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IL-10 producing intestinal macrophages prevent excessive anti-bacterial innate immunity by limiting IL-23 synthesis

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Abstract

Innate immune responses are regulated in the intestine to prevent excessive inflammation. Here we show that a subset of mouse colonic macrophages constitutively produce the anti-inflammatory cytokine IL-10. In mice infected with *Citrobacter rodentium*, a model for enteropathogenic *Escherichia coli* infection in humans, these macrophages are required to prevent intestinal pathology. IL-23 is significantly increased in infected mice with a myeloid cell-specific deletion of IL-10, and the addition of IL-10 reduces IL-23 production by intestinal macrophages. Furthermore, blockade of IL-23 leads to reduced mortality in the context of macrophage IL-10 deficiency. Transcriptome and other analyses indicate that IL-10-expressing macrophages receive an autocrine IL-10 signal. Interestingly, only transfer of the IL-10 positive macrophages could rescue IL-10 deficient infected mice. Therefore, these data indicate a pivotal role for intestinal

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AUTHOR CONTRIBUTIONS

M.M. and M.K. designed experiments. P.K., V.M., Y.P., U.B., Z.M., T.M. and M.M. performed experiments. M.M., P.K., J.G. and B.P. performed the bioinformatics analysis, J.S., G.K., H.C., Y.C.L. and B.P. helped with critical advice and discussion throughout. P.K., M.K. and M.M. wrote the manuscript.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interest.

macrophages that constitutively produce IL-10, in controlling excessive innate immune activation and preventing tissue damage after an acute bacterial infection.

Introduction

Interleukin 10 (IL-10) is an immunoregulatory cytokine that limits mucosal immune responses and minimizes immunopathology. Indeed, mutations in the IL-10 receptor gene (*Il10r*) were found in some patients with early-onset colitis^{1, 2}. Similarly, mice deficient for IL-10 (*Il10^{-/-}*) or IL-10R (*Il10rb^{-/-}*) developed spontaneous inflammation of the large intestine, a process that was T cell dependent and dominated by a pathogenic T helper type 1 and type 17 (Th1 and Th17) immune responses^{3, 4, 5}.

A number of cell types can produce IL-10, including lymphocytes, monocytes, macrophages, mast cells, keratinocytes, and intestinal epithelial cells. In several colitis models, the role of T cell-derived IL-10 has proven to be central^{6, 7, 8}, and in fact, mice with a conditional deletion of IL-10 in the CD4⁺ T cell subset develop spontaneous inflammation of the intestine, as do those with a deletion in regulatory T cells (Treg) mediated by a Foxp3 driven Cre^{9, 10}. However, we found IL-10 from macrophages plays an indispensable role in the maintenance of Foxp3 expression by regulatory T cells (Treg) and their function in mice with colitis¹¹. Despite this, mice with a conditional deletion of IL-10 or IL-10R in myeloid cell subsets^{12, 13}, or of IL-10 specifically in macrophages¹⁴, did not spontaneously develop aggressive colitis, although they were susceptible to excessive inflammation following systemic LPS exposure^{12, 13}. These data suggest that myeloid cell derived IL-10 might have a pivotal role in controlling mucosal immune responses after bacterial infection.

In the context of acute mucosal infections, the lack of IL-10 could be protective because of an enhanced inflammatory response with increased IL-12, TNF and other cytokines^{15, 16, 17, 18, 19, 20}. However, the absence of IL-10 also could lead to excessive inflammation^{17, 21, 22, 23}. Therefore, in the context of acute bacterial infection of the intestine, it remains to be determined which cell type(s) produce IL-10, if IL-10 is protective or harmful, and which pathways are activated or inhibited by IL-10 secretion. To explore these issues, we have analyzed mice with cell type-specific deletion of *Il10* that were infected with *Citrobacter rodentium* (*C. rodentium*). This is a gram-negative bacterium that is considered a model for enteropathogenic and enterohemorrhagic *E. coli* infections in humans. *C. rodentium* causes attaching and effacing mechanism of epithelial infection, leading to intestinal inflammation and diarrhea. The bacteria normally are cleared in wild type mice due to the actions of innate and adaptive immunity and the intestinal inflammation ultimately resolves^{24, 25, 26}. Here we show that a unique subset of macrophages in the colonic lamina propria that constitutively produces IL-10 plays a critical role in preventing excessive inflammation following acute bacterial infection by limiting innate immunity, and that a major pathway by which IL-10 acts is through controlling IL-23 production.

Results

Myeloid cell IL-10 is important for survival from *Citrobacter rodentium*

To assess the function of IL-10 in regulating mucosal immune responses after intestinal bacterial challenge, we used *Il10*^{-/-}, *Il10rb*^{-/-} and wild type (wt) recipient mice that were infected with a sublethal dose of *C. rodentium* by oral gavage. The onset and severity of colitis in *Il10*^{-/-} mice is strongly influenced by the genetic background³, and we used mice on the C57BL6/J background that are more protected from inflammation under steady state conditions. Indeed, none of the groups developed spontaneous colitis in our mouse colony (Supplementary Fig. 1). However, we found that all *Il10*^{-/-} and *Il10rb*^{-/-} mice died 7 to 12 days after infection, whereas all wt mice survived (Fig. 1a). At day 6 after infection, *Il10*^{-/-} and *Il10rb*^{-/-} mice showed severe colonic inflammation, characterized by epithelial cell destruction, infiltration of mononuclear cells (Supplementary Fig. 1). These indicate that the role of IL-10 in modulating the initial mucosal immune response against *C. rodentium* is indispensable for survival and mucosal damage.

To identify which cell type(s) produce the IL-10 that is essential for host protection, we analyzed mice with cell type-specific deletions of the *Il10* gene. These included *Il10*^{flx/flx}*LysM-Cre*, with deletion targeted primarily to CD11b⁺ myeloid cells, including macrophages, monocytes and neutrophils, *Il10*^{flx/flx}*Cd4-Cre* targeting mostly CD4⁺ T cells, and *Il10*^{flx/flx}*Cd11c-Cre* mice, which acts predominantly in dendritic cells (DC). We used *Il10*^{flx/flx}*Cre*⁻ (*Il10*^{flx/flx}) littermates for controls. We found that *Il10*^{flx/flx}*LysM-Cre* mice started to die early after *C. rodentium* infection, with less than half surviving beyond day seven. In contrast, most of the *Il10*^{flx/flx}*Cd11c-Cre* and all of the *Il10*^{flx/flx}*Cd4-Cre* recipients survived to 21 days after infection (Fig. 1b). The average histology scores of each group were similar before *C. rodentium* infection. With the dose of bacteria we used, at day 6 we observed mild inflammation in *Il10*^{flx/flx}*Cd4-Cre* and *Il10*^{flx/flx} recipients, manifested by epithelial cell hyperplasia and some cellular infiltration. However, *Il10*^{flx/flx}*LysM-Cre* recipients, most of which survived to day 6, developed significantly more severe colonic inflammation, characterized by epithelial cell destruction, infiltration of mononuclear cells and submucosal edema in the large intestine (Fig. 1c and d). In parallel with this, we found increased colonic epithelial permeability and bacterial dissemination to the spleen after infection in *Il10*^{flx/flx}*LysM-Cre* recipients (Fig. 1e and f). Despite this, bacterial numbers in the feces of *Il10*^{flx/flx}*LysM-Cre* recipients at day 6 after bacterial challenge were similar compared to *Il10*^{flx/flx}*Cd4-Cre* and *Il10*^{flx/flx} mice (Fig. 1g). These data suggest intestinal epithelial cell destruction by excessive mucosal immune responses after infection in *Il10*^{flx/flx}*LysM-Cre* recipients probably contributed to bacterial dissemination and lethality.

