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# IL-10 modulates DSS-induced colitis through a macrophage – ROS – NO axis

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## Abstract

Breakdown of the epithelial barrier due to toxins or other insults leads to severe colitis. IL-10 is a critical regulator of this, yet its cellular targets and mechanisms of action are not resolved. We address this here. Mice with a macrophage-selective deletion of IL-10Rα (IL-10Rα$^{Mdel}$) developed markedly enhanced DSS-induced colitis that did not significantly differ from disease in IL-10$^{-/-}$ or IL-10Rα$^{-/-}$ mice; no impact of IL-10Rα-deficiency in other lineages was observed. IL-10Rα$^{Mdel}$ colitis was associated with increased mucosal barrier disruption in the setting of intact epithelial regeneration. Lamina propria macrophages did not show numerical or phenotypic differences from controls, or a competitive advantage over wild type cells. Pro-inflammatory cytokine production, and particularly TNF-α, was increased, though TNF-α neutralization failed to reveal a defining role for this cytokine in the aggravated disease. Rather, IL-10Rα$^{Mdel}$ lamina propria macrophages produced substantially greater levels of NO and ROS than controls. Inhibition of these had modest effects in wild type mice, though dramatically reduced colitis severity in IL-10Rα$^{Mdel}$ mice, and largely eliminated the differential effect of DSS in them. Therefore, IL-10's palliative actions in DSS-induced colitis pre-dominantly results from its macrophage specific effects. Downregulation of NO and ROS production are central to IL-10's protective actions.

### Keywords

IL-10; Inflammatory Bowel Disease; macrophage; NO; ROS

## Introduction

Inflammatory bowel diseases (IBD), including Ulcerative Colitis (UC) and Crohn's disease, are characterized by mucosal damage and ulceration. Breech of the intestinal epithelial barrier by commensal bacteria triggers the inflammation that is responsible for IBD



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**Supplementary Materials**

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

**Disclosure/Conflicts of Interest**

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pathogenesis. Dextran sodium sulfate (DSS) administration has commonly been used to model UC. Ingested DSS concentrates in the colon where it disrupts the epithelial barrier and induces a secondary inflammatory response characterized by the production of proinflammatory cytokines, including IL-1β, IL-6, IL-12, IL-18 and TNF-α[1–6].

IL-10 is a pre-dominantly anti-inflammatory cytokine with an essential role in maintaining gastrointestinal homeostasis. Genetic variants in IL-10 or the IL-10 receptor are associated with IBD susceptibility. Older IL-10$^{-/-}$ mice develop spontaneous colitis, and IL-10 deficiency exacerbates colitis in several models, including DSS and T-cell transfer colitis[7–11]. Moreover, pharmacologically administered IL-10 ameliorates colitis in mice by inhibiting intestinal inflammation and suppressing proinflammatory cytokine production[12–14]. IL-10 is produced by hematopoietically-derived cells, including T cells, B cells, dendritic cells, and macrophages. Signaling through the IL-10 receptor (IL-10R) down-modulates TNF-α production and pro-inflammatory signaling through various mechanisms, including the induction of SOCS3, other anti-inflammatory proteins, and miRNA[15–17].

The cell types responsible for IL-10's anti-inflammatory effects and mucosal protection in colitis have not been resolved. The IL-10 receptor is a heterodimer comprised of an IL-10Rα chain that is specific for IL-10 and an IL-10Rβ that is shared with other IL-10-family cytokines, including IL-22, IL-26 and the IFN-λ family. Whereas IL-10Rα is largely restricted to hematopoietic cells, IL-10Rβ is broadly expressed. We recently described the production of mice allowing the conditional deletion of IL-10Rα, which we apply here to assess how lineage-specific IL-10 responsiveness influences colitis severity[18]. We identify a selective role for macrophage IL-10Rα, and find suppression of NO and ROS production to be critical downstream mechanisms.

## Results

### Macrophage IL-10 response limits the severity of DSS-induced colitis

To define the cellular lineages responsible for IL-10's protective effect in colitis, we first generated mixed chimeras in which bone marrow from mice with a germline deletion of IL-10Rα (CMV-Cre×IL-10Rα$^{fl/fl}$; IL-10Rα$^{-/-}$) or wild type (WT) controls was transplanted in a criss cross manner into lethally irradiated IL-10Rα$^{-/-}$ or IL-10Rα$^{WT}$ recipients. Colitis was induced by oral administration of 3% DSS for 5 d. No effect of IL-10Rα deficiency restricted to radioresistant host populations was seen (Supp. fig. S1). In contrast, mice with IL-10Rα deficiency in the bone marrow grafts developed significantly worse disease compared with those receiving WT bone marrow. This implies that radiosensitive hematopoietic populations are primarily responsible for IL-10's effects.

To further dissect the lineages responsible, we bred C57BL/6-IL-10Rα$^{fl/fl}$ mice with mice expressing Cre transgenes in macrophages (Lys-Cre; IL-10Rα$^{Mdel}$), T cells (CD4-Cre; IL-10Rα$^{Tdel}$), dendritic cells (CD11c-Cre; IL-10Rα$^{DCdel}$), or B cells (CD19-Cre; IL-10Rα$^{Bdel}$). Cells from the different lines demonstrated an anticipated absence of IL-10Rα on Cre-expressing lineages (Supp. fig. S2 and [18]).

After colitis induction, control IL-10Rα$^{fl/fl}$ mice typically lost ~15–20% of body weight by d 7–8, with subsequent restoration of initial weight (Fig. 1a–d). IL-10Rα$^{DCdel}$, IL-10Rα$^{Bdel}$, and IL-10Rα$^{Tdel}$ mice displayed identical disease kinetics and magnitude, indicating that T cell, B cell, and DC responsiveness to IL-10 did not affect clinical severity (Fig. 1a–c). In contrast, IL-10Rα$^{Mdel}$ mice developed more severe disease (Fig. 1d). Mean maximal weight loss was greater than for controls (25±5% vs 16±4%) and 2/10 IL-10Rα$^{Mdel}$ mice but no controls had to be culled due to their illness. At a higher dose of 4% DSS, 6/10 (60%) IL-10Rα$^{Mdel}$ though no control mice died or required euthanasia (Fig. 1e).

The Lys-Cre transgene is expressed in granulocytic cells in addition to macrophages[19]. Comparison of DSS-treated IL-10Rα$^{fl/fl}$ and IL-10Rα$^{Mdel}$ mice indicated that the IL-10R deficiency did not lead to differences in the percent or absolute numbers of granulocytes in the lamina propria (Supp. fig. S3). To more definitively exclude a role for these cells in the enhanced disease in IL-10Rα$^{Mdel}$ mice, we depleted them with anti-Ly6G antibody (Ab) prior to DSS administration[20]. Neutrophils remained undetectable for >9 days (data not shown). Despite this, the anti-Ly-6G Ab treatment did not significantly alter disease course or severity in either IL-10Rα$^{fl/fl}$ or IL-10Rα$^{Mdel}$ mice (Fig. 1f; $p>0.05$). This indicates that macrophage and not granulocytes are responsible for the protective effects of IL-10 in colitis.

To better delineate the contribution of macrophage, we compared disease in IL-10Rα$^{Mdel}$, IL-10$^{-/-}$ and IL-10Rα$^{-/-}$ mice. The latter two lines have global deficiency of IL-10 or its specific receptor. All three lines developed more severe disease than IL-10Rα$^{fl/fl}$ controls (Fig. 1g). However, disease in IL-10Rα$^{Mdel}$ mice did not significantly differ at any time point from that in 10Rα$^{-/-}$ or IL-10$^{-/-}$ mice. Therefore, colitis in IL-10Rα$^{Mdel}$ mice is comparable to that in mice ubiquitously deficient in the IL-10 response. Cumulatively, these results implicate the macrophage lineage as the primary mediator of IL-10's effects in colitis induced by barrier disruption.

