rag1$^{-/-}$ and Rag1$^{-/-}$ mice respectively. Moreover, fewer IL-10Rα^{Mdel}Rag1$^{-/-}$ mice survived with extended disease times (Fig. 1d), and these developed an elevated incidence of rectal prolapse (75% IL-10Rα^{Mdel}Rag1$^{-/-}$ mice vs 0% Rag1$^{-/-}$ controls at 12 wk).

In a recent study, mice deleting IL-10R using a CX3CR1-driven Cre that is expressed by a large proportion of LPMφs developed spontaneous colitis. We did not observe overt spontaneous disease in our IL-10Rα^{Mdel}Rag1$^{-/-}$ mice during the time frame of our assays. To further assess this, cohorts of these and IL-10Rα$^{fl/fl}$Rag1$^{-/-}$ controls (n=10 for each) were aged for 6 months. There was no difference in weight gain, or presence of clinical

signs of colitis or other illness in either population (Supplementary Fig. 2c). Histologic analysis of 5 aged IL-10Rα^{Mdel}Rag1$^{-/-}$ mice failed to identify evidence for colitis. Similarly, spontaneous colitis was not observed in 6 month aged IL-10Rα^{Mdel}Rag1$^{+/+}$ mice.

IL-10's protective role in colitis is well established. To determine whether the Mφ-specific response to IL-10 can account for this, we also compared disease in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice with Rag1$^{-/-}$ mice harboring a germline deletion in IL-10Rα (IL-10Rα$^{-/-}$Rag1$^{-/-}$; Supplementary Fig. 2a, b). No difference in clinical disease or histopathology was evident in these two recipient lines, implying that IL-10-mediated immunoprotection can largely be accounted for by its Mφ-specific effects.

The Lys-M-Cre transgene used to ablate IL-10Rα in the IL-10Rα^{Mdel}Rag1$^{-/-}$ mice is expressed in granulocytes as well as Mφs[27]. Neutrophils have a modest protective role in T cell transfer colitis[28], and loss of IL-10Rα signaling there, rather than in Mφs, may have impacted disease. To isolate the Mφ-specific effect, we depleted neutrophils with specific Ab beginning prior to T cell transfer. Neutrophils were undetectable in the peripheral blood from αLy6G Ab but not control Ab treated animals throughout the experimental interval. In Rag1$^{-/-}$ recipients, weight loss was mildly increased on some disease days in neutrophil-depleted mice, though no significant difference in histopathology was identified (Fig. 1e, f). No significant change in disease severity was apparent with neutrophil depletion in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice. Further, the prominent disease exacerbation in IL-10Rα^{Mdel}Rag1$^{-/-}$ compared with Rag1$^{-/-}$ recipients persisted after neutrophil depletion, indicating that the intensified immunopathology in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice is primarily attributable to the Mφ rather than granulocyte IL-10 response.

Increased numbers and activation of IL-10Rα^{Mdel}Rag1$^{-/-}$ LPMφs

To identify causes of the enhanced IL-10Rα^{Mdel}Rag1$^{-/-}$ disease, we first analyzed lamina propria macrophages (LPMφs). LPMφs, typed as CD11b^{hi}CD11c$^{-/lo/mod}$F4/80^{+}Ly6G$^{-/lo}$SiglecF^{-}, were significantly increased in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice with colitis compared with Rag1$^{-/-}$ controls (Fig. 2a). This population was uniformly CD64^{+} and predominantly CX3CR1^{+}, further delineating them as LPMφs[29, 30] (Supplementary Fig. 3a). The cells were further characterized by a uniformly elevated surface expression of activation markers, including CD40, CD80, and CD86 (Fig. 2b). Segregation of CD11b^{+}CD64^{+}CD103^{-} and CD45^{+} cells based on Ly6C and class II MHC expression can distinguish pro-inflammatory (Ly6C^{hi}) and anti-inflammatory (Ly6C^{lo}) macrophage populations[20, 30]. Most LPMφs analyzed using this alternative gating strategy were phenotypically pro-inflammatory, and the proportions of Ly6C^{hi} and Ly6C^{lo} macrophages did not differ between IL-10Rα^{Mdel}Rag1$^{-/-}$ mice and Rag1$^{-/-}$ controls (Supplementary Fig. 3b).

The increased IL-10Rα^{Mdel}Rag1$^{-/-}$ versus Rag1$^{-/-}$ LPMφ numbers were further associated with an increased proliferation rate, as determined by incorporation of the nucleotide analog BrdU. Unlike for LPMφs, no differences in BrdU incorporation were evident in colonic CD4 T cells, DCs, and neutrophils, or in Mφs from other locations (Fig. 2c). Therefore, numbers, proliferation, and activation state of LPMφs are increased in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice.

Unimpaired IL-10Rα^{Mdel}Rag1$^{-/-}$ T regulatory response

A subset of T cells transferred into Rag1$^{-/-}$ mice upregulate Foxp3, and IL-10 signaling into Mφs could contribute to the generation and maintenance of these regulatory T cells[21, 22]. Indeed, recent findings with IL-10Rβ$^{-/-}$Rag2$^{-/-}$ mice have identified defective iTreg formation and Treg function. Treg co-transferred with naïve T cells even at a 1:1 ratio were incapable of preventing disease[20]. To assess Treg activity here, we quantified Foxp3^{+} iTreg forming in the LP, mesenteric lymph nodes (MLNs), and spleen of IL-10Rα^{Mdel}Rag1$^{-/-}$ and control Rag1$^{-/-}$ colitic mice. No differences were observed (Fig. 3a). We next analyzed whether sorted Treg transferred into IL-10Rα^{Mdel}Rag1$^{-/-}$ and Rag1$^{-/-}$ mice with the induction of colitis could prevent disease. The transferred cells were able to fully suppress disease development in both IL-10Rα^{Mdel}Rag1$^{-/-}$ and Rag1$^{-/-}$ recipients, indicating that a macrophage-specific defect in IL-10 response does not overtly impact Treg suppressive activity in this setting (Fig. 3b). Treg transfers led to a >2-fold increase in Treg in the spleens, MLNs, and colons of recipient mice and Treg percentages did not differ between IL-10Rα^{Mdel}Rag1$^{-/-}$ and Rag1$^{-/-}$ recipients in any of the locations (Fig. 3c). These results indicate that Treg are able to suppress disease in the absence of a macrophage-specific response to IL-10, and that Treg-specific mechanisms independent of IL-10 actions on macrophages are employed in this system.

Alternative regulatory T cell populations are demarcated by IL-10 production, and IL-10 itself may impact these directly or indirectly. To evaluate this, we induced colitis by transferring naïve T cells from IL-10-GFP knock-in reporter mice. Here too, no differences in population sizes were seen (Fig. 3d). To determine if T cell IL-10 production was itself functionally dispensable for the differential colitis in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice, we performed transfers using IL-10$^{-/-}$ or WT T cells. Disease severity, measured clinically and histologically, was exclusively associated with recipient type. IL-10 production by transferred T cells did not influence disease magnitude clinically or histologically (Fig. 3e, f). Therefore, the Mφ IL-10 response does not identifiably impact regulatory T cell presence. Further, while Mφ response to IL-10 is critical in attenuating disease, T cells are not a significant source of this IL-10 here.

Pro-inflammatory cytokine production by IL-10Rα^{Mdel} LPMϕs

To further evaluate the heightened disease severity in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice, we measured in whole colons the levels of cytokines implicated in its pathogenesis, including IL-1β, IL-6, TNF-α, MCP-1, IL-10, IL-17, and IFN-γ[31]. With early disease (wk 4), IL-1β, IL-6, and MCP-1 were increased in IL-10Rα^{Mdel}Rag1$^{-/-}$ colon compared with Rag1$^{-/-}$ controls (Fig. 4a). As disease progressed (wk 8), the quantity of cytokine produced was altered. Additional elevations in IL-17 and TNF-α were identified at this time. Differences in IL-10 and IFN-γ were not seen.

T cell transfer colitis is associated with colonic infiltration by both Th1 and Th17 cells, and the Th17 response is required for disease development. Our identification of increased colonic IL-6 and IL-1β in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice, cytokines associated with Th17 induction, coupled with elevated IL-17 but not IFN-γ levels implied an intensification of the Th17 response. To test this, we enumerated IFN-γ and IL-17 producing T cells in the colonic

infiltrate. Significantly increased percents and absolute numbers of $CD4^+IL\text{-}17A^+$ T cells were identified in IL-10Rα^{Mdel} Rag1$^{-/-}$ compared with Rag1$^{-/-}$ colons (Fig. 4b). Likewise, after the induction of disease with $CD4^+CD25^-CD45RB^{hi}$ RORγT-GFP reporter T cells, increased GFP^+ T cells were identified in the colon at 4 and 8 wk (Supplementary Fig. 4a). No differences in $CD4^+$IFN-γ^+ T cell quantities were evident (Fig. 4c).