Macrophages are the principal IL-10 producing cell

We assessed the effectiveness and specificity of the deletion of *Il10* in the various cell populations by measuring the decrease in *Il10* mRNA at steady state. Indeed, we detected a decrease of approximately 90% in *Il10* mRNA transcripts in the CD11c^{int}CD11b⁺ macrophage population from the colon of *Il10*^{flx/flx}*LysM-Cre* mice compared to *Il10*^{flx/flx} controls, with little decrease in CD11c⁺CD11b⁻ cells or CD4⁺ T cells

(Supplementary Fig. 2). Similarly, we detected a specific and nearly complete loss of *Il10* transcripts in CD4⁺ T cells in *Il10^{flox/flox}Cd4-Cre* mice and a similar decrease in the CD11c⁺CD11b⁻ population in *Il10^{flox/flox}Cd11c-Cre* mice. There also was a 15–20% decrease in *Il10* mRNA in the CD11c^{int}CD11b⁺ population in *Il10^{flox/flox}Cd11c-Cre* mice (Supplementary Fig. 2). The intermediate level of CD11c expression by macrophages in the CD11c^{int}CD11b⁺ population likely is responsible for the incomplete disruption of IL-10 expression and the preservation of protective function in the *Il10^{flox/flox}Cd11c-Cre* strain. Therefore, we conclude that the absence of a strong effect on survival and inflammation in the *Il10^{flox/flox}Cd4-Cre* and *Il10^{flox/flox}Cd11c-Cre* strains is not due to inefficient removal of the *Il10* gene.

Because the *LysM* promoter drives Cre recombinase expression in macrophages, monocytes and neutrophils, we determined which of these cell types produced IL-10 after infection. Consistent with a recent report²⁰, IL-10 mRNA was most highly expressed in CD11b⁺CD11c^{int} macrophages, which also express F4/80 and MHC class II, but was undetectable in granulocytes (Fig. 1h) in the large intestine. Therefore, the reduced survival in *Il10^{flox/flox}LysM-Cre* mice is very likely due to the loss of IL-10 production by macrophages.

A significant proportion of macrophages were IL-10 competent, GFP⁺ cells in the large intestine before infection. By flow cytometry, these cells were more frequent in the colon compared to the small intestine (Supplementary Fig. 3a). When analyzed by immunohistology, both the GFP⁺ (IL-10⁺) and GFP⁻ (IL-10⁻) macrophages were scattered from the top of villi to the crypt in both small and large intestine (Supplementary Fig. 3b). This population is maintained through day 6 of infection (Supplementary Fig. 4), although the frequency tended to decrease.

To confirm that IL-10 producing macrophages regulate mucosal innate immune responses independently of adaptive immunity, we analyzed intestinal inflammation triggered by *C. rodentium* using *Rag^{-/-}* and *Il10^{-/-}Rag^{-/-}* mice. *Il10^{-/-}Rag^{-/-}* mice had more severe mortality and severe colonic inflammation at day 6 after infection compared to *Rag^{-/-}* mice (Supplementary Fig. 5). Together, these data suggest that macrophage derived IL-10 plays a nonredundant role in limiting the innate mucosal immune responses against intestinal infection with *C. rodentium*.

Macrophage IL-10 regulates myeloid cell recruitment to the colon

To determine if macrophage derived IL-10 regulates inflammatory cell recruitment to the large intestine after *C. rodentium* infection, we examined the kinetics of cell accumulation in the colon lamina propria in *Il10^{flox/flox}LysM-Cre*, *Il10^{flox/flox}Cd4-Cre* and *Il10^{flox/flox}* recipients during *C. rodentium* infection, and we characterized the infiltrating cell types by flow cytometric analysis. The total cell number in the colon lamina propria was comparable in all groups before *C. rodentium* injection, and CD11b⁺Ly6G⁻Ly6C^{high} monocytes were undetectable (Fig. 2a). Six days after infection, we detected a significant increase in total cell numbers in *Il10^{flox/flox}LysM-Cre* mice compared to *Il10^{flox/flox}Cd4-Cre* and *Il10^{flox/flox}* recipients, and the population in the large intestine lamina propria consisted mainly of CD11b⁺Ly6G⁻Ly6C^{high} monocytes and CD11b⁺Ly6G^{high}Ly6C^{low} granulocytes (Fig. 2a).

There was no increase in CD4⁺ T cells in any of these groups at this early time point (Fig. 2a).

Because myeloid cell infiltrates were increased in infected *Il10^{fllox/fllox}LysM-Cre* mice, we tested if chemokines that would attract these cells were also increased. Because *Il10^{fllox/fllox}Cd4-Cre* and *Il10^{fllox/fllox}* recipients were similar in survival and degree of inflammation, we compared *Il10^{fllox/fllox}LysM-Cre* mice to *Il10^{fllox/fllox}Cd4-Cre* mice. Although CCL2 and CXCL1, known to recruit monocytes and granulocytes, respectively, were similar between *Il10^{fllox/fllox}LysM-Cre* mice and *Il10^{fllox/fllox}Cd4-Cre* mice (Fig. 2b), other chemokines that recruit monocytes and neutrophils, including CCL3, CCL4 and CCL5, were significantly increased in colon fragment culture supernatants from *Il10^{fllox/fllox}LysM-Cre* mice. This was true even before *C. rodentium* infection (Fig. 2b), although this increased level before infection apparently was not sufficiently high to recruit monocytes and granulocytes and cause detectable inflammation. These data suggest that macrophage-derived IL-10 regulates the chemokine dependent recruitment of monocytes and granulocytes to the large intestine during intestinal bacterial infection.

Increased pro-inflammatory cytokines in *Il10^{fllox/fllox}LysM-Cre* mice

We examined if the increased colitis in *C. rodentium* infected *Il10^{fllox/fllox}LysM-Cre* mice was associated with excessive production of pro-inflammatory cytokines using a Bio-Plex multi-cytokine assay. As illustrated (Fig. 3), IL-10 production was reduced before infection and early after infection in *Il10^{fllox/fllox}LysM-Cre* compared to *Il10^{fllox/fllox}Cd4-Cre* mice, suggesting that the majority of IL-10 in the colon under these conditions is produced by macrophages. Conversely, the production of TNF and IL-12p40 was increased in *Il10^{fllox/fllox}LysM-Cre* recipients in colon fragment cultures before, and more strongly during infection, while IL-6 and IFN- γ levels were comparable. We used a different method, ELISA, to explore expression of IL-12 family cytokines further. We detected a striking increase in IL-23p19 by day 6 after infection in *Il10^{fllox/fllox}LysM-Cre* mice. However neither IL-23 nor IL-12p70 were significantly increased in *Il10^{fllox/fllox}LysM-Cre* before infection, suggesting that IL-12p40 monomers or dimers, known to be produced in other contexts^{27, 28}, likely were produced in the intestine under lack of macrophage derived IL-10, although the function of IL-12p40 homodimers is uncertain.

It has been reported that IL-23 induces IL-22 production from innate lymphoid cells^{29, 30}. Indeed, consistent with the significant increase in IL-23 by day six after infection, in colonic fragment cultures from infected *Il10^{fllox/fllox}LysM-Cre* mice, we detected a profound increase in IL-22 production as well (Fig. 3).