## Increased immunopathology in IL-10Rα$^{Mdel}$ colonic mucosa

We anticipated that the enhanced disease in IL-10Rα$^{Mdel}$ mice would be associated with increased mucosal damage and hence gastrointestinal blood loss. Indeed, significantly increased bleeding was seen in IL-10Rα$^{Mdel}$ compared with IL-10Rα$^{fl/fl}$ cohorts on each day that blood was detectable (Fig. 2a).

This was correlated with histologic changes. IL-10Rα$^{Mdel}$ colons, isolated at d 7, showed a more extensive cellular infiltrate, increased submucosal edema, and increased epithelial erosion compared with IL-10Rα$^{fl/fl}$ controls (Fig. 2b). Observer-blinded scoring demonstrated a significantly greater area of tissue destruction and severity of inflammation, ulceration, and hyperplasia in the IL-10Rα$^{Mdel}$ colons. Total histologic score was 2.7±2.3 and 7.6±1.8 (scale 0–12) for IL-10Rα$^{fl/fl}$ and IL-10Rα$^{Mdel}$ mice respectively (Fig. 2c). Consistently, IL-10Rα$^{Mdel}$ colons were shorter than controls (5.8±0.6 vs 7.2±0.8 cm, Fig. 2d, e). Therefore, by clinical, gross pathologic, and histopathologic measures, IL-10Rα$^{Mdel}$ mice develop colitis that is increased in severity.

Importantly, we did not observe clinical evidence of spontaneous colitis in our IL-10Rα$^{Mdel}$ colony, which was housed under helicobacter spp.-free conditions, arguing against the development of subclinical disease prior to DSS administration. We verified this histologically. Colon tissue sections from unmanipulated IL-10Rα$^{fl/fl}$ and IL-10Rα$^{Mdel}$ mice were equivalent, and abnormalities indicating incipient colitis were not observed (data not shown).

## Unimpaired epithelial regeneration in IL-10Rα$^{Mdel}$ mice

To assess for alterations in barrier integrity with loss of macrophage IL-10 responsiveness, we administered FITC-dextran by gastric lavage and measured its passage into the blood stream. Levels of FITC-dextran were greater in IL-10Rα$^{Mdel}$ than IL-10Rα$^{fl/fl}$ blood (Supp. fig. S4a), consistent with increased barrier disruption.

Impaired epithelial regeneration from crypt progenitors has been associated with enhanced colitic inflammation[21], and may have contributed to the barrier disruption. We analyzed this by pulsing unmanipulated or colitic IL-10Rα$^{Mdel}$ or IL-10Rα$^{fl/fl}$ mice with BrdU for 2 h, then measuring its incorporation into the colonic epithelium. No significant difference was detected between IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ colons, either in untreated mice or mice receiving DSS (Supp. fig. S4b), indicating that differential epithelial turnover is not responsible for the different disease susceptibilities.

## Macrophage infiltrate in DSS colitis

As an alternative explanation for the enhanced IL-10Rα$^{Mdel}$ colitis, we looked for changes in the number and maturation state of IL-10Rα$^{Mdel}$ lamina propria macrophages (LPMφs). Surprisingly, gated colonic CD11b$^{+}$F4/80$^{+}$Ly6G$^{-/lo}$CD11c$^{-/dim}$ LPMφs, a population we also characterized as CD45$^{+}$ and SiglecF$^{-}$, were not significantly increased in colitic IL-10Rα$^{Mdel}$ compared with IL-10Rα$^{fl/fl}$ mice (Fig. 3a and Supp. Fig. S5).

LPMφ are functionally diverse. Takada and colleagues separated CD11b$^{+}$ F4/80$^{+}$CD11c$^{-}$ LPMφs into a SSC$^{hi}$ population, referred to as LPMφ1, and a SSC$^{lo}$ population, LPMφ2, with distinct cytokine production and chemokine response properties[22]. LPMφ subsets expressing CD11c have been more recently identified during intestinal inflammation[23]. We assessed LPMφs, gated to include CD11c$^{-}$ and CD11c$^{dim}$ cells, in IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice with colitis. These did segregate into discrete SSC$^{hi}$ and SSC$^{lo}$ populations (Fig. 3b). However, the proportions of SSC$^{hi}$ and SSC$^{lo}$ cells did not significantly differ (Fig. 3c). Further, markers associated with LPMφ activation and subset assignment, including CD40, CD80, CD86 and TLR2, were comparably expressed in IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ LPMφs (Supp. Fig. S6). Therefore, despite the difference in disease severity, IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ LPMφs are phenotypically similar and present in similar numbers.

## IL-10Rα$^{Mdel}$ macrophage actively promote colitis and do not outcompete wild type cells

To gain insight into whether IL-10Rα$^{Mdel}$ macrophages play a dominant role in increasing colitis severity, we generated hematopoietic chimeras in which lethally irradiated wild type (WT) recipients received stem cell rescue with WT, IL-10Rα$^{Mdel}$, or a mixture of WT and

IL-10Rα$^{Mdel}$ bone marrow. Cell origins were distinguishable by the alternative expression of CD45.1 and CD45.2, allowing us to compare the populations in a competitive manner. As anticipated, recipients of IL-10Rα$^{Mdel}$ marrow developed more severe colitis than those receiving WT marrow. However, mice receiving a mixture of WT and IL-10Rα$^{Mdel}$ marrow developed disease essentially identical to those receiving IL-10Rα$^{Mdel}$ marrow alone (Supp. Fig. S7) despite equivalent proportions of IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ cells among transferred bone marrow cells, blood macrophage prior to colitis induction, and macrophage in the LP, spleen, and blood in diseased mice (data not shown). Implicitly, altered macrophage function rather than competitiveness acts to worsen disease in IL-10Rα$^{Mdel}$ mice.

## Increased proinflammatory cytokine production in IL-10Rα$^{Mdel}$ colons

To functionally analyze the impact of the IL-10Rα$^{Mdel}$ mutation, we next measured levels of IL-1β, IL-18, IL-6, MCP-1 and TNF-α, pro-inflammatory cytokines associated with colitis, in whole colons from d 7 colitic mice. Each was increased in IL-10Rα$^{Mdel}$ compared with IL-10Rα$^{fl/fl}$ controls (Fig. 4a, $p<0.05$). IL-10 is also produced by activated macrophages, initiating an autocrine and paracrine negative feedback loop. However, IL-10 levels were unaffected, indicating that the inability of macrophages to respond to IL-10 did not influence its overall quantity.

We further characterized specific cytokine production by macrophages themselves. Cytokine transcription was measured in neutrophil-depleted flow sorted CD11b$^{+}$F4/80$^{+}$CD11c$^{-/dim}$Ly6G$^{-/lo}$ LPMφs. A 4.4±0.6, 2.8±0.4 and 1.5±0.2 fold increase in IL-1β, TNF-α and IL-12p35 message respectively was seen in IL-10Rα$^{Mdel}$ compared with IL-10Rα$^{fl/fl}$ LPMφs (Fig. 4b). In contrast, TGF-β message was decreased in IL-10Rα$^{Mdel}$ LPMφs by 0.42±0.08 fold, while IL-6, IL-10, and IL-23p19 mRNA were essentially unchanged. Therefore, loss of macrophage responsiveness to IL-10 leads to an overall shift toward increased pro-inflammatory cytokine production.