To more specifically determine whether elevated production of Th17-promoting cytokines was specific to the Mφ population, we sorted colonic $CD11b^{hi}CD11c^{-/lo/mod}F4/80^+Ly6G^{-/lo}SiglecF^-$ LPMφs from colitic mice and assayed their cytokine expression profiles by qRT-PCR. A particularly prominent elevation in IL-1β production was apparent in IL-10Rα^{Mdel}Rag1$^{-/-}$ compared with Rag1$^{-/-}$ LPMφs (mRNA expression ratio: 7.5±1.2). Lesser elevations in IL-6 (3.4±0.5), IL-23 (2.9±0.3) and other pro-inflammatory cytokines were also identified (Fig. 4d, e). IL-10 itself was unchanged and a modest decline in arginase with a corresponding increase in iNOS further indicated enhanced pro-inflammatory function of the IL-10Rα^{Mdel}Rag1$^{-/-}$ LPMφs. For IL-1β, IL-6, IL-23p19, and iNOS, relative expression was compared in sorted colonic LPMφs, DCs, neutrophils, and epithelial cells (Fig. 4d). Elevated expression was specific to the Mφs, indicating that Mφs are the primary source for the increased Th17-associated cytokines and implying that IL-10 acts directly on these cells to suppress their production.

As an alternative gating strategy, we also sorted $CD11b^+CD64^+CD103^-CD45^+Ly6C^{hi}$ LPMφs and assessed similarly for IL-1β, IL-6, IL-23, IL-10, iNOS, and arginase. These were differentially expressed in a manner paralleling results above (Supplementary Fig. 4b). CD163 was additionally assessed as a marker for anti-inflammatory macrophages and found to be similar in the IL-10Rα^{Mdel}Rag1$^{-/-}$ and control Rag1$^{-/-}$ populations.

Colitis in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice is Th17 dependent

Th17 cells have been shown to be essential to T cell transfer colitis in Rag1$^{-/-}$ mice[32]. The elevated colonic inflammation in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice was correlated with an increased Th17 response, but may also have been mediated by alternative pathologic pathways. To verify a role for Th17 cells, we transferred RORγT$^{-/-}$ T cells into Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ recipients (Supplementary Fig. 5). RORγT, and hence Th17 cells, proved essential for colitis in both recipient types.

Colitis is IL-6 independent in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice

Though IL-6 is well established as a key inducer of Th17 cells, its isolated role in fostering the Th17 response fundamental to T cell transfer colitis has not been addressed. The increased IL-6 in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice might have driven the increased immunopathology there. To assess this, we induced colitis by transfers of IL-6Rα$^{-/-}$ or WT naive T cells into IL-10Rα^{Mdel}Rag1$^{-/-}$ and Rag1$^{-/-}$ recipients. IL-6Rα$^{-/-}$ T cells were ineffective in inducing disease in Rag1$^{-/-}$ mice (Fig. 5a, b). Mice gained weight after T cell transfer and histologic lesions were mild. In contrast, IL-6Rα$^{-/-}$ and WT T cells proved equipotent in mediating severe clinical disease in IL-10Rα^{Mdel}Rag1$^{-/-}$ recipients. Although there was a trend toward modestly diminished histologic severity after IL-6Rα$^{-/-}$ transfer,

this was not significant. Thus, the Mφ response to IL-10 creates a dependency for IL-6 in disease pathogenesis.

To better understand this, we analyzed the T cell responses leading to colonic injury. Th17 cell percentages among $CD4^+TCR^+$ T cells were reduced in the MLNs of mice receiving IL-6Rα$^{-/-}$ T cells (Fig. 5c). This was true for both IL-10Rα^{Mdel}Rag1$^{-/-}$ and Rag1$^{-/-}$ mice. Thus IL-6Rα signaling supports but is not essential for Th17 formation in the MLNs of both of these recipient lines. In the colon, the representation of Th17 cells was significantly decreased in Rag1$^{-/-}$ but not IL-10Rα^{Mdel}Rag1$^{-/-}$ recipients. This indicates a relative expansion of Th17 cells at the site of autoimmune inflammation specifically in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice.

Absolute numbers of Th17 cells in all organs sampled dramatically differed between Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ recipients of IL-6Rα$^{-/-}$ T cells. Few Th17 cells were present in the spleens, MLNs, and colons of Rag1$^{-/-}$ recipients, whereas large numbers were present in IL-10Rα^{Mdel}Rag1$^{-/-}$ recipients (Fig. 5c). This difference reflected a pervasive decrease in total T cell number with the IL-6Rα$^{-/-}$→Rag1$^{-/-}$ transfers (Fig. 5d). Therefore, IL-6 is broadly necessary for T cell expansion and colitis in Rag1$^{-/-}$ but dispensable in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice. IL-10's actions on Mφs generate an IL-6 requirement for autoinflammatory disease and robust T cell expansion.

In contrast to Th17 development, no differences were identified in the proportions of Th1 or $Foxp3^+$ $CD4^+$ T cells in any of the transfer combinations tested, indicating that T cell IL-6 and Mφ IL-10 responses do not similarly skew these maturation pathways (Fig. 5e, f). However, absolute numbers of Th1 and $Foxp3^+$ cells were diminished in the IL-6Rα$^{-/-}$ →Rag1$^{-/-}$ combination, again reflecting the impaired T cell expansion in the absence of disease development.

IL-1 response is required for IL-10Rα^{Mdel}Rag1$^{-/-}$ colitis

IL-1β plays an essential role in the steady-state development of intestinal Th17 cells in healthy mice[33] and further promotes the Th17 response during colitis[34]. Considering this, the lack of a requirement for IL-6 in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice (Fig. 5a, b), and the dramatically elevated IL-1β expression in IL-10Rα^{Mdel}Rag1$^{-/-}$ LPMφs (Fig. 4), we hypothesized that IL-1β plays a pathologic role in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice that is able to supersede the IL-6 requirement for colitis. To test IL-1's impact, we induced colitis by transferring IL-1R$^{-/-}$ T cells into IL-10Rα^{Mdel}Rag1$^{-/-}$ and Rag1$^{-/-}$ recipients.

IL-1R$^{-/-}$ T cell transfers into Rag1$^{-/-}$ mice did not lead to the virtually complete disease protection seen after IL-6Rα$^{-/-}$ T cell transfers. However IL-1R$^{-/-}$ →Rag1$^{-/-}$ transfers did show diminished clinical and histologic measures of colitis compared with control WT T cell transfers (Fig. 6a, b). IL-1R$^{-/-}$ T cells also provoked significantly milder disease than WT T cells In IL-10Rα^{Mdel}Rag1$^{-/-}$ recipients (Fig. 6a, b). This contrasts with IL-6Rα$^{-/-}$ T cells, which did not detectably alter disease severity. Therefore, while T cell IL-6 responsiveness is clinically important only in Rag1$^{-/-}$ colitis, T cell IL-1 response modulates both Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ disease.

We performed similar analyses of the impact on T cell responses after IL-1R$^{-/-}$ transfers as for the IL-6Rα$^{-/-}$ transfers above. Whereas a decreased percent and absolute number of Th17 cells was seen in the MLNs, a site of T cell priming, after IL-6Rα$^{-/-}$ transfers (Fig. 5c), no differences in the MLNs were seen for IL-10Rα^{Mdel}Rag1$^{-/-}$ or Rag1$^{-/-}$ recipients receiving IL-1R$^{-/-}$ compared with WT T cells (Fig. 6c). However, substantially diminished proportions and absolute numbers of Th17 cells were seen in the colons of mice receiving IL-1R$^{-/-}$ T cells. The actions of IL-1 were specific to Th17 effectors; no differences were identified in Th1 and Foxp3^{+} Treg populations (Fig. 6d, e). This indicates that there is a selective defect in the Th17 response in the colon. T cell IL-1 but not IL-6 response in IL-10Rα^{Mdel}Rag1$^{-/-}$ mice promotes colonic inflammation by supporting Th17 cells at the site of autoinflammatory disease. In Rag1$^{-/-}$ mice, where IL-10 suppresses LPMφ IL-1β production, IL-6 plays a more critical role.