Macrophage IL-10 influences mortality through regulation of IL-23p19

We assessed if IL-10 acts on the IL-23p19 producing cells activated by *C. rodentium*. It has been reported IL-23 is produced by macrophages and DCs^{29, 31, 32}. To determine the dominant source of IL-23 producing cells, we sorted CD11b⁺CD11c^{int}F4/80⁺ colonic macrophages and CD11b⁻CD11c⁺F4/80⁻ DCs from two strains of IL-10 deficient mice at day 6 after infection, and compared the *Il23p19*, *Il12p40* and *Il12p35* mRNA transcripts. Macrophages tended to have higher amounts of each of these transcripts, but importantly,

mice with a LysM Cre-mediated deletion of IL-10 had increased amounts of *Il23p19* and *Il12p40* mRNA, while *Il12p35* mRNA transcripts were similar to mice with a CD4 Cre-mediated deletion (Fig. 4a). To determine if IL-10 by macrophages regulates IL-23 production, we sorted GFP⁺ (IL-10⁺) and GFP⁻ (IL-10⁻) macrophages from *Il10^{gfp}* mice before and after *C. rodentium* infection. We detected significantly increased amounts of *Il23p19* mRNA in GFP⁻ macrophages compared to those in GFP⁺ cells after infection, suggesting an autocrine role for IL-10 in the reporter positive cells. There were very few *Il23p19* mRNA transcripts in either type of macrophages before infection, but even in the GFP⁺ macrophages there was a small increase in *Il23p19* mRNA following exposure to *C. rodentium* (Fig. 4b). We also sorted macrophages from the large intestine of mice with a germline *Il10* deficiency at day three after infection, cultured them with or without the addition of recombinant IL-10, and analyzed the supernatants by ELISA. Exogenous IL-10 completely abrogated IL-23p19 production by colonic macrophages from infected mice; but had little effect on IL-6 production (Fig. 4c). IL-12p70 was undetectable at this time, with or without the addition of IL-10 (data not shown). These data suggest that IL-10 might inhibit IL-23 production *in vivo* following infection, in part by acting directly on the IL-23 producing macrophages.

To elucidate if increased IL-23 production in the absence of IL-10 is involved in intestinal damage and mortality, independent of adaptive immunity, we analyzed *C. rodentium* infected *Rag*^{-/-} and *Il10*^{-/-}*Rag*^{-/-} recipients that were treated with IL-23p19 blocking or control antibodies. *Il10*^{-/-}*Rag*^{-/-} recipients treated with control antibodies died between 5 and 10 days after infection, however, a neutralizing IL-23p19 antibody treatment rescued the survival of the infected mice (Fig. 4d). Furthermore, IL-23p19 antibody treatment prevented tissue damage at day 6 after infection compared to control antibodies (Fig. 4e). These data show that in the absence of IL-10 the innate immune response is unchecked, and excessive IL-23 contributes to early morbidity and death.

To verify that blocking IL-23 also can reduce mortality in fully immune competent mice lacking only macrophage-derived IL-10, we analyzed *C. rodentium* infected *Il10^{flox/flox}LysM-Cre* mice, and as a control, *Il10^{flox/flox}Cd4-Cre* mice. *Il10^{flox/flox}LysM-Cre* mice treated with the isotype control antibody began to die at day 3 after infection, and none survived beyond day 11. However IL-23p19 antibody treatment had a significant effect on promoting the survival and preventing the epithelial cell damage of the infected *Il10^{flox/flox}LysM-Cre* mice (Fig. 4f and g). Altogether, these data indicate macrophage derived IL-10 plays a dominant role in controlling the amount of IL-23 production by macrophages in the large intestine during the early phases of *C. rodentium* infection, and IL-23 is responsible, at least in part, for morbidity and mortality and is a marker for the excessive response by innate immune cells.

Gene signature of IL-10 competent large intestine macrophages

Macrophages are heterogeneous, and therefore we determined which subset was responsible for preventing excessive intestinal damage after *C. rodentium* infection. We reported previously that IL-10 competent GFP⁺ cells in the large intestine from *Il10^{gfp}* reporter mice were mainly CD11b⁺, CD11c^{int} and F4/80⁺ ¹¹. Therefore we first gated GFP⁺ or GFP⁻ cells,

then sorted IL-10 competent, GFP⁺ CD11b⁺CD11c^{int}F4/80⁺ macrophages (IL-10⁺) or their GFP⁻ counterparts (IL-10⁻) from the large intestine of *Il10^{gfp}* reporter mice, and performed microarray analyses to obtain insight into their degree of relatedness (Fig. 5a). F4/80, CD11b and MHC class II expression levels in post sorted GFP⁺ (IL-10⁺) and GFP⁻ (IL-10⁻) populations were similar. While CD11c expression in the GFP⁻ population had a broad distribution, we sorted those cells that had an amount of CD11c comparable to the GFP⁺ population. Heatmap and MA plots showed that these two populations were closely related, with 242 mRNA transcripts that were differentially expressed (Fig. 5b and c). 211 genes were upregulated in the GFP⁺ cells, including the cell surface markers, *Vcam1*, *Cd209f* and *Cd163*. In addition, 31 genes, including *Il12b*, were down regulated (Fig. 5c).

An annotation search in DAVID yielded unexpectedly enriched functional categories among upregulated genes in GFP⁺ macrophages ($P < 0.05$), including: extracellular structure organization, extracellular matrix organization, biological adhesion, regulation of peptidyl-tyrosine, vasculature development and behavior. However, no pathways were identified for the downregulated genes, likely reflecting the small number of genes with decreased expression (Fig. 5d and Supplementary Fig. 6).

We validated increased expression of *Il10* and *Cd163* in GFP⁺ colonic macrophages by real-time RT-PCR. In contrast, both macrophage populations produced *Tgfb1* mRNA (Fig. 5e). We also confirmed increased expression of CD163 in GFP⁺ macrophages by flow cytometry (Fig. 5f).

It has been reported CD163 is an IL-10 target gene *in vitro*³³. Therefore we compared IL-10 receptor expression in IL-10⁺ and IL-10⁻ colonic macrophages. However there was no difference in receptor expression. To determine if IL-10 provides an autocrine signal in macrophages *in vivo*, we crossed *Il10^{gfp}* mice with *Il10rb^{-/-}* mice. Interestingly, we found a similar frequency of GFP⁺ colonic macrophages in mice that had not received any IL-10R signals *in vivo* (Fig. 5f). However, intestinal macrophages from *Il10rb^{-/-}* mice showed reduced CD163 cell surface expression (Fig. 5f). To determine if an autocrine IL-10 signal could be responsible for the increased expression of CD163 by intestinal macrophages, we analyzed cells from *Il10^{lox/lox}LysM-Cre* and control mice. Intestinal macrophages expressed increased CD163 in *Il10^{lox/lox}Cd4-Cre* and *Il10^{lox/lox}* mice compared to *Il10^{lox/lox}LysM-Cre* (Fig. 5g). These data suggest that autocrine IL-10 may play a role in the induction of the phenotype of the IL-10 producing colonic macrophage subset, but IL-10 signals, either autocrine or paracrine, are not required for the induction of IL-10 competence in these cells.