The role of Th17 cells in DSS colitis is unclear, with one study indicating positive and negatives roles for IL-17F and IL-17A respectively, and another identifying disease promoting effects of IL-17A[4, 24]. We did not observe differences in IL-17A levels in whole colons or in the numbers of IL-17A or IL-17F-positive infiltrating T lymphocytes during DSS colitis when comparing IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice (data not shown). Considering this and the similar IL-23 mRNA expression in IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ LPMφs, modulation of Th17 cells does not appear to play a role in the differential disease.

TNF-α is among the earliest macrophage biomarkers produced in DSS colitis. It promotes secondary secretion of other pro-inflammatory cytokines, and is potently down-modulated by IL-10[25]. These features, together with the increased production of TNF-α in IL-10Rα$^{Mdel}$ colons, potentially implicate it in the exacerbated disease. To test this, we blocked its activity using anti-TNF-α Ab. Treatment reduced disease severity in both IL-10Rα$^{fl/fl}$ and IL-10Rα$^{Mdel}$ mice (Supp. fig. S8). However, the extent of this was similar in each line, and treated IL-10Rα$^{Mdel}$ mice still developed more severe disease than even untreated IL-10Rα$^{fl/fl}$ controls ($p<0.01$). Therefore, increased TNF-α production may play a role but is inadequate in itself to explain the heightened IL-10Rα$^{Mdel}$ disease.

## Nitric oxide modulation of IL-10Rα$^{Mdel}$ colitis

IL-10 potently inhibits iNOS, and thereby NO production. The integrated effects of NO's antimicrobial activity, toxic actions on the barrier, and cell signaling activity may alternatively promote or diminish colitis. In DSS colitis, excessive NO production worsens disease, though protective effects of NO have also been identified[26]. To assess iNOS activity, we sorted LPMφs from colitic mice and quantified iNOS mRNA. Levels were 4.7±0.8 fold higher in IL-10Rα$^{Mdel}$ compared with IL-10Rα$^{fl/fl}$ macrophages. Arginase, which inhibits iNOS by degrading NO's nitrogen source, was reciprocally though less strongly decreased (0.52±0.07 fold, Fig. 5a).

We also analyzed the accumulation of iNOS in homogenized LP cells from colitic mice using an assay for iNOS functional activity. This indicated a >2 folder increased activity in IL-10Rα$^{Mdel}$ than IL-10Rα$^{fl/fl}$ colons (Fig. 5b, 10.9±1.4 vs 4.8±0.6 μmol nitrite produced/μg protein).

To assess the impact of this NO, cohorts of IL-10Rα$^{Mdel}$ or IL-10Rα$^{fl/fl}$ mice were treated with aminoguanidine hydrochloride (AG), a selective iNOS inhibitor. Consistent with the mixed roles of NO in DSS colitis, treatment of IL-10Rα$^{fl/fl}$ mice led to only a mild and non-significant trend toward reduced disease severity (Fig. 5c). In contrast, a more substantial protective effect was apparent in IL-10Rα$^{Mdel}$ mice. Mean weight loss at disease peak in AG-treated IL-10Rα$^{Mdel}$ mice was 15±3% versus 24±4% for untreated mice ($p<0.05$). A similar pattern was observed when comparing bleeding scores and colon lengths for the different treatments (Fig. 5d and Supp. Fig. S9). There was a non-significant trend toward diminished bleeding in AG-treated versus untreated IL-10Rα$^{fl/fl}$ mice, however this proved significant and more substantial in IL-10Rα$^{Mdel}$ mice.

As an alternative approach to address the role of NO production, we selectively inhibited arginase with BEC. BEC increased peak weight loss in IL-10Rα$^{fl/fl}$ mice (17±3% treated versus 12±2% untreated, $p<0.05$, Fig. 5e). Further, bleeding scores in IL-10Rα$^{fl/fl}$ mice were significantly greater in the mice receiving BEC (Fig. 5f, $p<0.05$) than untreated controls. Therefore, arginase inhibition promotes disease in WT mice. In contrast, BEC did not significantly impact weight loss or bleeding score in IL-10Rα$^{Mdel}$ mice, though a non-significant trend toward increased bleeding was apparent. Colon length measurements showed similar trends (Supp. Fig. S9). Therefore arginase inhibition, which will increase NO production, preferentially promotes colitis in IL-10Rα$^{fl/fl}$ mice. In IL-10Rα$^{Mdel}$ mice, where NO production is already elevated and arginase diminished, an effect of further reduction in arginase activity is not detected. Cumulatively, these results indicate a role for elevated NO production in the aggravated colitis in IL-10Rα$^{Mdel}$ mice.

## Increased reactive oxygen generation in IL-10Rα$^{Mdel}$ colitis

ROS production is regulated by IL-10 and is implicated in colitis. Analysis of p47phox$^{-/-}$ mice demonstrated no difference from controls in DSS colitis severity. Nevertheless, as for NO, the role of ROS in colitis is multifaceted. ROS is important in mediating protection against bacteria entering the mucosa[27]. At the same time, excess production may be

damaging. Indeed, ROS production defects or anti-oxidant treatments can potentiate disease protection in some circumstances[26, 28].

We measured ROS production in LPMφs by staining with CM-H2DCFDA. ROS was undetectable in LPMφs from untreated mice (not shown). LPMφs from IL-10Rα$^{fl/fl}$ mice with colitis stained positively for ROS (Fig. 6a). However, IL-10Rα$^{Mdel}$ LPMφs displayed a significant increase in this (IL-10Rα$^{fl/fl}$ MFI=42.9±14.1, IL-10Rα$^{Mdel}$ MFI=134.0±24.4, $p<0.01$). This increase in ROS was specific for macrophages; LP-derived DCs, B cells, and T cells did not show this difference. Some splenic macrophages showed detectable ROS production, however quantities were substantially decreased compared to the LP and did not differ between IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice. Therefore, ROS production is markedly and selectively elevated in IL-10Rα$^{Mdel}$ LPMφs.

To clarify the role of the increased IL-10Rα$^{Mdel}$ ROS, we treated the mice with an ROS scavenger, NAC. NAC did not affect the weight loss or colon length in IL-10Rα$^{fl/fl}$ mice (Fig. 6b, c and Supp. Fig. S9). Comparison of treated and untreated mice did show a trend toward a decrease in bleeding scores, but this was not significant (Fig. 6f). The limited effect of NAC in WT mice was not unexpected considering the similar previously documented results with p47phox deficiency. However, in IL-10Rα$^{Mdel}$ mice, where ROS production is elevated, NAC led to a more substantial attenuation of disease. Maximal weight loss was decreased from 27±2% to 21±2% (Fig. 6b, d, $p<0.05$) A trend toward decreased bleeding score was seen, but as for IL-10Rα$^{fl/fl}$ mice was not significant (Fig. 6f). Therefore, anti-oxidant treatment shows greater effectiveness in IL-10Rα$^{Mdel}$ than IL-10Rα$^{fl/fl}$ mice.