Il-10 inhibits pro-IL-1β production and maturation

Our data indicated that IL-1β, increased in the colon of IL-10Rα^{Mdel} mice, supports the pathologic Th17 response mediating colitis. IL-10 inhibits this IL-1β production thereby attenuating disease. To determine whether IL-1β protein in the colon is primarily produced by LPMφs, we used immunohistochemistry to colocalize IL-1β and CD11b in colon sections from diseased IL-10Rα^{Mdel}Rag1$^{-/-}$ and Rag1$^{-/-}$ mice (Supplementary Fig. 6a). This demonstrated that IL-1β is largely associated with the CD11b^{+} population.

IL-1β is generated from an inactive cytosolic precursor (pro-IL-1β). Caspase-1 cleaves pro-IL-1β, converting it into its active form which is then released from the cell[35]. Caspase-1, in turn, is activated by inflammasome stimulation[36]. To better evaluate the impact of IL-10 signaling on IL-1β production by LPMφs, we first quantified pro-IL-1β levels by flow cytometry in gated CD11b^{hi}CD11c$^{-/lo/mod}$F4/80^{+}Ly6G$^{-/lo}$SiglecF^{-} Mφs. This demonstrated a 1.7-fold elevation in the percent of LPMφs expressing pro-IL-1β in the colons of diseased IL-10Rα^{Mdel}Rag1$^{-/-}$ compared with Rag1$^{-/-}$ mice (mean±s.d.: 84.6±6.0% vs 48.6±8.4%; Fig. 7a). Further, the MFI of positive cells from IL-10Rα^{Mdel}Rag1$^{-/-}$ mice was nearly 2-fold greater than that from control mice (131.4±9.3 vs 76.6±5.7). In contrast to the colons of diseased mice, pro-IL-1β levels were low to undetectable in MLN and bone marrow macrophages, and levels did not differ between IL-10Rα^{Mdel}Rag1$^{-/-}$ mice and Rag1$^{-/-}$ controls. Differences in pro-IL-1β were also not apparent in mice in which disease was not induced (Supplementary Fig. 6b).

We verified that pro-IL-1β was directly regulated in Mφ by IL-10, analyzing its production in cultured and LPS and ATP-stimulated Mφ by Western analysis, and simultaneously assessing for mature IL-1β formation (Fig. 7b and Supplemenatary Fig. 7). Substantially lower amounts of both pro-IL-1β and IL-1β were present in cultures of Rag1$^{-/-}$ than IL-10Rα^{Mdel}Rag1$^{-/-}$ Mφ. After treatment with IL-10, pro-IL-β was diminished and mature IL-1β undetectable in Rag1$^{-/-}$ cultures, while this manipulation had no effect on IL-10Rα^{Mdel}Rag1$^{-/-}$-derived Mφs. In contrast, treatment with blocking anti-IL-10R antibody elevated Rag1$^{-/-}$ pro-IL-1β and IL-1β levels to those seen with IL-10Rα^{Mdel}Rag1$^{-/-}$ Mφs.

The complete suppression of IL-1β maturation by IL-10 suggested that Mφ IL-10R signaling also suppressed caspase-1-activation. To test this we assessed for caspase-1 cleavage to its activated form. LPS and ATP stimulation of Rag1$^{-/-}$ Mφs led to a modest increase in activated caspase-1 (p20, Fig. 7b and Supplementary Fig. 7). Pre-treatment of Rag1$^{-/-}$ Mφs with IL-10, however, abrogated this induction. In contrast, pre-treatment with anti-IL-10Rα Ab markedly increased activated caspase-1, indicating that autocrine IL-10 normally restrains caspase-1 activation. In the IL-10Rα^{Mdel}Rag1$^{-/-}$ Mφs, p20 formation was similarly increased regardless of IL-10 or anti-IL-10Rα Ab treatment.

To determine whether increased IL-1 and IL-6 from LPMφs can also directly impact Th17 cell maturation, we sorted LPMφs from diseased IL-10Rα^{Mdel}Rag1$^{-/-}$ and control Rag1$^{-/-}$ mice, and co-cultured them with naïve T cells in the presence of added TGFβ but not IL-6. Some Th17 formed in the presence of Rag1$^{-/-}$ LPMφs, however, this was markedly elevated with IL-10Rα^{Mdel}Rag1$^{-/-}$ LPMφs (Fig. 7c). Addition of αIL-6Rα blocking antibody largely abrogated Th17 formation with Rag1$^{-/-}$ LPMφs. However, consistent with our *in vivo* findings after IL-6R$^{-/-}$ T cell transfer, this effect was modest with IL-10Rα^{Mdel}Rag1$^{-/-}$-derived LPMφs. Blocking IL-1 signaling led to more substantial inhibition of Th17 development in IL-10Rα^{Mdel} Rag1$^{-/-}$-derived LPMφs and inhibition of signaling by both cytokines essentially abrogated Th17 formation. Therefore, IL-10 can act on LPMφs to directly impair their support of Th17 production. This occurs through the downregulation of IL-1 and to a lesser extent IL-6.

Discussion

IL-10's anti-inflammatory signals maintain intestinal homeostasis. Yet the essential targets and mechanisms of IL-10 action are incompletely understood. Recent data has provided support for a myeloid response to IL-10 in restraining colonic inflammation, and indicated a critical role for IL-10 in the production of anti-inflammatory LPMφs that are essential for maintaining immune homeostasis[19, 20]. We demonstrate here that Mφs are the primary targets of IL-10 limiting colitis after naive T cell transfer into immunodeficient mice. We further identify how the macrophage-specific response to IL-10 skews the production and expansion of pathologic T cells, thereby promoting disease exacerbation. Colitis is Th17 dependent regardless of Mφ IL-10 response. However, we show that IL-10 converts the disease from one that is independent of the Th17 inducing cytokine IL-6 to one that is highly dependent. In Rag1$^{-/-}$ recipients of CD4^{+}CD45RB^{hi} T cells, T cell responsiveness to IL-6 is necessary for Th17 formation and colitis development. However, in IL-10Rα^{Mdel}Rag1$^{-/-}$ recipients of IL-6Rα$^{-/-}$ T cells, a strong Th17 amplification occurs. Colitis develops that is clinically indistinguishable from that in recipients of IL-6Rα^{WT} T cells.

Our results further indicate that the Mφ response to IL-10 downmodulates multiple pro-inflammatory cytokines in the colon, but its impact on IL-1β, recently documented to regulate Th17 formation during colitis[34], appears paramount. IL-10 therefore shifts the cytokine requirements for the Th17 response. In IL-10's absence, redundancy between IL-1β and IL-6 supports persistent colitis. Implicitly, though monotherapy with anti-IL-6 may be promising, tandem inhibition of the IL-1 pathway may be necessary for optimal suppression of the Th17 response in IBD, particularly where IL-10 signaling is attenuated.

Previous studies have indicated a role for IL-1β in promoting Th17 development both in humans and mice[34, 37, 38]. IL-1β levels in IL-10$^{-/-}$ mice with colitis are elevated[39] and we extend this finding here to show that a Mφ-selective deficit in IL-10 response is sufficient for this. Prior *in vitro* findings have also indicated that IL-1β synergizes with IL-23 to promote Th17 expansion[7], and we did observe increased IL-23 along with IL-1β production by IL-10Rα-deficient macrophages.

Though we and others have identified an essential role for RORγT^{+} Th17 cells in colitis and elevated Th17 cells and/or cytokines have been observed in patients with IBD, the actual role of IL-17 itself is controversial and in a randomized control trial anti-IL-17A proved ineffective in Crohn's disease[40]. Identifying effector mechanisms responsible for Th17 mediated immunopathology during colitis will be important as new therapeutic strategies are developed.

Zigmond, et. al., recently found that mice with a CX3CR1-restricted IL-10Rα deficiency develop spontaneous colitis[19]. This was hypothesized to be mediated by defective macrophage regulation by IL-10. Our findings are consistent with this and with a unique defining role for macrophages in colitis susceptibility. CX3CR1 is also expressed by DCs[41] and monocytes[42], and by using IL-10Rα^{DCdel}Rag1$^{-/-}$, IL-10Rα^{Mdel}Rag1$^{-/-}$, IL-10Rα$^{-/-}$Rag1$^{-/-}$ and neutrophil specific depleted mice, our findings support a dominant role for IL-10 action on macrophages in colitis development. This identification of Mφ as a key target of IL-10 is consistent with a recently published report showing that ATP derived from commensal bacteria promotes Th17 differentiation through a subset of CD11c$^{-/low}$ LP cells[43]. Unlike Zigmond, et. al., we did not observe spontaneous autoimmunity in our animals, Differences in microflora, and particularly the presence of *Helicobacter* spp., may account for this. Our colony is maintained under helicobacter-free conditions, whereas Zigmond, et. al. report the presence of helicobacter. Consistently, helicobacter-free IL-10 deficient mice are protected from spontaneous disease[44]. Alternatively, differences in the subsets of Mφs expressing the CX3CR1 versus Lys-M promoters, may distinguish spontaneous disease susceptibility in our two systems, and this needs to be further explored.