IL-10 competent macrophages are protective

To determine if the reporter positive subset of large intestinal macrophages is required for preventing excessive inflammation after *C. rodentium* infection, we transferred sorted, reporter expressing GFP⁺ (IL-10⁺) or GFP⁻ (IL-10⁻) colonic macrophages from *Il10^{gfp}* mice into *C. rodentium* infected *Il10^{-/-}Rag^{-/-}* recipients (Fig. 6a). When GFP⁺ (IL-10⁺) macrophages were transferred, we detected a dramatic increase in survival and protection of tissue damage in the intestine compared to recipients of GFP⁻ cells (Fig. 6b). Indeed we detected significant reduction of inflammatory cell recruitment, including monocytes and

granulocytes, in the large intestine of recipients of GFP⁺ colonic macrophages after *C. rodentium* infection (Fig. 6c). Furthermore, chemokines potentially involved in the recruitment of monocytes and neutrophils to the large intestine, including CCL3, CCL4 and CCL5, were significantly decreased in the recipients of GFP⁺ cells (Fig. 6c). These data show that IL-10 from macrophages in the large intestine is indispensable for limiting excessive inflammation by regulating the inflammatory cell recruitment during the innate response.

DISCUSSION

A balanced innate immune response is required for protection from infection, and preventing excessive inflammation and tissue damage. Here we address what mediators control excessive inflammation and mortality during innate immune responses against mucosal bacteria, and the cell type and the mechanism responsible for the resolution of inflammatory response early during infection.

IL-10 is a potent inhibitor of the production of tumor necrosis factor (TNF), IL-1, IL-6 and IL-12 *in vitro*^{34, 35}, and is therefore a good candidate for limiting excessive inflammation in the intestinal mucosae. In the context of mucosal infectious disease, studies using *Salmonella choleraesuis*¹⁵, *Klebsiella pneumoniae*¹⁶, *Listeria monocytogenes*¹⁸, and *Candida albicans*¹⁹ have reported an increase in pathogen clearance, because the lack of IL-10 enhanced pro-inflammatory cytokines. In these situations the lack of IL-10 was beneficial to the host. In contrast, during infection with *Toxoplasma gondii*^{17, 21, 22} and *Trypanosoma cruzi*²³, infection in the absence of IL-10 caused excessive and often lethal inflammatory responses. These data highlight the importance of IL-10 in influencing the proper balance of innate immunity following infection with a variety of pathogens.

We observed that *C. rodentium* was cleared slightly faster in mice deficient for IL-10, but these animals suffered from high morbidity and mortality, indicative of increased inflammation. A recent study, however, reported only a beneficial effect of the lack of IL-10 following *C. rodentium* infection²⁰. The discordant findings could reflect the four-fold increased dose of bacteria we used, but more likely reflect aspects of the endogenous microbiota in different mouse colonies, as the virulence of *C. rodentium* is dependent on other components of the microbial flora³⁶. By analyzing mouse strains with T cell or myeloid cell specific deletion of IL-10, we could implicate macrophage IL-10 in limiting the destructive immune response. Consistent with this, intestinal tissue from *Il10^{fllox/fllox}Cd4-Cre* mice produced more IL-10 protein than tissue from *Il10^{fllox/fllox}LysM-Cre* mice, both at steady state and at early time points after infection, suggesting that macrophages are major IL-10 producing cells in the colon, both before and after bacterial infection. Indeed, colonic macrophages were GFP⁺ before and after infection in *Il10^{gfp}* reporter mice.

Macrophage IL-10 is indispensable for maintaining a balance in adaptive immune responses in the mucosae^{11, 37, 38, 39, 40}, and here we demonstrate its importance during the innate immune response. It is possible, however, that IL-10 producing intestinal macrophages acquire inflammatory features during bacterial infection, or alternatively, that many intestinal macrophages can rapidly acquire the ability to produce IL-10. We showed that

adoptive transfer of IL-10 producing intestinal macrophages from reporter mice, but not macrophage populations that did not express IL-10, rescued the survival of infected, IL-10 deficient mice. Therefore the GFP⁻ colonic macrophages could not induce sufficient IL-10 production to attain a protective function during this acute infection. Although we have shown that IL-10 from colonic macrophages is protective, we cannot formally exclude the possibility that the GFP⁻ cells were not protective because they could not home to the intestine or survive well after transfer. We consider this unlikely, because we did not see an increase in GFP⁺ cells after infection. Therefore, while we suggest that the IL-10 producing intestinal macrophages could be a relatively stable subset, further experiments will be required to prove that this is the case. Interestingly, while the transcriptomes of the two colonic macrophage subsets are similar, the GFP⁺ cells have increased expression of genes involved in extracellular matrix organization, vascular development, and others involved in tissue regeneration, such as the epidermal growth factor receptor and the platelet derived growth factor receptor. These data suggest that the GFP⁺ macrophages may have a more prominent role in tissue homeostasis and repair.

Here we have demonstrated that after a bacterial challenge the absence of IL-10 production by macrophages led to increased IL-23 synthesis, predominantly by macrophages, an enhanced innate immune response and fatal colitis. It is curious that IL-12 synthesis was not greatly affected, but indeed, it has been reported that IL-23 is associated with innate immune pathology in the intestine^{41, 42}. Consistent with this, IL-23 transgenic mice developed systemic inflammation that included the terminal ileum and colon, with infiltration of neutrophils and macrophages⁴³. A few other studies have demonstrated that IL-10 negatively regulates IL-23 production, and therefore the absence of IL-10 caused an increase in IL-17 producing T cells and spontaneous intestinal inflammation^{5, 44}. We note, however, that the *Il10^{flx/flx}LysM-Cre* did not have increased IL-23 in the intestine at steady state nor did they spontaneously develop colitis.

Our data demonstrate that exogenous IL-10 reduced *in vitro* IL-23 production by intestinal macrophages from *C. rodentium* infected mice. *In vivo*, there is evidence that IL-10 acts directly on the IL-10 producing macrophage subset, although IL-10 competence did not depend on any IL-10 signals. IL-10 producing macrophages expressed increased CD163, however, which was dependent on IL-10R expression by these cells. Furthermore, GFP⁻ macrophages from infected mice produced more IL-23p19 mRNA more than GFP⁺ macrophages. However, in our analyses less than 40% of colonic macrophages were GFP⁺, and therefore the reduced inflammation and improved survival in wild type mice suggests a paracrine role for IL-10 is also possible.

Recent studies showed that IL-10 signals drive macrophages to express tolerogenic functions that prevent colitis^{14, 45}. However, those investigations focused on either steady state or adaptive mucosal immune responses, not on innate mucosal immune responses, and they did not provide a mechanism or tie the protective role of IL-10 to macrophage IL-23 production.

In humans, there is a study showing myeloid DCs from Crohn's disease patients produced a higher amount of IL-23 and a lower amount of IL-10 after a bacterial stimulation when

compared to ulcerative colitis patients⁴⁶, leading us to propose that intestinal IL-10 producing myeloid cells might regulate IL-23 production not only in Crohn's disease, but also in bacterial infection in humans.

In summary, our data show that IL-10 synthesis by intestinal macrophages has a dramatic effect on regulating the innate immune response in mice through regulating macrophage IL-23 production after mucosal bacterial infection. Furthermore, intestinal macrophages are heterogeneous, and although IL-10 competent and IL-10⁻ macrophages are similar, we suggest that they may not rapidly interconvert *in vivo*.