NAC may also protect against reactive nitrogen species, such as peroxynitrites, formed by the reaction of ROS and NO. Therefore part of its activity may be secondary to its effects on NO-derived species. To determine if NAC's actions were still discernible after inhibiting iNOS, we treated mice with both AG and NAC (Fig. 6b–e). These demonstrated complementary effects. Dually treated control IL-10Rα$^{fl/fl}$ mice showed a limited improvement over untreated or NAC-only treated mice (Fig. 6b, c). Their bleeding scores were not significantly improved compared with mice treated with NAC alone but were compared with untreated animals (Fig. 6f). In contrast, IL-10Rα$^{Mdel}$ mice treated with both inhibitors showed markedly diminished weight loss, with a significant effect compared with NAC treatment by itself (Fig. 6b, d, e). Peak weight loss of treated IL-10Rα$^{Mdel}$ mice did not significantly differ from that of untreated IL-10Rα$^{fl/fl}$ controls and was only mildly more severe than similarly treated IL-10Rα$^{fl/fl}$ mice. Treated IL-10Rα$^{Mdel}$ bleeding scores did not significantly differ from untreated IL-10Rα$^{fl/fl}$ mice, though did remain elevated compared with NAC or NAC and AG treated IL-10Rα$^{fl/fl}$ controls (Fig. 6f). Therefore, dual inhibition of the ROS and iNOS pathways substantially alleviates the enhanced disease in IL-10Rα$^{Mdel}$ mice while more modestly affecting disease in IL-10Rα$^{fl/fl}$ controls.

## Discussion

Intestinal immune inflammatory and regulatory pathways exist in a highly dynamic balance, ensuring that inevitable disruptions in the mucosal barrier are repaired without undo inflammation or the development of a self-perpetuating colitic process. IL-10 plays a critical

role in this and governs IBD susceptibility. Macrophages may serve as a specific control point. MyD88 deletion in macrophages or DCs but not intestinal epithelial cells impacts spontaneous colitis in IL-10$^{-/-}$ mice[29]. Macrophage-specific deletion of Stat3, which signals downstream of multiple cytokines including IL-10[30], leads to chronic IBD. However, the cell types directly responsible for IL-10's protective effects in colitis have not been definitively resolved.

We did not identify a role for non-hematopoietic IL-10Rα expression in IL-10 mediated colitis protection. Likewise, IL-10Rα$^{Tdel}$, IL-10Rα$^{Bdel}$, and IL-10Rα$^{DCdel}$ mice developed DSS colitis with a kinetics and severity identical to WT controls. In contrast, IL-10Rα$^{Mdel}$ mice manifested more severe disease with increased mortality. This was comparable to that of mice wholly deficient in IL-10 or IL-10Rα. Granulocyte depletion further implicated macrophages as the primary cellular target for IL-10 after mucosal breech with DSS. Histopathologic changes in IL-10Rα$^{Mdel}$ mice were consistent with an increase in disease magnitude compared with WT controls, but not an altered disease quality.

The absence of a T-specific IL-10 effect is notable considering the documented effect of IL-10 in Treg maintenance and disease severity in colitis induced by T cell transfer into Rag$^{-/-}$ mice[31]. Similarly, T cell response to IL-10 has been implicated in the immunoregulation of intestinal inflammation after αCD3 treatment[32]. Contrasting with these models, T cells appear to have a limited involvement in DSS colitis, reflecting the acute toxic influence of DSS on the colonic barrier and subsequent innate inflammatory response. Although changes in the T cell compartment are evident after DSS treatment, this lineage is not essential for the colitis which may be comparably induced in Rag$^{-/-}$ mice and immunoreplete mice[33, 34].

It is also notable that IL-10Rα$^{DCdel}$ mice did not develop exacerbated DSS colitis. Many macrophages identified in the colon during DSS colitis demonstrated a CD11c$^{dim}$ immunophenotype (Suppl. Fig. S5). Further, sorted CD11c$^{-}$ and CD11c$^{dim}$ LPMφs from IL-10Rα$^{Mdel}$ mice both showed elevated levels of iNOS, TNFα, and IL-1β relative to controls (data not shown). This suggests that both CD11c$^{-}$ and CD11c$^{dim}$ populations contribute to the increased IL-10Rα$^{Mdel}$ disease severity. The time course for disease in DSS colitis is highly abbreviated. One possible explanation for the lack of a CD11c-Cre effect is that as monocytes enter the colon, mature into LPMφ, and some upregulate CD11c, there is insufficient time to induce Cre and delete the IL-10Rα gene, and for pre-existing expressed IL-10Rα protein to be depleted. Further comparisons of the IL-10Rα$^{DCdel}$ and IL-10Rα$^{Mdel}$ mice are, however, warranted to clarify the mechanism(s) underlying the distinct impacts of these different Cre transgenes.

LPMφs are activated in all commonly studied colitis models[31, 35, 36], and we further assessed how their inability to respond to IL-10 is linked to exacerbated colitis. DSS disrupts the mucosal barrier. Defective epithelial regeneration may aggravate colitis[21], though was not observed here.

IL-10 suppresses macrophage pro-inflammatory cytokine production, and colitic IL-10Rα$^{Mdel}$ LPMφs produced more IL-1β and TNF-α than controls, though IL-10 itself

was unaltered. The T cell response is not essential to DSS colitis[34], and in pilot studies we found no differences in IFN-γ, IL-17 and IL-23 production by qRT-PCR (data not shown). Although pro-inflammatory cytokines are broadly elevated in IL-10Rα$^{Mdel}$ colitis, we focused on TNF-α, due its direct cytopathic effects and prominent regulation by IL-10 in macrophage. TNF-α inhibition proved protective in both IL-10Rα$^{Mdel}$ mice and controls, but to a similar extent, and disease in treated IL-10Rα$^{Mdel}$ mice remained more severe than in even untreated IL-10Rα$^{fl/fl}$ controls. Therefore, though TNF-α plays a role in colitis development, it cannot in itself explain the increased IL-10Rα$^{Mdel}$ disease susceptibility.

We did not observe differences in macrophage numbers, phenotype, or segregation into SSC$^{hi}$ and SSC$^{lo}$ LPMφ1 and LPMφ2 populations. Likewise, mixed chimeras demonstrated that IL-10Rα$^{Mdel}$ macrophages do not outcompete WT macrophages during colitis development, indicating that IL-10 is not impacting cellular localization, migration, or expansion. However, IL-10Rα$^{Mdel}$ macrophages showed markedly elevated NO and ROS production, which are important for clearing bacteria that traverse the disrupted mucosal barrier[37, 38], though in excess may also mediate direct tissue damage[26, 28, 39–41].

Importantly, inhibition of either NO or ROS led to no or modest effects on colitis severity in WT (IL-10Rα$^{fl/fl}$) mice. This is consistent with these agents' mixed protective and pathologic functions. In contrast, colitis was more substantially alleviated by their inhibition in IL-10Rα$^{Mdel}$ mice. Indeed, weight loss in NAC/AG treated IL-10Rα$^{Mdel}$ was only mildly increased compared with similarly treated WT controls, indicating that inhibition of these pathways converts the more extreme disease in IL-10Rα$^{Mdel}$ mice to one similar to that of WT mice. Limited studies have been performed in DSS colitis to identify immunopathologic mechanisms of ROS and NO, and it will be important in the future to further clarify how IL-10 impacts the effects of these molecules. Nevertheless, our results are consistent with a model in which intestinal IL-10 acts to downregulate macrophage NO and ROS production after barrier insult. In the absence of adequate IL-10 signaling, damage produced by these mediators amplifies the toxic insult from DSS treatment and aggravates disease. The limited impact of NO and ROS inhibition in WT mice implies that IL-10 normally reduces these compounds to a level where their direct toxic effects are roughly balanced by their protective functions.