Shouval, et. al. recently identified a prominent role for IL-10Rβ signaling into myeloid cells in suppressing colitis development. A significant diminution of the Foxp3^{+} regulatory T cell response was seen. We did not identify a discernible effect of Mφ IL-10Rα deficiency on iTreg development or transferred nTreg function. This will need resolution. IL-10Rβ is also utilized by IL-22, IL-26 and IFN-λ, which might explain the differences. Alternatively, cell types besides Lys-M^{+} macrophages may provide critical signals supporting Treg formation and maintenance, and IL-10 may be necessary for this. Regardless of these differences, Shouval, et. al. demonstrated a strongly pro-inflammatory phenotype of IL-10Rβ$^{-/-}$ BMDM, and extended this to Mφs from IL-10R deficient patients, implying that these regulatory mechanics are translatable to human IBD. Cumulatively, these data provide strong evidence for macrophage as the critical target of IL-10, and assert several mechanisms through which this occurs.

After transfers of IL-6Rα-deficient T cells, we identified a decreased percent of Th17 cells in the MLN but not colon of IL-10Rα^{Mdel}Rag1$^{-/-}$ recipients. This may imply that Th17

cells primed in the MLN are amplified by IL-1β in the colon. However, the site(s) of priming of the Th17 response in colitis is not defined and it is possible that additional priming occurs within gut-associated lymphoid tissue itself. In this regards, we show that isolated LPMφs from IL-10Rα^{Mdel}Rag1$^{-/-}$ mice have an enhanced ability to support Th17 development from naïve T cells. Hence, although without being able to track the initial site of pathologic T cell development we cannot distinguish whether IL-1β in our system is acting at priming or expansion of Th17 cells, increased colonic Mφ IL-1β production can support either or both of these mechanisms.

Our results here are consistent with data demonstrating that IL-10 acts via STAT3 to suppress pro-IL-1β production[45]. Moreover, we extend these findings, showing that IL-10 further inhibits caspase-1 activity. Caspase-1 is activated through inflammasome induced oligomerization and autocatalytic cleavage and it will be important to identify the precise site at which IL-10 acts within the inflammasome cascade.

Though our study specifically interrogates the IL-10 response during colitis, its implications may extend beyond this. IL-1β also promotes the Th17 response in *Helicobacter hepaticus* colitis[34]. Administration of rIL-1-β selectively induced Th17 cells in the steady-state intestinal environment[33]. High levels of IL-1β are associated with an amplified Th17 response in autoimmune RA and EAE models[46–48]. These effects of IL-1β on Th17 cells may be similarly IL-10 and Mφ dependent, and this can be assessed using the conditional knock-out mice we have developed.

Our results, as those using a Tg DN IL-10R[24], do not support transferred naïve T cells as a significant target of IL-10 in this model. This does not indicate that IL-10 is not a significant T cell regulator. The effects of IL-10 on T cells is complex, and system and T cell subset dependent. Thus, the Treg response to IL-10 was found to sustain Treg in colitis, and mice with a Treg-selective deletion of IL-10R developed spontaneous colitis[18, 21]. Likewise, we have identified direct effects of IL-10 on T cells in regulating myelin-specific responses during experimental autoimmune encephalomyelitis[25].

It will also be important to identify the source of IL-10 relevant to Mφ targets. Our findings here and those of others indicate that neither effector nor the adaptive regulatory T cells that form after naïve T cell transfer are relevant sources[49]. In contrast, purified and adoptively transferred IL-10$^{-/-}$ Tregs were seen to be less potent than WT Tregs in preventing and treating established colitis, indicating that Treg IL-10 is significant in some circumstances. However, Treg only account for a portion of IL-10's effects[49–51]. The absence of a physiologically relevant T cell IL-10 source here may suggest a myeloid source. Mφ themselves are strong IL-10 producers and autocrine IL-10 signaling may well provide necessary signals that prevent overzealous Mφ reactions. Indeed, we identified substantial IL-10 mediated autoregulation of pro-IL-1β production and caspase-1 activation in cultured Mφ (Fig. 7). In contrast, Zigmond et. al. found no role for CX3CR1^{+} macrophage-produced IL-10 in the spontaneous colitis that they observed[19], potentially implying that multiple sources may be relevant.

In summary, we show that the Mφ response to IL-10 is critical for Th17 development during colitis. Further, IL-10's suppression of pro-inflammatory cytokine production does not obviate the essential role for Th17 cells but does shift the cytokine requirements for that response from one primarily governed through IL-1β to one that is IL-6 dependent.

Methods

Mice

We previously produced and verified correct targeting of IL-10Rα$^{fl/fl}$ mice and lineage specific IL-10Rα deletions on a C57BL/6 background as described in prior publications[25, 26]. These were bred with B6.129S7-Rag1^{tm1Mom}/J mice. B6.129P2-IL-10^{tm1Cgn}/J, B6.129P2-Rorc^{tm1Litt}/J, B6.129S7-Il1r1^{tm1mx}/J, B6.129(Cg)-*Foxp3*$^{tm4(YFP/cre)Ayr}$/J, and B6;SJL-Il6Rα$^{tm1.1Drew}$/J mice were obtained from The Jackson Laboratories. Colonies were maintained under spf, including detectable *Helicobacter spp*.-free, conditions. Mice of either sex were between 8 and 12 weeks of age at the time of study, and paired between experimental and control groups. Experimental protocols were approved by the St. Jude Animal Care and Use Committee.

Induction of colitis

Flow cytometrically sorted CD4$^+$CD25$^-$CD45RB^{hi} T cells (5×10^5/mouse) derived from pooled splenocytes and LN cells of indicated mice were transferred i.v. into Rag1$^{-/-}$ or IL-10Rα^{Mdel}Rag1$^{-/-}$ mice. Body weight was monitored on a weekly basis. For neutrophil depletion studies, 1 mg anti-Ly6G mAb 1A8 (Bio-X-Cell) or control IgG[26] was administered per mouse i.p. 1 d before cell transfer and weekly thereafter. Depletion was confirmed by flow cytometry. For analyses of Treg-mediated disease suppression, splenocytes from Foxp3-YFP reporter mice were collected and naïve T cells, defined as CD4$^+$CD45RB^{hi}YFP$^-$, and Treg cells, defined as CD4$^+$CD45RB^{low}YFP$^+$, sorted. Purity after sorting was >99%. Age-matched Rag1$^{-/-}$ mice or IL-10Rα^{Mdel}Rag1$^{-/-}$ mice received 2×10^5 naive T cells with or without Treg cells at a 1:1 ratio i.v.

Histology

Colons were stained with hematoxylin and eosin. Three independent sections were assessed per mouse by a blinded reviewer. Inflammation scoring: 0, no or occasional inflammatory cells in the lamina propria (LP); 1, increased LP inflammatory cells; 2, confluence of inflammatory cells extending into the submucosa; 3, transmural infiltrate extension of the infiltrate. Ulceration scoring: 0, no ulceration; 1, mild (1–2 ulcers per 40 crypts analyzed); 2, moderate (3–4 ulcers); 3, severe (> 4 ulcers). Hyperplasia scoring: 0, normal; 1, crypts up to twice normal thickness with normal epithelium; 2, crypts >2 times normal thickness, hyperchromatic epithelium; reduced goblet cells, scattered arborization; 3, Crypts >4 times normal thickness, marked hyperchromasia, few to no goblet cells, high mitotic index, frequent arborization. Disease area scoring: 0, 0–5% involvement; 1, 5–30%; 2, 30–70%; 3, >70%. Total score is the sum of individual scores.

Cytokine levels

Frozen colon samples were homogenized in ice-cold PBS containing 1% NP-40 and complete protease inhibitor cocktail (Roche). Cytokines and chemokines were measured by Luminex (Bio-Rad) or ELISA (R&D Systems).

LP cell isolation

LP cells were isolated using a modification of a previously described protocol[26]. Large intestines were carefully excised, mesentery and fat removed, and intestines then opened longitudinally, rinsed in HBSS and cut into 1-cm pieces. Colon segments were vigorously shaken twice in medium with 1 mM EDTA (Sigma-Aldrich) for 20 min at 37°C, and suspended cells collected and filtered through a cell strainer. Tissue was further minced and incubated at 37°C for 1 h in medium with 1 mM collagenase type IV (Sigma-Aldrich) and 40 U ml^{-1} DNase I (Roche) with agitation. Cells were filtered, washed, and isolated over a percoll step gradient.