Material and Methods

Animals

Mice were bred and housed under SPF conditions in the vivarium of the La Jolla Institute for Allergy & Immunology (LJI, La Jolla, CA). Female 8 week old *Il10*^{-/-}, *Il10rb*^{-/-} and *Rag*^{-/-} were purchased from Jackson laboratory. Female 8 week old *Il10*^{gfp} (*VertX*) were obtained from Christopher Karp (Cincinnati Children's Hospital, OH) and also purchased from Jackson Laboratory. *Il10*^{-/-}*Rag*^{-/-} mice were described previously⁴⁷. *Il10*^{lox/lox} mice were obtained from Alexander Rudensky (Memorial Sloan-Kettering Cancer Center, NY) with the permission of Axel Roers (Dresden University of Technology, Germany). *Il10*^{lox/lox} mice were bred to *Cd4-Cre*, *LysM-Cre* and *Cd11c-Cre* mice, which were all purchased from Jackson laboratory. All mouse strains were generated on the C57BL6/J background or backcrossed at least 10 generations. All procedures were approved by the LJI Animal Care and Use Committee.

Citrobacter rodentium infection

A chloramphenicol-resistant variant of the wild-type *Citrobacter rodentium* strain DBS100 was cultured as previously described³⁰. Briefly, bacteria were cultured for 15–16 hours in Luria-Bertani broth at 37°C. Mice were infected with 2–3×10⁹ c.f.u. *C. rodentium* by oral gavage. In some experiments functional grade IL23p19 (G23-8) antibody or control rat IgG were injected intravenously at 100µg per mouse at the time of infection. Antibodies were purchased from eBiosciences (San Diego, CA). GFP⁺ (IL-10⁺) and GFP⁻ (IL-10⁻) macrophages were sorted from colon tissue of uninfected *Il10*^{gfp}(*VertX*) mice as CD45⁺, MHC class II⁺, CD11b⁺, F4/80⁺, CD11c^{int} cells. One day after infection, 5×10⁵ macrophages were injected intravenously per mouse. For c.f.u. determination, fecal pellets and spleen were weighed, homogenized and plated on chloramphenicol-containing MacConkey agar plates in serial dilutions.

FITC-dextran permeability assay

In vivo permeability assays to assess intestinal barrier function were performed using FITC-labelled dextran (Sigma) as described previously³⁰. Briefly, mice were infected with *C. rodentium*. At day 0 or 6 after infection, mice were gavaged with FITC-dextran (60mg per 100 g body weight) and serum was collected retro-orbitally 4 hours later. Blood cells in serum samples were removed and the fluorescence intensity of FITC-dextran was measured.

FITC-dextran concentrations were determined from standard curves generated by serial dilution.

Reagents and antibodies

For flow cytometry and cell sorting the following antibodies were used: CD11b (M1/70), CD11c (N418), F4/80 (BM8), CD45 (30-F11), IA/IE (M5/114.15.2), Ly6C (HK1.4), Ly6G (1A8), CD4 (RM4-5), TCR β (H57-597), CD163 (polyclonal). Antibodies were purchased from BD Biosciences (San Diego, CA), eBiosciences (San Diego, CA), BioLegend (San Diego, CA) or Antibodies-online.com. Cell sorting was performed on an Aria II instrument (BD Biosciences). Cytokines and chemokines were quantified using either the Bio-plex multicytokine assay (Biorad, Hercules, CA), as described previously⁴⁸, IL-23p19, IL-22 or IL-12p70 ELISA kits (eBiosciences, San Diego, CA), or a IL-6 ELISA kit (BD, San Diego, CA).

Colon fragment cultures

Large intestines were harvested and 3mm colon fragments were cultured for 24h in DMEM with 10% FBS and antibiotics. Supernatants were analyzed for cytokines and chemokines in ratio to the weight of the corresponding tissue fragment.

Isolation of lamina propria cells

Large intestines were harvested, opened longitudinally and washed to remove fecal content. Intestines were cut into small pieces and incubated in a horizontal shaker at 37°C in the presence of 2.5mM EDTA three times for 20 min to remove epithelial cells. Colon pieces were minced and digested for 20min with 1mg/ml Collagenase type VIII (Sigma, St. Louis, MO) at 37°C. Lamina propria cells were filtered and stained for flow cytometric analysis or cell sorting.

Histology

Tissue sections were taken from the middle colon and fixed in 10% formalin. After embedding in paraffin, 5 μ m sections were cut and stained with hematoxylin- eosin (H&E). Histological scores were assigned in a blinded manner according to the following criteria: (i) mucosal architecture (0, normal; 1, focally abnormal; 2, diffusely abnormal; 3, severely abnormal); (ii) inflammatory cell infiltration of mucosa (0, normal; 1, mild infiltration; 2, moderate infiltration; 3, severe infiltration), submucosa (0, normal; 1, mild infiltration; 2 moderate infiltration; 3, severe infiltration), muscle (0, normal; 1 moderate to severe), and serosa (0, absent; 1, present); (iii) Epithelial erosions and ulcerations (0, absent; 1, present); (iv) crypt abscesses (0, absent; 1, present); and (v) goblet cell loss (0, absent; 1, present). Maximal total score for colitis severity is 14.

Relative mRNA quantification

Total RNA was extracted from sorted cells or 2–3mm long colon tissue fragments using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was digested with DNase I (Qiagen), cDNA was synthesized using iScript (Biorad, Hercules, CA) and real-time PCR was performed using SYBRgreen (Roche,

Indianapolis, IN) on a LightCycler instrument (Roche). The primers were as follows: L32, 5'-GAA ACT GGC GGA AAC CCA-3' (sense), 5'-GGA TCT GGC CCT TGA ACC TT-3' (antisense), CD163, 5'-GGT GGA CAC AGA ATG GTT CTT C-3' (sense), 5'-CCA GGA GCG TTA GTG ACA GC-3' (antisense), TGF β 1, 5'-ACC ATG CCA ACT TCT GTC -3' (sense), 5'-CGG GTT GTG TTG GTT GTA GA -3' (antisense), *Il10*, 5'-GCC ACA TGC TCC TAG AGC TG-3' (sense), 5'-CAG CTG GTC CTT TGT TTG AAA-3' (antisense), *Il23p19*, 5'-AAT AAT GTG CCC CGT ATC CA-3' (sense), 5'-GGA TCC TTT GCA AGC AGA AC-3' (antisense), *Il12p35*, 5'-GTG AAG ACG GCC AGA GAA A-3' (sense), 5'-GGT CCC GTG TGA TGT CTT C-3' (antisense), *Il12p40*, 5'-AGC AGT AGC AGT TCC CCT GA-3' (sense), 5'-AGT CCC TTT GGT CCA GTG TG-3' (antisense).

Microarray data processing and analysis

All microarray datasets were processed in R, using customized scripts and Bioconductor modules⁴⁹. Raw intensity data were normalized with the 'rma' function, with default parameters. Differential expression analysis was performed using the 'limma' package. Probe sets were deemed significantly differentially expressed only if their corrected P values were less than 0.05 and the difference was at least 3-fold. Heatmaps containing the differentially expressed genes in each set were built using the 'regHeatmap' function of the 'Heatplus' module and sorted by average fold-change. The 242 probe sets that were significantly differentially expressed were mined for enriched GO biological process terms using the database for Annotation, Visualization and Integrated Discovery (DAVID) Functional Classification Tool (<http://david.abcc.ncifcrf.gov>).