In summary, we demonstrate an indispensable and dominant role for macrophage IL-10 responsiveness in IL-10's protective effects in colitis development. We further demonstrate that IL-10 does not alter the competitive fitness of macrophages themselves, but rather impairs their effector functions, and most particularly their excessive production of pathologic reactive oxygen and nitrogen species.

## Materials and Methods

### Mice

IL-10Rα$^{fl/fl}$ mice were generated on a C57BL/6 background as described[18], and bred with B6.129P2-Lyzs$^{tm1(cre)Ifo}$/J (Lys-cre, Jackson), B6.Cg-Tg(Cd4-Cre)1Cwi*/BfluJ* (CD4-cre, gift of H. Chi), CD11c-cre (gift of H. Chi), B6.129P2-CD19$^{tm1(cre)Cgn}$/J (CD19-cre, Jackson); and B6.C-Tg(CMV-cre)1Cgn/J (CMV-cre, Jackson). B6.129P2-IL-10$^{tm1Cgn}$/J

mice were obtained from The Jackson Laboratories. Mice were maintained under SPF conditions negative for detectable *Helicobacter spp*. Experimental protocols were approved by the St. Jude Animal Care and Use Committee.

### Induction of colitis and clinical scoring

Dextran sodium sulfate (DSS, m.w. 40,000; ICN Biomedicals) was administered ad libitum in the distilled water at 3% concentration or as indicated for 5 d followed by normal drinking water. For inhibition experiments, N-acetyl-L-cysteine (NAC, 100 mg/kg, Sigma), aminoguanidine hydrochloride (AG, 100 mg/kg, Calbiochem), or *S*-(2-boronoethyl)-l-cysteine (BEC, 20 mg/kg, Sigma) was administered i.p. Neutrophils were depleted using anti-Ly6G MAb 1A8 (Bio X Cell). 1 mg antibody per mouse was administered i.p. 1 d before DSS treatment. Depletion was confirmed by flow cytometry. Body weight and gross blood were analyzed on a daily basis[42]. Bleeding scores were: 0, hemoccult negative (Beckman Coulter), 1, hemoccult positive, 2, blood traces in stool, 3, gross rectal bleeding.

### Histology

Colons (d 7) 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 in samples were directly measured by Luminex (Bio-Rad) or ELISA (R&D Systems).

### LP cell isolation

Lamina propria (LP) cells were isolated using a modification of a previously described protocol [43]. Briefly, colon segments were twice vigorously shaken 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 DNase I (Roche) with agitation. Cells were filtered, washed, and isolated over a percoll step gradient.

### Bone marrow chimeras

Chimeras were produced as previously described[44]. Briefly, ~$5\times10^6$ donor bone marrow cells were transplanted into lethally irradiated C57BL/6J recipients. Reconstitution was

verified after 4 wk by staining peripheral blood for the transplanted cells. Colitis was induced at 8 wk.

### Intestinal permeability

Epithelial barrier permeability was assessed using FITC-labeled dextran as described[21]. Briefly, mice were gavaged with FITC-dextran (Sigma-Aldrich, 1 g/ kg) on d 7. After 6 h, blood was collected and the plasma FITC-dextran quantified by fluorescence spectrophotometry.

### Epithelial cell proliferation

Proliferating intestinal epithelial cells were quantified as described[45]. Briefly, BrdU (20 mg/kg) was administered i.p. to mice with colitis (d 7) or untreated mice. After 2 h, colons were removed and cells incorporating BrdU quantified by immunohistochemistry (IHC). The average number of BrdU-positive epithelial cells per intact and well-oriented crypt was determined (minimum of 50 crypts assessed per mouse).

### Cyotkine PCR

Total RNA was isolated from sorted LPMφ 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[46]. Primer sequences are: TGF-β: F, CACAGTACAGCAAGGTCCTTGC; R, AGTAGACGATGGGCAGTGGCT; IL-12p35: F, ATGACCCTGTGCCTTGGTAG; R, GATTCTGAAGTGCTGCGTTG; IL-23p19: F, AGCGGGACATATGAATCTACTAAGAGA; R, GTCCTAGTAGGGAGGTGTGAAGTT; IL-12p40: F, GACCATCACTGTCAAAGAGTTTCTAGAT; R, AGGAAAGTCTTGTTTTTGAAATTTTTTAA; IL-10: F, GTGAAAATAAGAGCAAGGCAGTG; R, ATTCATGGCCTTGTAGACACC; TNF-α: F, AATGGCCTCCCTCTCATCAGT; R, CTACAGGCTTGTCACTCGAA; iNOS: F, TGACGGCAAACATGACTTCAG; R, GCCATCGGGCATCTGGTA; IL-6: F, TATGAAGTTCCTCTCTGCAAGAGA; R, TAGGGAAGGCCGTGGTT; Arginase: F, TCACTTTCCACCACCTCTTGA; R, TCTCCACCGCCTCACGACTC; HPRT: F, GACCGGTCCCGTCATGC; R, TCATAACCTGGTTCATCATCGC. F, forward primer; R, reverse primer.

### Flow cytometry

LPMφs were stained with mAbs against mouse F4/80, CD11b, CD11c, CD40, CD80, CD86, MHC class II, CD103, TLR2, CD45.1, CD45.2, Ly6G, Siglec-F or with isotype-matched control Abs (BD Pharmingen or eBiosciences), and analyzed using a FACSCalibur or LSRII flow cytometer with Cell Quest (BD Biosciences) or Flowjo (TreeStar) software.

### NOS activity

LP cells were isolated, homogenized in 200 μl lysis buffer (80 mM sodium phosphate, pH 6, containing 0.5% hexadecyltrimethyl ammonium bromide, Sigma-Aldrich) for 60 s, centrifuged at 14,000 × g for 15 minutes, and supernatant protein determined by Bradford

assay (Bio-Rad). Nitric oxide synthase (NOS) activity was measured by an ultrasensitive colorimetric assay (Oxford Biomedical Research, cat. #NB 78) per manufacturer's instructions.

### ROS staining

LP cells were stained for surface markers, incubated for 30 min at 37°C with 10 μM CM-H2DCFDA (Invitrogen) and analyzed by flow cytometry[47].

### Statistics

Statistics were calculated using Prism5 (GraphPad Software). Group comparisons were by 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 |
| **DSS** | dextran sodium sulfate |
| **IL-10R** | interleukin 10 receptor |
| **ROS** | reactive oxygen species |
| **NO** | nitric oxide |
| **LPMφ** | lamina propria macrophage |

| | |
|---|---|
| **WT** | wild type |
| **AG** | aminoguanidine hydrochloride |
| **BEC** | *S*-(2-boronoethyl)-l-cysteine |
| **NAC** | N-acetyl-L-cysteine |
| **TNF** | tumor necrosis factor |