Cytokine PCR

Total RNA was isolated from sorted LPMφs using the RNeasy mini kit (Qiagen), and cDNA synthesized using superscript III and oligo (dT) primers (Invitrogen). Expression levels of were normalized to HPRT (ΔCt) and compared with littermate controls using the ΔΔCt method[52]. Primer sequences are: TGF-β: F, CAC AGT ACA GCA AGG TCC TTG C; R, AGT AGA CGA TGG GCA GTG GCT; IL-12p35: F, ATG ACC CTG TGC CTT GGT AG; R, GAT TCT GAA GTG CTG CGT TG; IL-23p19: F, AGC GGG ACA TAT GAA TCT ACT AAG AGA; R, GTC CTA GTA GGG AGG TGT GAA GTT; IL-12p40: F, GAC CAT CAC TGT CAA AGA GTT TCT AGA T; R, AGG AAA GTC TTG TTT TTG AAA TTT TTT AA; IL-1β: F, GAT CCA CAC TCT CCA GCT GCA; R, CAA CCA ACA AGT GAT ATT CTC CATG; IL-10: F, GTG AAA ATA AGA GCA AGG CAG TG; R, ATT CAT GGC CTT GTA GAC ACC; TNF-α: F, AAT GGC CTC CCT CTC ATC AGT; R, CTA CAG GCT TGT CAC TCG AA; iNOS: F, TGA CGG CAA ACA TGA CTT CAG; R, GCC ATC GGG CAT CTG GTA; IL-6: F, TAT GAA GTT CCT CTC TGC AAG AGA; R, TAG GGA AGG CCG TGG TT; Arginase: F, TCA CTT TCC ACC ACC TCT TG AY; R, TCT CCA CCG CCT CAC GAC TC; IL-17A: F, GCT CCA GAA GGC CCT CAG, R, CTT TCC CTC CGC ATT GAC A; CD163: F, CCT TGG AAA CAG AGA CAG GC; R, TCC ACA CGT CCA GAA CAG TC; HPRT: F, GA CCG GTC CCG TCA TGC; R, TCA TAA CCT GGT TCAT CAT CGC. F, forward primer; R, reverse primer.

Flow cytometry

Cells were stained with Abs specific for mouse TCRβ, F4/80, CD11b, CD11c, CD40, CD64, CD80, CD86, Ly6G, Siglec-F, CD4, Foxp3, IL-17, IFN-γ, pro-IL-1β, or with isotype-matched controls (1:100 dilution for each antibody; BD Pharmingen or eBiosciences), and analyzed using a FACSCalibur or LSRII flow cytometer with Cell Quest (BD Biosciences) or FlowJo (TreeStar) software.

BRDU staining

Mice were injected i.p. with 150 μl BrdU (10 mg ml^{-1}) in sterile 1× DPBS. After 16–20 h, lymphocytes were isolated, stained with Abs to cell surface markers, fixed and permeabilized with Cytofix/Cytoperm Buffer (BD Biosciences), treated with DNase (300 μg ml^{-1}) at 37°C for 1 h, stained with anti–BrdU-APC (BD BRDU flow kit), and analyzed by flow cytometry.

BMDM culture and LPS stimulation

BMDMs were generated by culturing mouse bone marrow cells in L-cell-conditioned IMDM. The L-cell conditioned medium comprised supernant from cultures of L929 cells secreting M-CSF mixed at a 1:2 ratio with IMDM and then supplemented with 10% FBS, 1% non-essential amino acids and 1% penicillin–streptomycin. After 6 days of culture, cells were seeded in 12-well plates, and the next day treated with IL-10 (50ng ml^{-1}) or anti-IL-10Rα Ab (1μg ml^{-1}), and 4 h later stimulated with or without LPS (20 ng ml^{-1}) for 12 h. For the final 30 min, 5mM ATP was added into the medium[53].

Western blot

Culture samples were denatured in loading buffer containing SDS and 100 mM DTT, and boiled for 5 min. SDS-PAGE–separated proteins were transferred to polyvinylidene difluoride membranes and immunoblotted with primary Abs against caspase-1 (Adipogen; AG-20B-0042 or kind gift of Dr Peter Vandenabeele, Ghent University), IL-1β (R&D Systems), and GAPDH (Cell Signaling Technology; D16H11), followed by secondary anti-rabbit, anti-rat, anti-mouse, or anti-goat HRP Abs (Jackson ImmunoResearch Laboratories)[54]. Images have been cropped for presentation. Full size images are presented in Supplementary Fig. 7.

Th17 culture

Naive ($CD4^+CD45RB^{high}CD25^-$) T cells were purified by cell sorting to a purity >99%. These (5×10^5) were co-cultured at a ratio of 5:1 with or without sorted LPMφs from 8 week diseased $IL\text{-}10R\alpha^{Mdel}$ $Rag1^{-/-}$ or $Rag1^{-/-}$ mice in 96-well plates pre-coated with 1 μg ml^{-1} anti-CD3 and 2 μg ml^{-1} anti-CD28. Cells were cultured in complete RPMI 1640 media containing 5 ng ml^{-1} TGF-β, 10 μg ml^{-1} anti-IL-4 and 10 μg ml^{-1} anti-IFN-γ Abs. 20 ng ml^{-1} IL-6 was added to a positive control Th17 culture condition only. After 4 days, cells were washed and restimulated with cytokine stimulation cocktail containing PMA, Ionomycin and Brefeldin A (Cell stimulation cocktail, eBioscience) for 4 hours at 37°C. Cells were washed and stained for the indicated cytokines.

Immunohistochemistry

Tissue cryosections were fixed in 4% PFA at 4°C overnight, embedded in optimal cutting temperature (OCT) compound, and sectioned in a cryostat (12 μm). For IL-1β immunostaining, sections were incubated with a polyclonal goat anti-mouse IL-1β primary antibody (1:200, R&D Systems) and monoclonal rat anti-mouse CD11b antibody (1:500, AbD Serotec). After washing 3 times with TBST, sections were incubated with Cy3-labeled donkey-anti-rat IgG antibody (1:200, Jackson Lab) and Alexa 488-labeled donkey anti-goat

IgG antibody (1:200,Molecular Probes). Sections were mounted with mounting medium containing DAPI (Invitrogen), and confocal microscopy was performed.

Statistics

Statistics were calculated using Prism5 (GraphPad Software). Group comparisons were by two-sided Student's t-test or, when multiple cohorts were present, ANOVA with Bonferroni correction. A $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IBD	inflammatory bowel disease
IL-10Rα	interleukin 10 receptor α
LPMφ	lamina propria macrophage
WT	wild type
BMDM	Bone marrow derived macrophage
RORγT	retinoic acid receptor-related organ receptor-γT

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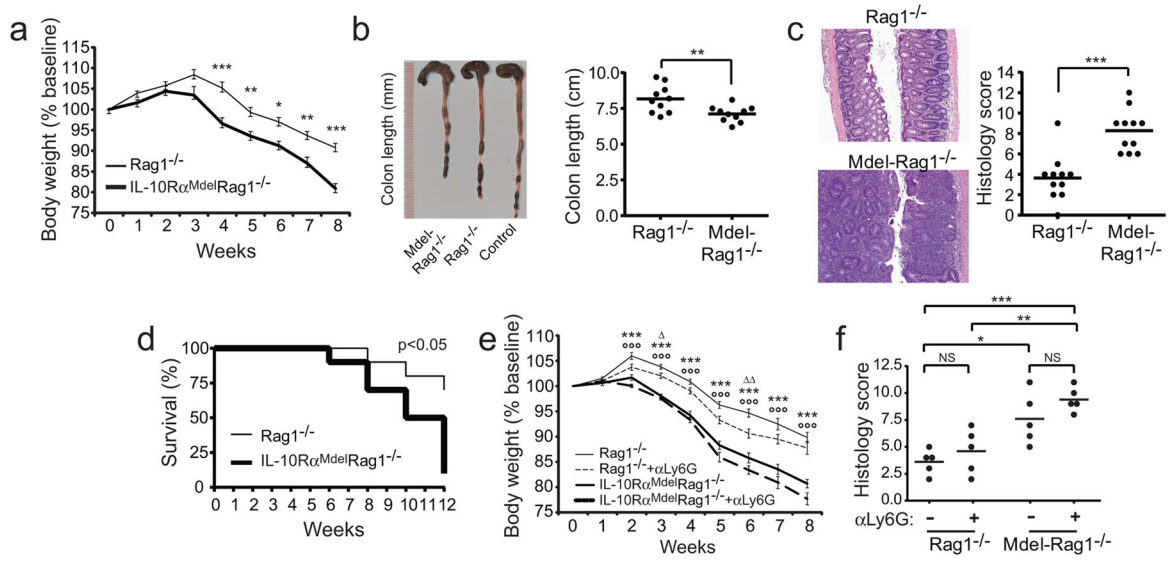