Immunofluorescence

Colons and ilea were harvested, flushed with phosphate buffered saline (PBS, Life Technologies, Carlsbad, CA) and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS overnight at 4°C and then equilibrated in 30% w/v sucrose solution in PBS for another 24 hours. Tissues were briefly washed in PBS, frozen in OCT and stored at -80 °C. Cryostat sections were cut at 16 μ m thickness, and air-dried for 30 minutes. Sections were rehydrated for 10 minutes in PBS and non-specific binding sites were saturated with 10% normal goat serum, 0.5% bovine serum albumin (both from Gemini Bio-Products, West Sacramento, CA) in the presence of 0.1% Triton X-100 in PBS for 1 hour. Tissues were incubated with anti-F4/80 rat monoclonal antibodies (50 μ g/ml, BM8, eBiosciences) and anti-GFP rabbit antiserum (5 μ g/ml, ab290, Abcam, Boston, MA) overnight at 4 °C. Following washing, sections were reacted with donkey anti-rat DyLight 549-labeled (1.4 μ g/ml, Jackson ImmunoResearch) and goat anti-rabbit F(ab')₂ fragment Alexa Fluor 647-labeled secondary antibodies (4 μ g/ml, Invitrogen) for 1h at room temperature. Slides were washed, mounted in Prolong Gold antifade reagent (Invitrogen) with a cover slip and examined with FluoView FV10i confocal microscope (Olympus). Imaging was performed at room temperature, using 60x/1.35 NA oil objective. To improve feature visibility images were processed by contrast stretching using identical procedures.

Statistical analysis

The Student's t test was used for statistical analysis except for survival and histological scores, for which the Log-Rank test and the Mann Whitney test were used respectively. Differences were considered significant at $P < .05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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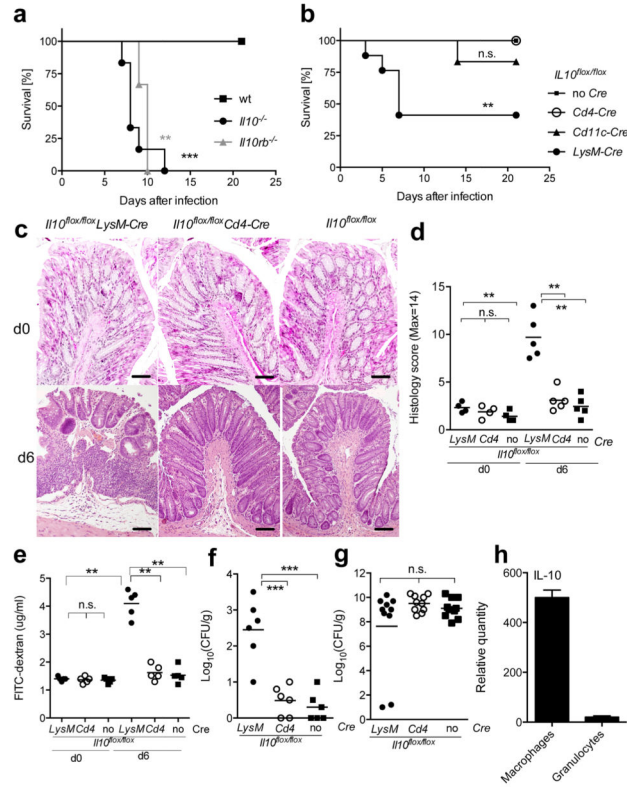


Figure 1. Myeloid cell-derived IL-10 is crucial for survival after *Citrobacter rodentium* infection (a) *Il10*^{-/-} (black circle), *Il10rb*^{-/-} (gray triangle) and C57BL/6J (black square) mice or (b) *Il10*^{flx/flx}*LysM-Cre* (black circle), *Il10*^{flx/flx}*Cd4-Cre* (open circle), *Il10*^{flx/flx}*Cd11c-Cre* (black triangle) and *Il10*^{flx/flx} (black square) mice were infected orally with *C. rodentium* and survival was monitored. (a) Two independent experiments with 6 mice per group and (b) 3 independent experiments with 12–17 mice per group are presented. (a,b) Log-Rank test, **p<.01, ***p<.001 (c,d) Inflammation of middle colon was assessed by histology before and 6 days after infection. Scale bars represent 100 µm. (e) Epithelial barrier permeability was determined before and 6 days after infection. (f,g) Titers of *C. rodentium* in (f) spleen and (g) feces were quantified 6 days of infection. (eg) Each symbol represents a measurement from a single mouse. Student’s t test, **p<.01, ***p<.001 (h) Analysis of *Il10* mRNA in macrophages or granulocytes at day 3 after infection. Results were representative of two independent experiments.