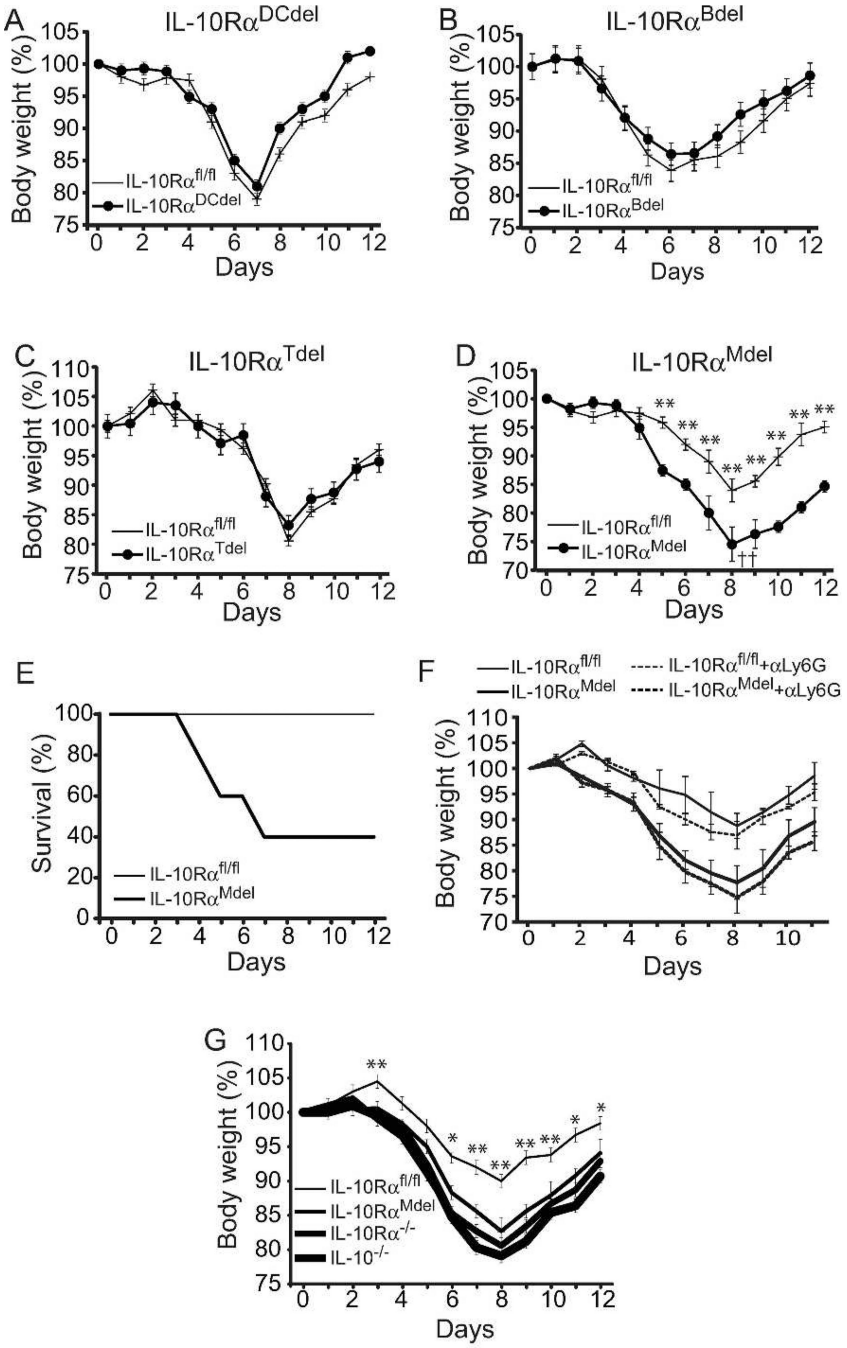


**Figure 1. Macrophage IL-10Rα expression protects mice from DSS-induced colitis**

IL-10Rα$^{DCdel}$ (A), IL-10Rα$^{Bdel}$ (B), IL-10Rα$^{Tdel}$ (C), and IL-10Rα$^{Mdel}$ (D) mice or littermate Cre$^{-}$ (IL-10Rα$^{fl/fl}$) controls (n=10/cohort) received 3% DSS solution in drinking water ad libitum for 5 d. Mean ± s.e.m. percent of initial body weight is plotted. **, $p<0.01$; †, death event. (E) IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice (n=10/cohort) were treated with 4% DSS for 5 d and survival monitored. (F) IL-10Rα$^{Mdel}$ mice and IL-10Rα$^{fl/fl}$ controls (n=10/cohort) were depleted of neutrophils with 1A8 antibody or received control rat IgG 1 d prior to 3% DSS administration. Data are representative of three independent experiments. (G)

IL-10$^{-/-}$, IL-10Rα$^{-/-}$, and IL-10Rα$^{fl/fl}$ mice were treated with 3% DSS for 5 d and body weight monitored. Data are representative of three independent experiments. *, $p<0.05$; **, $p<0.01$ for IL-10Rα$^{Mdel}$ vs IL-10Rα$^{fl/fl}$. Significant differences between IL-10Rα$^{Mdel}$, IL-10Rα$^{-/-}$, and IL-10$^{-/-}$ cohorts were not seen.

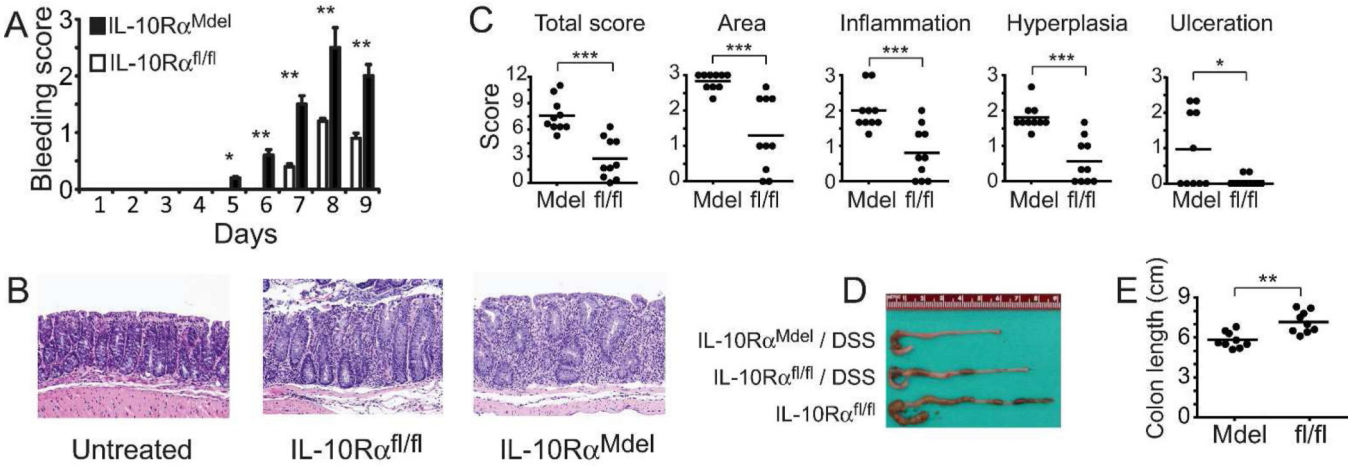


**Figure 2. DSS-induced colitis in IL-10Rα$^{Mdel}$ mice**

(A) IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice received 3% DSS for 5 d, and rectal bleeding was scored daily. (B, C) Representative photomicrographs and tallied scores for disease parameters from H&E stained colon sections obtained 7 d after initiating DSS treatment. Scoring for individual parameters is scaled from 0–3 (0–12 total) and criteria are listed under Methods. Mean values for individual mice (circles) and cohorts (lines) are plotted; (D, E) Colons were removed at d 7 and colon length measured. Individual mice (circles) and cohort means (lines) are plotted. *, P<0.05; **, p<0.01, ***, p<0.001.