Figure 1. Mφ IL-10Rα expression attenuates T cell-induced colitis

(A) $CD4^+CD25^-CD45RB^{hi}$ C57BL/6 T cells (5×10^5) were transferred into C57BL/6 $Rag1^{-/-}$ and $IL\text{-}10R\alpha^{Mdel}Rag1^{-/-}$ mice to induce colitis. Mean±1 s.e.m. percent of initial body weight is plotted. *, p<0.05; **, p<0.01; ***, p<0.001 (by t-test); (B) Colons were removed at week 8 and colon length measured from the indicated recipients or mice not receiving T cells (control). (C) Representative photomicrographs and tallied scores for disease parameters from H&E stained colon sections obtained 8 wk after naive T cells transfer. (D) Kaplan-Meier survival curves of $Rag1^{-/-}$ and $IL\text{-}10R\alpha^{Mdel}Rag1^{-/-}$ mice (n=10). (E) Colitis was induced in $Rag1^{-/-}$ and $IL\text{-}10R\alpha^{Mdel}Rag1^{-/-}$ mice with 5×10^5 $CD4^+CD25^-CD45RB^{hi}$ T cells from C57BL/6 mice. Anti-Ly6G or control Ab was administered i.p. 1 d pre-transfer and then weekly to deplete neutrophils. Mean±1 s.e.m. initial body weight is plotted (n=10/cohort). △, $Rag1^{-/-}$ vs $Rag1^{-/-}$+ αLy6G; ○, $Rag1^{-/-}$ vs $IL\text{-}10R\alpha^{Mdel}Rag1^{-/-}$; *, $Rag1^{-/-}$+ αLy6G vs $IL\text{-}10R\alpha^{Mdel}Rag1^{-/-}$+ αLy6G; p<0.05, p<0.01, and p<0.001 for 1, 2, and 3 symbols (by ANOVA). No significant difference was seen in $IL\text{-}10R\alpha^{Mdel}Rag1^{-/-}$ vs $IL\text{-}10R\alpha^{Mdel}Rag1^{-/-}$+ αLy6G. (F) Histology scores for 8 week colon from mouse cohorts treated as in (e). Data are representative of three independent experiments, n=5–10 per cohort.

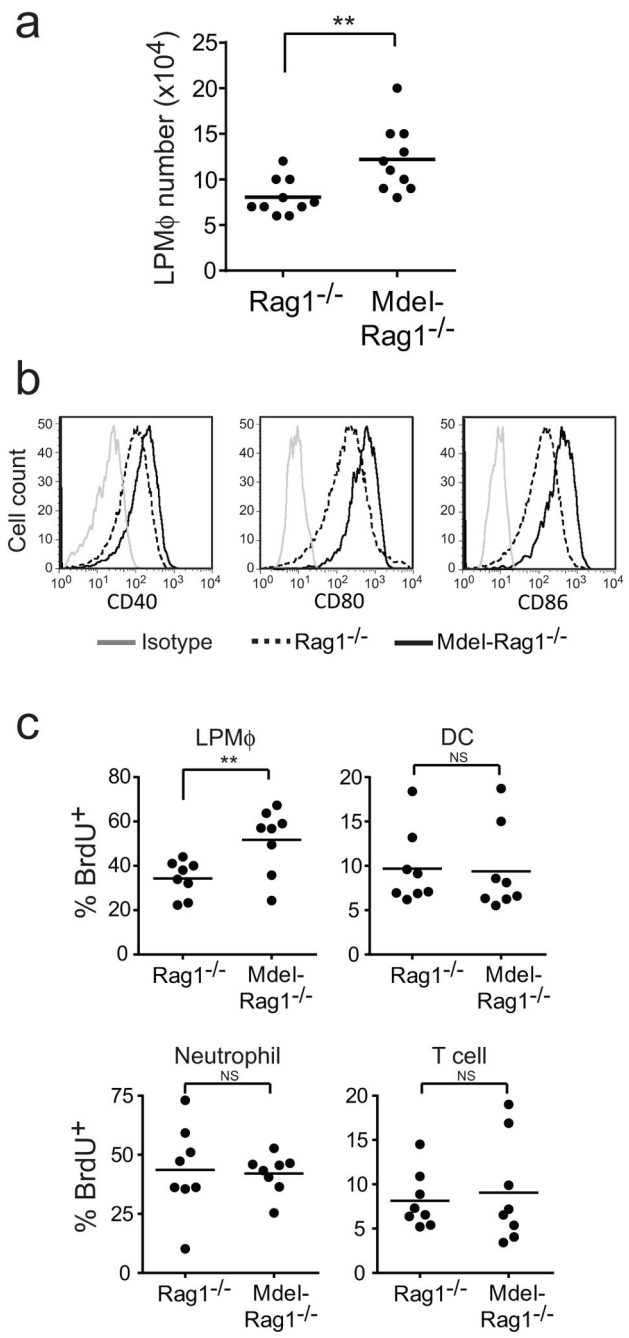

Figure 2. Analysis of lamina propria macrophages

(A) Cells were isolated from large intestine lamina propria of Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice at wk 8 after colitis induction. Absolute numbers of LPMφ (CD11b^{hi}CD11c$^{-/lo/mod}$F4/80^{+}Ly6G$^{-/lo}$Siglec-F^{-}) were quantified. (B) Staining of LPMφ gated as above, for CD40, CD80 and CD86. Gray line, isotype control; dashed black line, LPMφs from Rag1$^{-/-}$ mice; Solid black line, LPMφs from IL-10Rα^{Mdel}Rag1$^{-/-}$ mice. (C). Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice received 5×10^{5} CD4^{+}CD25^{-}CD45RB^{hi} C57BL/6 T cells. After 8 wk, BrdU was administered i.p. and the mice sacrificed 24 h later. Colon LP

cells were isolated and BrdU positive Mφs, DCs, neutrophils and $CD4^+$ T cells measured by intracellular flow cytometry. Data are representative of three independent experiments, n=8–10 per cohort. **, $p<0.01$ (by t-test).