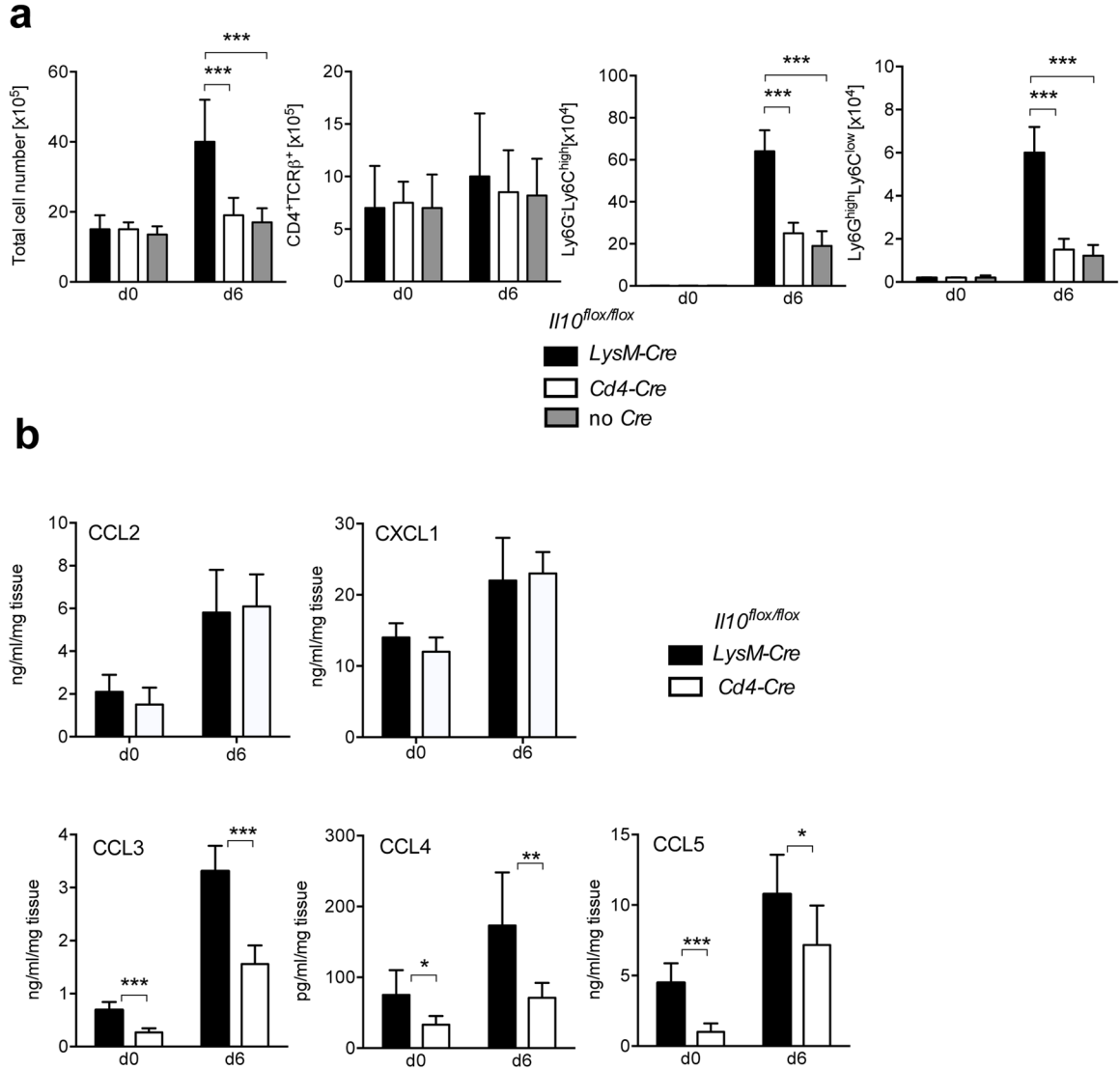


Figure 2. Macrophage IL-10 regulates myeloid cell recruitment to the colon

Il10^{fllox/fllox}LysM-Cre (black), *Il10^{fllox/fllox}Cd4-Cre* (white) and *Il10^{fllox/fllox}* (grey) mice were analyzed before a day 6 after *C. rodentium* infection. (a) Lamina propria cells were isolated at the indicated times and total cell numbers were counted. Frequencies of CD4⁺ T cells (CD4⁺TCRβ⁺), monocytes (Ly6G⁻Ly6C^{high}) and granulocytes (Ly6G^{high}Ly6C^{low}) were measured by flow cytometry and cell numbers were calculated according to the respective total cell number. Cell numbers are presented as averages and SD from 9 mice per group from 3 independent experiments. (b) At the indicated time points, 3mm fragments of the middle colon of *Il10^{fllox/fllox}LysM-Cre* (black), *Il10^{fllox/fllox}Cd4-Cre* (white) mice were cultured for 24h. Chemokine production was measured in the supernatants by Multi-Plex cytokine assay and normalized to the weight of the corresponding tissue fragments. Data are presented as averages with SD from two independent experiments with 6 mice in each group. Student's t test, * p<.05, ** p<.01, *** p<.001

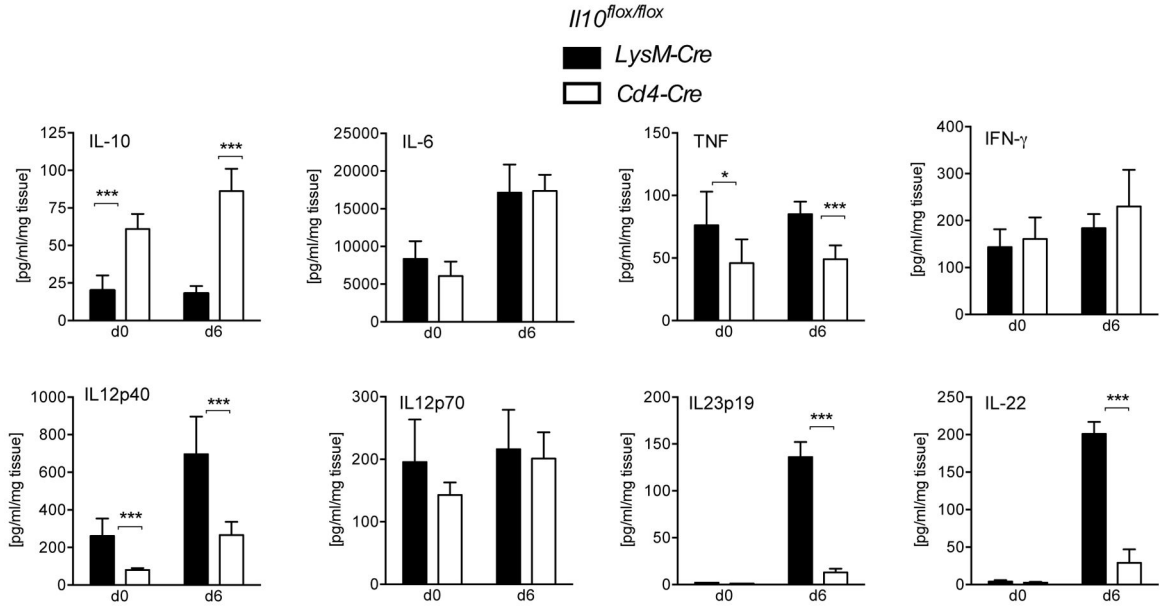


Figure 3. Increased pro-inflammatory cytokines in the large intestine in *Il10^{lox/lox}LysM-Cre* mice

Il10^{lox/lox}LysM-Cre (black) and *Il10^{lox/lox}Cd4-Cre* (white) mice were analyzed at day 0 or day 6 after *C. rodentium* infection. At the indicated time points, 3mm fragments of the middle colon were cultured for 24h. Cytokine production was measured in the supernatants by Multi-Plex or ELISA and normalized to the weight of the corresponding tissue fragments. Averages and SD from two independent experiments with 6 mice in each group are presented. Student's t test, * p<.05, *** p<.001

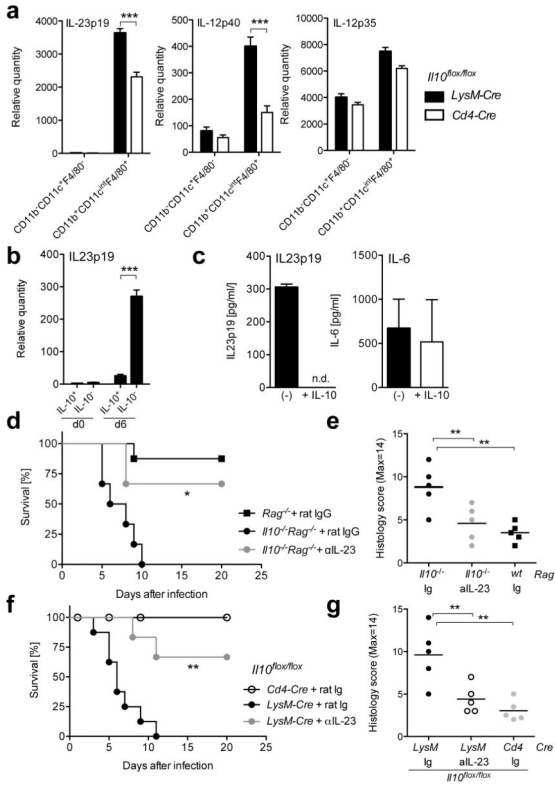


Figure 4. Macrophage-derived IL-10 influences mortality through regulation of IL-23p19
(a) Analysis of gene expression by RT-PCR in sorted CD11b⁻CD11c⁺F4/80⁻ DC or CD11b⁺CD11c⁺F4/80⁺ macrophages from large intestines in *Il10^{lox/flox}LysM-Cre* or *Il10^{lox/flox}Cd4-Cre* mice at day 6 after *C. rodentium* infection. Averages and SD from two independent experiments with 6 mice in each group are shown. **(b)** Analysis of *Il23p19* mRNA transcripts by RT-PCR in large intestine lamina propria macrophages, including GFP⁺(IL-10⁺) and GFP⁻(IL-10⁻), sorted from five to eight *Il10^{gfp}* mice before and after 6 days after *C. rodentium* infection. These data are from 2 independent experiments. **(c)** Large intestine lamina propria macrophages were sorted from five to eight infected *Il10^{-/-}Rag^{-/-}* mice and cultured for 24h in the absence (black bars) or presence (white bars) of 100ng/ml recombinant mouse IL-10. Supernatants were analyzed for IL23p19 and IL-6 by ELISA. These data are from 2–3 independent experiments. **(d,e)** *Il10^{-/-}Rag^{-/-}* and *Rag^{-/-}* mice or **(f,g)** *Il10^{lox/flox}LysM-Cre* and *Il10^{lox/flox}Cd4-Cre* mice were infected with *C. rodentium*. **(d–g)** At the time of infection, 100µg anti-IL-23 or rat IgG were injected per mouse intravenously. **(d, f)** Survival curve and **(e,g)** histology score from middle colon at day 6 after infection are shown. These are from 2 independent experiments with 3–4 mice per group. Student’s t test, * p<.05, ** p<.01, *** p<.001.