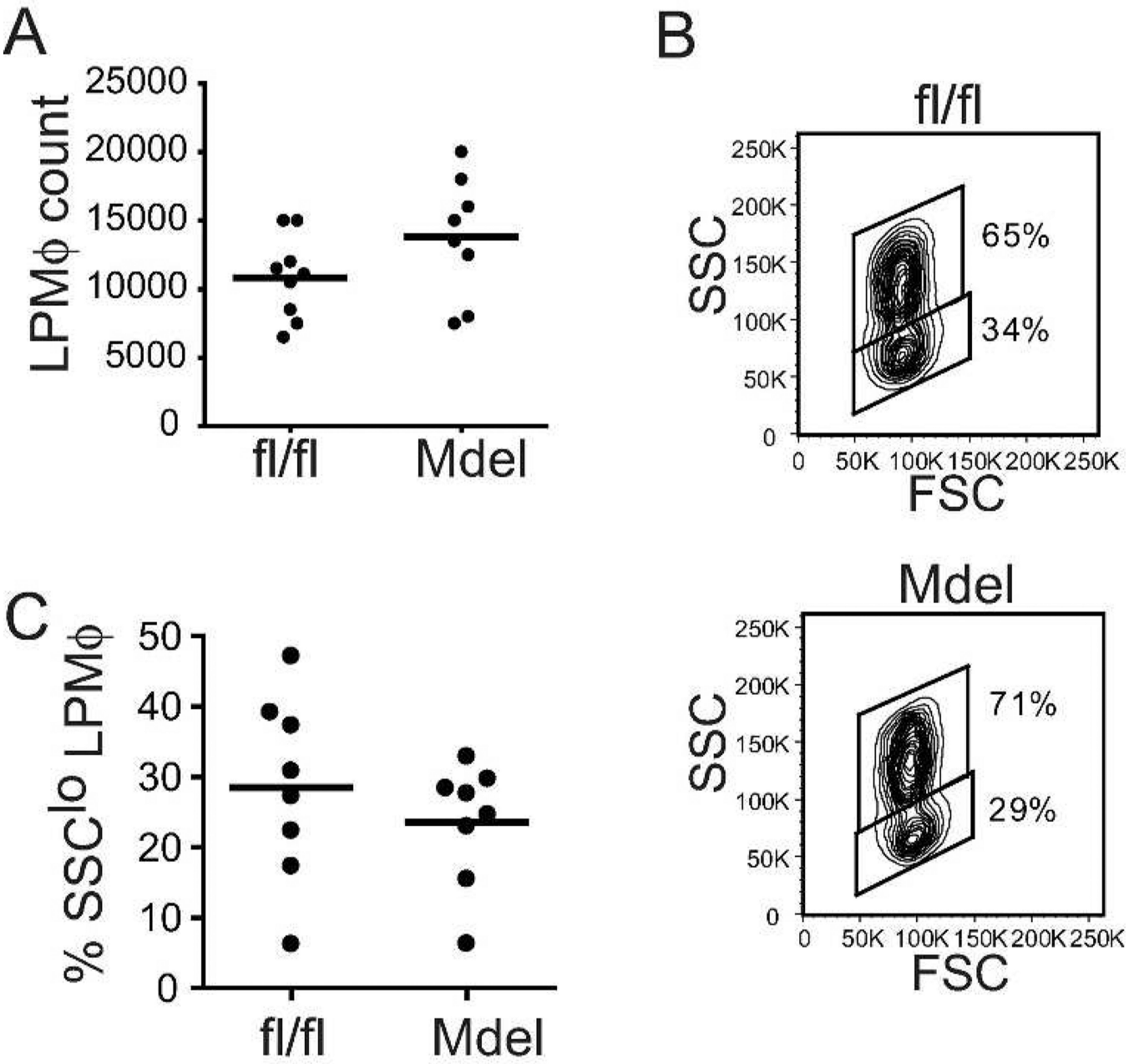


**Figure 3. Analysis of lamina propria macrophages**

(A) Cells were isolated from large intestine lamina propria of IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice on d 7 after colitis induction. Absolute numbers of macrophages (CD11b$^{+}$F4/80$^{+}$Ly6G$^{lo/-}$CD11c$^{-/dim}$) were calculated. (B and C) CD11b$^{+}$F4/80$^{+}$Ly6G$^{lo/-}$CD11c$^{-/dim}$ SSC$^{hi}$ and SSC$^{lo}$ LPMφs were distinguished by flow cytometry (B). The proportion of SSC$^{lo}$ cells of total (SSC$^{hi}$ + SSC$^{lo}$) LPMφs is plotted (C). Results from individual mice (circles) and population means (lines) are plotted. Differences are not significant.

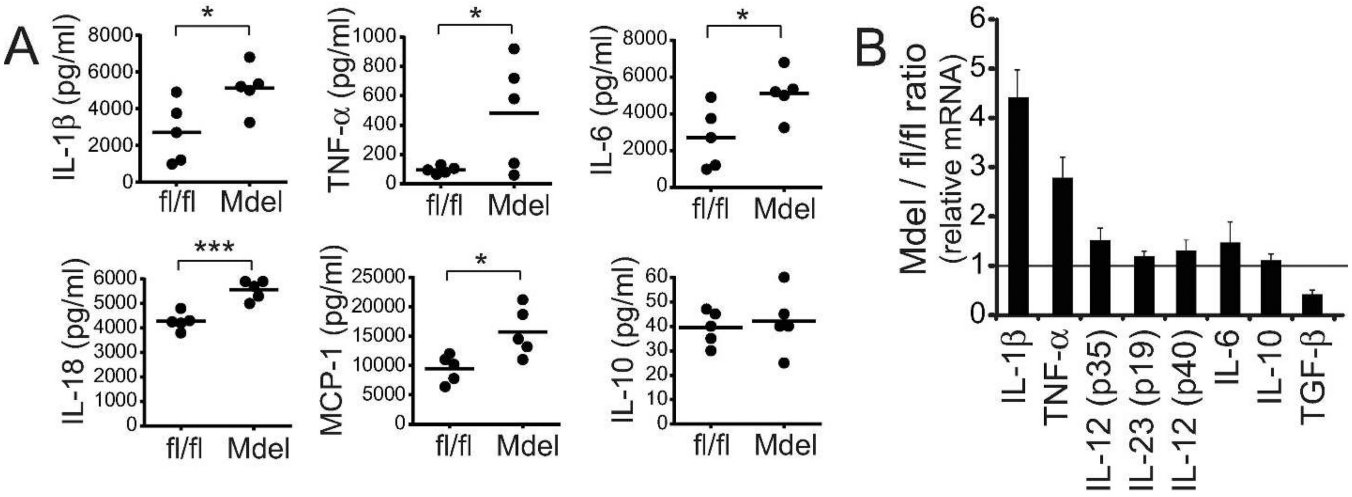


**Figure 4. Competitiveness and inflammatory cytokine production by IL-10Rα-deficient macrophages**

(A) Colons from IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice, 7 d after colitis induction, were homogenized and cytokine content measured by ELISA or multiplex assay. Results from individual mice (circles) and cohort means (lines) are plotted. (B) Relative expression levels (mean + 1 s.d.) of the indicated mRNAs from macrophages sorted from colon tissue was measured by qRT-PCR. *, $p<0.05$; ***. $p<0.001$. Data are representative of three independent experiments.