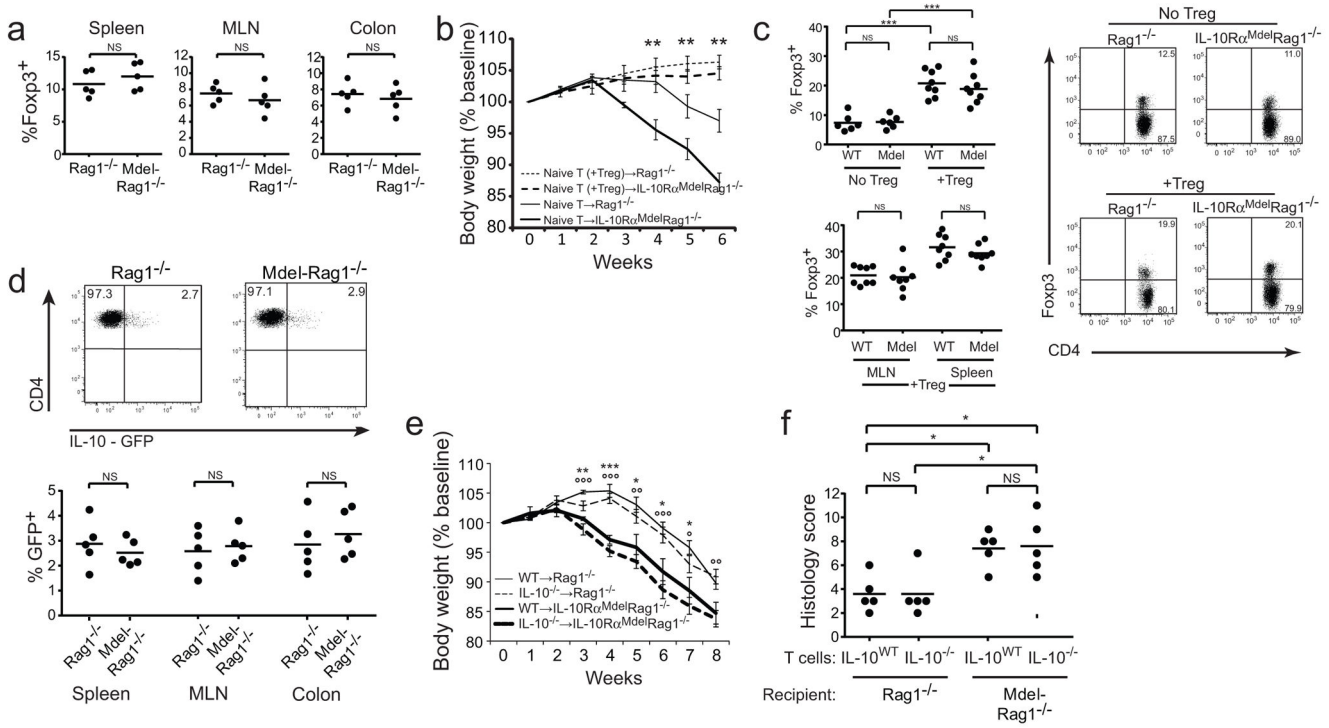

Figure 3. T cell Foxp3 expression and IL-10 production

(A) Colitis was induced in Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice by the transfer of 5×10^5 CD4$^+$CD25$^-$CD45RB^{hi} C57BL/6 T cells. The frequency of Foxp3$^+$ Treg cells among CD4$^+$ T cells in the spleen, MLN and colon 8 wk after initial T cell transfer is plotted. (B) Mice received sorted CD4$^+$CD45Rb^{lo}Foxp3-YFP$^+$ Treg with naive T cells at a 1:1 ratio or naive (CD4$^+$CD45Rb^{hi}Foxp3-YFP$^-$)T cells alone, and were monitored for weight loss. **, p<0.01 for naïve T→Rag1$^{-/-}$ vs naïve T→IL-10Rα^{Mdel}Rag1$^{-/-}$ (by ANOVA). Differences were not significant between naïve T+Treg→Rag1$^{-/-}$ vs naïve T+Treg →IL-10Rα^{Mdel}Rag1$^{-/-}$ cohorts. (C) Splenic, MLN, and colonic Foxp3$^+$ T cells were identified by flow cytometry in mice receiving or not receiving Treg as in (B).Upper-left and right plots depict results for colonic Treg. Lower-left plot depicts results for MLN and splenic Treg only in mice receiving supplemental Treg. ***, p<0.001 (by ANOVA). (D) Similar transfers were performed using CD4$^+$CD25$^-$CD45RB^{hi} T cells from IL-10-GFP donors. Representative dot plots and summary analysis of the frequency of IL-10-GFP$^+$ cells among CD4$^+$ T cells in the spleen, MLN, and colon at 8 wk is shown. (E) Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice received 5×10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 or IL-10$^{-/-}$ mice. Mean±1 s.e.m. percent initial body weight is plotted. *, WT→Rag1$^{-/-}$ vs WT→IL-10Rα^{Mdel}Rag1$^{-/-}$; ●, IL-10$^{-/-}$→Rag1$^{-/-}$ vs IL-10$^{-/-}$→IL-10Rα^{Mdel}Rag1$^{-/-}$; p<0.05, p<0.01, and p<0.001 for 1, 2, and 3 symbols (by ANOVA). Representative of 3 independent experiments, n=5 per cohort. (F). Histological scores for colons from the indicated mice at 8 wk.

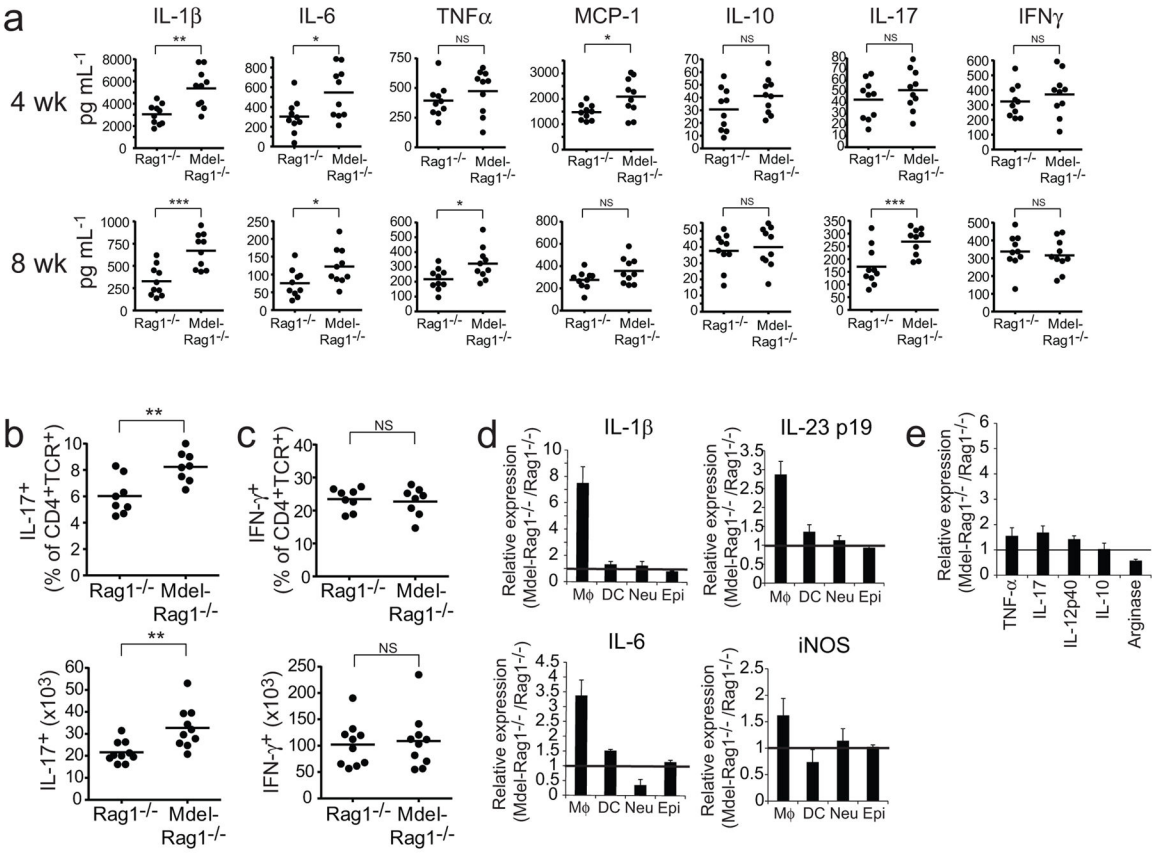

Figure 4. Cytokine production by colonic macrophages

(A) Colons from Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice, 8 wk after colitis induction, were homogenized and cytokines (IL-1β, IL-6, TNF-α, MCP-1, IL-10, IL-17 and IFN-γ) measured by ELISA or multiplex assay. Results from individual mice (circles) and cohort means (lines) are plotted. (B and C) Percent and absolute number of IL-17^{+} and IFN-γ^{+} cells among CD4^{+} T cells from colons of diseased mice (8 wk). (D) Relative expression of the indicated mRNAs (IL-1β, IL-6, IL-23p19 and iNOS) from LPMφs, DCs, neutrophils and epithelial cells sorted from colon tissue and measured by qRT-PCR. (E) Relative expression of the indicated mRNAs (TNF-α, IL-17, IL-12p40, IL-10, and arginase) from LPMφs sorted from colon tissue and measured by qRT-PCR. Data are representative of three independent experiments, n=10 per cohort. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ (by t-test).

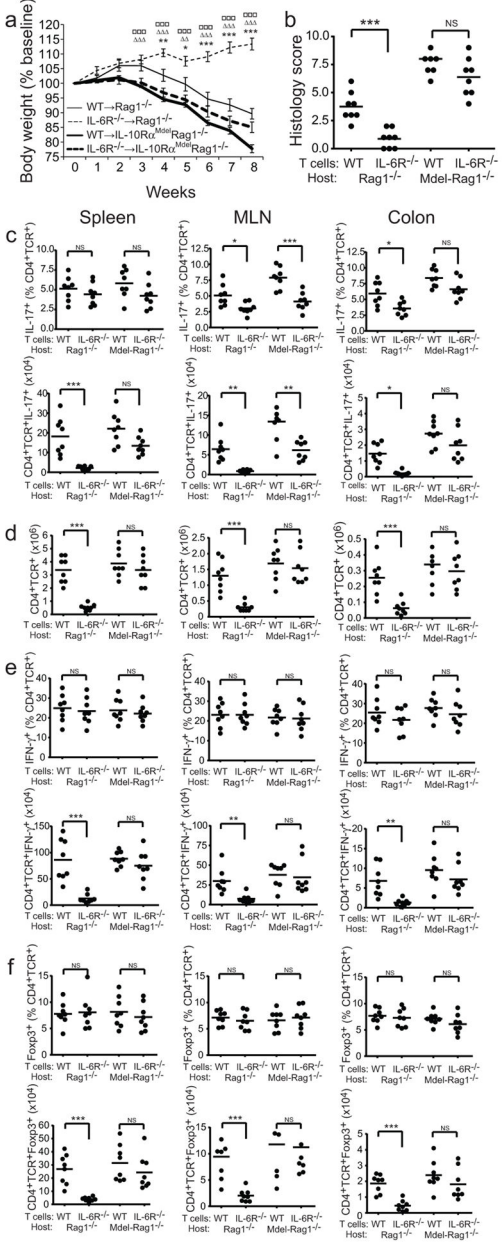

Figure 5. Role of T cell IL-6 response in colitis development in Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice