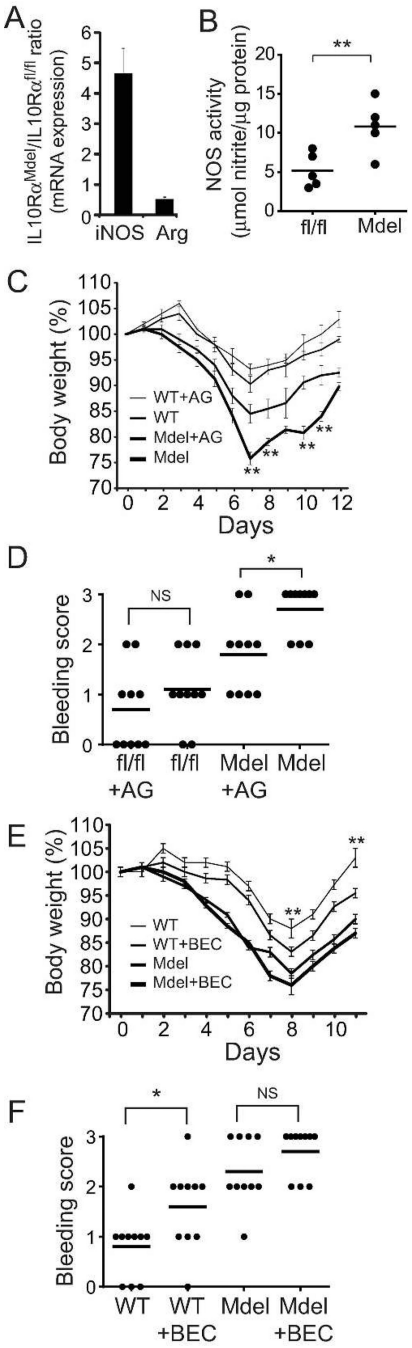


**Figure 5. Role of NO and arginase in colitis exacerbation**

(A) Macrophages were sorted from colons of mice with colitis (d 7) and iNOS and arginase expression were measured by qRT-PCR. The ratio of expression in IL-10Rα$^{Mdel}$ to IL-10Rα$^{fl/fl}$ was measured. Mean + 1 s.d. is plotted. (B) Colon tissue from mice with colitis (d 7) was homogenized and tissue NOS activity measured using a colorimetric assay. Results from individual mice (circles) and cohort means (lines) are shown. (C) Colitis was induced in IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice with 3% DSS. AG or saline was administered i.p. Mean±1 s.e.m. weight change from d 0 is plotted (n=10/cohort). (D) Rectal bleeding

scores measured on d 7 after colitis induction. (E, F) Analyses are similar to (C, D) except IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice (n=10/cohort) were treated with BEC or saline by i.p. injection. *; $p < 0.05$, **, $p < 0.01$. For experiments C-F, statistical significance is only shown comparing drug treated and untreated IL-10Rα$^{Mdel}$ or IL-10Rα$^{fl/fl}$ mice. Significance levels between IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ cohorts are not shown. Data are representative of two independent experiments.

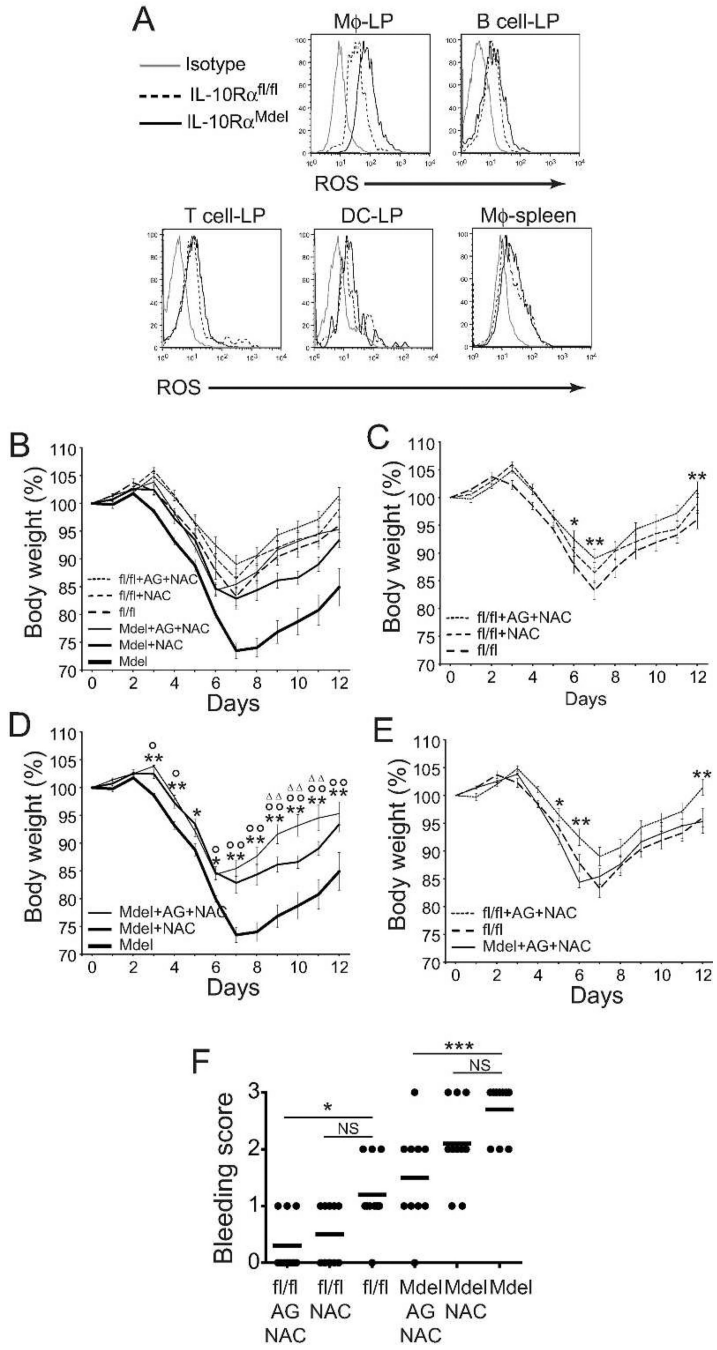


**Figure 6. Role of ROS in colitis exacerbation**

(A) On d 7 after colitis induction, gated T cells, B cells, DCs, and macrophages were analyzed by flow cytometry in the LP or spleen as indicated. Gray line, isotype control staining of IL-10Rα$^{Mdel}$ cells; dashed black line, IL-10Rα$^{fl/fl}$ cells; Solid black line, IL-10Rα$^{Mdel}$ cells. (B-E) Colitis was induced with 3% DSS in IL-10Rα$^{Mdel}$ and IL-10Rα$^{fl/fl}$ mice that were treated with NAC with or without AG or saline i.p. Mean ± 1 s.e.m. weight change is measured. Plots B-E show results for all cohorts, IL-10Rα$^{fl/fl}$ cohorts, IL-10Rα$^{Mdel}$ cohorts, and a comparison of NAC+AG treated IL-10Rα$^{Mdel}$ with untreated or

NAC+AG treated IL-10Rα$^{fl/fl}$ cohorts respectively. *, p< 0.05; **, p<0.01 comparison of AG+NAC vs untreated mice in (C-D), and AG+NAC treated IL-10Rα$^{fl/fl}$ vs IL-10Rα$^{Mdel}$ cohorts in (E). °, p<0.05; °°, p<0.01 for NAC vs untreated cohorts in (C-D). (F) Rectal bleeding scores on d 7 after DSS treatment. *, p < 0.05; **, p < 0.01; ***, p<0.001; NS, not significant. Comparisons are only shown for treated vs. untreated IL-10Rα$^{fl/fl}$ or IL-10Rα$^{Mdel}$ cohorts and not between IL-10Rα$^{fl/fl}$ and IL-10Rα$^{Mdel}$ mice. Data are representative of two independent experiments.