Colitis was induced in Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice by the transfer of 5×10^5 C57BL/6 or IL-6Rα$^{-/-}$ CD4$^+$CD25$^-$CD45RB^{hi} T cells. Mice were sacrificed after 8 wk. (A) Representative weight curves, mean ±1 s.e.m., are plotted. *, WT→Rag1$^{-/-}$ vs IL-6Rα$^{-/-}$ →Rag1$^{-/-}$; △, WT→Rag1$^{-/-}$ vs WT→IL-10Rα^{Mdel}Rag1$^{-/-}$; □, IL-6Rα$^{-/-}$ →Rag1$^{-/-}$ vs IL-6Rα$^{-/-}$ →IL-10Rα^{Mdel}Rag1$^{-/-}$; p<0.05, p<0.01, and p<0.001 for 1, 2, and 3 symbols (by ANOVA). No significant differences were observed for WT→IL-10Rα^{Mdel}Rag1$^{-/-}$ vs IL-6Rα$^{-/-}$ →IL-10Rα^{Mdel}Rag1$^{-/-}$. (B) Histologic scores for colons analyzed at 8 wk. (C) Percent and absolute number of IL-17$^+$ cells among CD4$^+$ T cells in the spleen, MLN and colon. (D) Absolute numbers of CD4$^+$ T cells. (E) Percent and absolute number of IFN-γ$^+$

cells among $CD4^+$ T cells. (F) Percent and absolute number of $Foxp3^+$ Treg cells among $CD4^+$ T cells. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (by t-test). Data are representative of three independent experiments, n=8 per cohort.

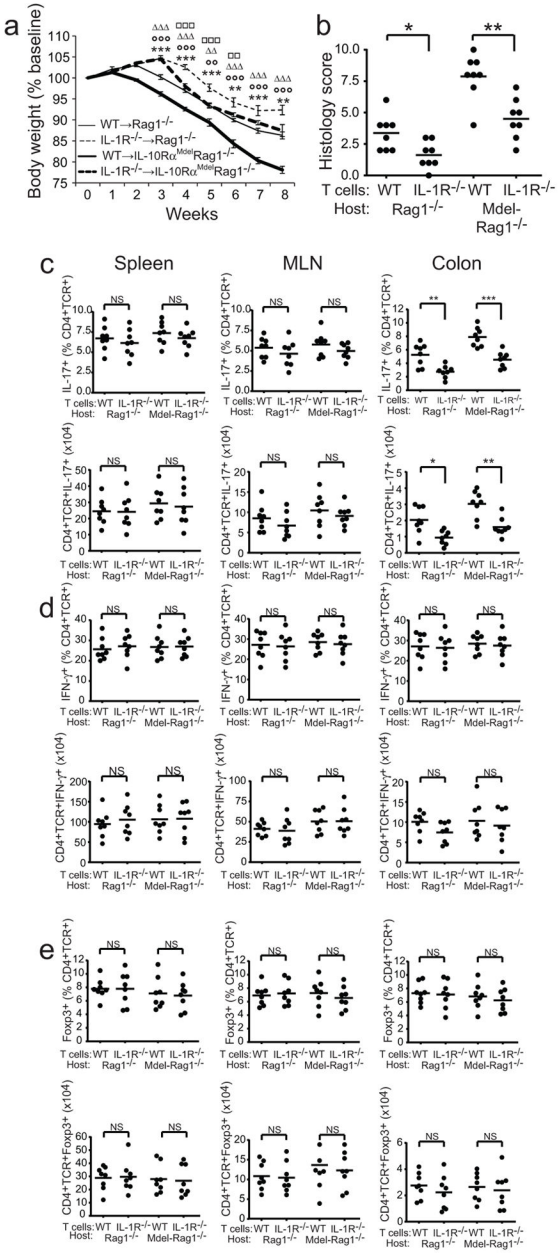

Figure 6. Role of T cell IL-1 response in colitis development in Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice

Colitis was induced in Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice by the transfer of 5×10^5 C57BL/6 or IL-1R$^{-/-}$ CD4$^+$CD25$^-$CD45RB^{hi} T cells. Mice were sacrificed after 8 wk. (A) Representative weight curves, mean ±1 s.e.m., are plotted. *, WT→Rag1$^{-/-}$ vs IL-1R$^{-/-}$ →Rag1$^{-/-}$; ○, WT→IL-10Rα^{Mdel}Rag1$^{-/-}$ vs IL-1R$^{-/-}$ → IL-10Rα^{Mdel}Rag1$^{-/-}$ △, WT→Rag1$^{-/-}$ vs WT→IL-10Rα^{Mdel}Rag1$^{-/-}$; □, IL-1R$^{-/-}$ →Rag1$^{-/-}$ vs IL-1R$^{-/-}$ →IL-10Rα^{Mdel}Rag1$^{-/-}$; $p<0.01$, and $p<0.001$ for 2 and 3 symbols (by ANOVA). (B) Histologic scores for colons analyzed at 8 wk. (C) Percent and absolute number of IL-17$^+$ cells among CD4$^+$ T cells in the spleen, MLN and colon. (D) Percent and absolute number of IFN-γ$^+$ cells among CD4$^+$ T cells. (E) Percent and absolute number of Foxp3$^+$ Treg cells

among $CD4^+$ T cells. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (by t-test). Data are representative of three independent experiments, n=8 per cohort.

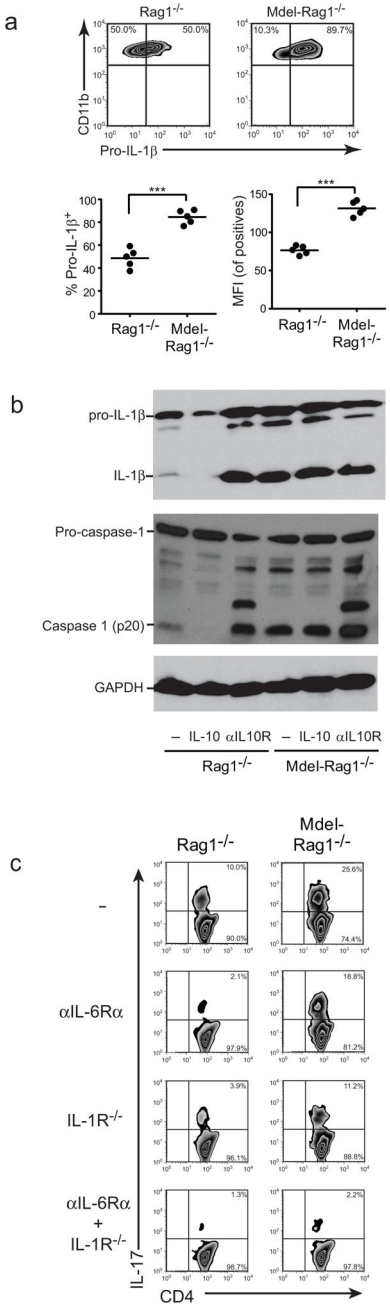

Figure 7. IL-10 inhibition of Mφ inflammasome activity and IL-1β production

(A) Colitis was induced in Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice by transfer of 5×10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from WT mice. After 8 wks, LPMφ pro-IL-1β expression was assessed by intracellular staining. Percent of Mφs expressing pro-IL-1β and mean fluorescence intensity (MFI) of positive cells are plotted. Data are representative of three independent experiments, n=5 per cohort. ***, p<0.001 (by t-test). (B) Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ BMDMs were pretreated with recombinant murine IL-10 or blocking anti-IL-10Rα Ab for 4 h prior to the addition of LPS for 12 h and ATP for the final 30 minutes. Lysates were immunoblotted for IL-1β, caspase-1, and GAPDH. (C) Naïve CD4$^+$ T

cells from WT or IL-1R$^{-/-}$ mice and LPMφs from colitic Rag1$^{-/-}$ and IL-10Rα^{Mdel}Rag1$^{-/-}$ mice were purified by flow cytometric sorting. The T cells were stimulated with anti-CD3 and anti-CD28 in the presence of anti-IL-4, anti-IFN-γ, and TGF-β. LPMφs and T cell source, and the addition of blocking anti-IL-6R Ab are indicated. After 4 d, IL-17-producing cells were analyzed by intracellular staining.