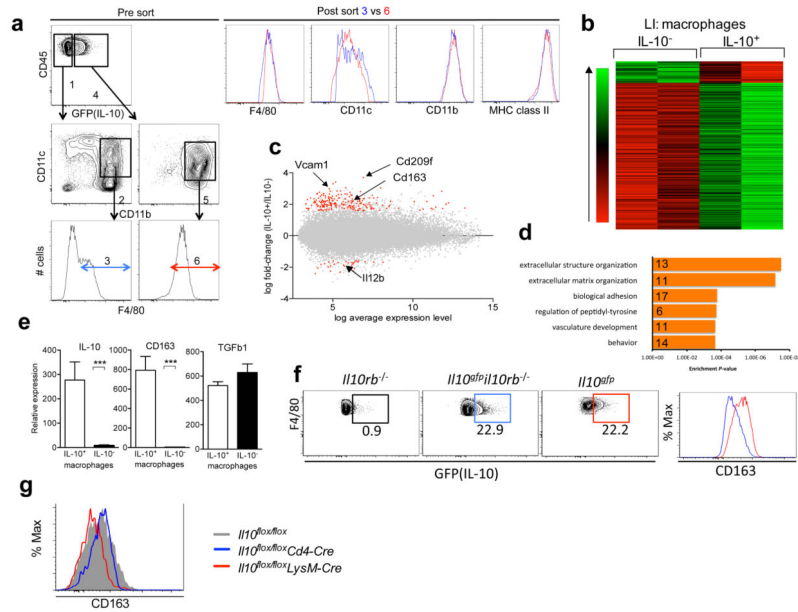


Figure 5. Comparison of IL-10-producing and non-producing large intestinal macrophages

(a) Gating strategy for the microarray analysis of GFP⁺(IL10⁺) and GFP⁻(IL-10⁻) colonic lamina propria macrophages. Cells that were CD45⁺, MHC class II⁺, CD11b⁺, CD11c^{int} and F4/80⁺ were analyzed. As depicted, the GFP⁻ cells (gate #1) used to provide mRNA were the subset falling in gates #2 and #3, CD11b⁺, CD11c^{int} and which were F4/80⁺ (gate #3). The corresponding GFP⁺ cells (gate #4) were less heterogeneous as they were almost entirely, CD11b⁺CD11c^{int} (gate #5) and F4/80⁺ (gate #6). After the sort, F4/80, CD11c, CD11b and MHC class II expressions of cell in gate 3 (blue) and gate 6 (red) were examined by flow cytometry. (b) Heatmap of differentially expressed probe sets (n=242) between IL-10⁺ and IL-10⁻ macrophages from the large intestine lamina propria in *Il10^{sfp}* reporter mice, ordered by average difference in intensity. (c) MA plot of all probe sets on the array. Red points significantly above zero represent higher expression in IL-10⁺ macrophages compared to IL-10⁻ macrophages (n=211), while points significantly below zero represent the inverse relationship (n=31). Gray points indicate probe sets that did not exhibit a significant difference in expression levels, according to criteria described in Methods. (d) Enriched GO biological processes among upregulated genes, as determined by DAVID (e) IL-10⁺ and IL-10⁻ macrophages (CD11b⁺CD11c^{int}F4/80⁺) were sorted from large intestine lamina propria of *Il10^{sfp}* reporter mice and analyzed for expression of *Il10*, *Cd163* and *Tgfb1* by real time PCR. Data are presented relative to L32 expression and are presented as average and SD of two independent experiments. (f) (Left) GFP (IL-10) expression by colonic macrophages in *Il10rb^{-/-}*, *Il10^{sfp}il10rb^{-/-}* and *Il10^{sfp}* mice. Gated on CD45⁺ MHC classII⁺ CD11b⁺ CD11c^{int} F4/80⁺ cells. (Right) CD163 expression by IL-10 positive macrophages in *Il10^{sfp}* mice (red) and in *Il10^{sfp}il10rb^{-/-}* mice (blue). (g) CD163 expression in large intestinal macrophages (CD11b⁺CD11c^{int}F4/80⁺ cells) from *Il10^{lox/lox}LysM-Cre* (red), *Il10^{lox/lox}Cd4-Cre* (blue) and *Il10^{lox/lox}* (gray) mice. Data are representative of one of two independent experiments.

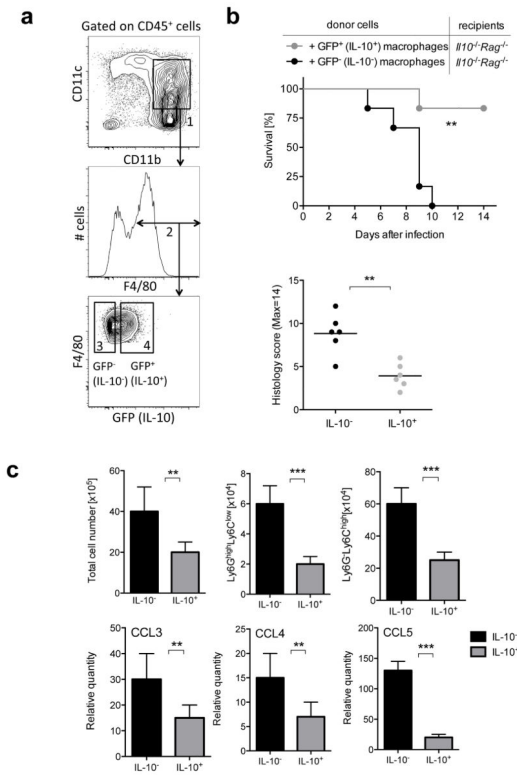


Figure 6. IL-10 competent macrophages can rescue survival after infection

Il10^{-/-} Rag^{-/-} mice were infected with *C. rodentium*. On day 1 after infection, macrophages (CD11b⁺CD11c^{int}F4/80⁺ cells) were sorted from large intestine of *Il10^{sfp}* reporter mice and 5×10⁵ cells per mouse of GFP⁺ (IL-10⁺) or GFP⁻ (IL-10⁻) populations were injected intravenously. (a) Gating strategy for obtaining GFP⁺ (IL-10⁺) and GFP⁻ (IL-10⁻) colonic macrophages for adoptive cell transfer. CD45⁺ colonic lamina propria cells were selected on the basis of CD11b⁺, CD11c^{int} expression (gate #1) and F4/80 expression (gate #2) and further separated into GFP⁺(IL-10⁺) (gate #4) or GFP⁻ (IL-10⁻)(gate #3) populations. (b) Survival and Histology score at day 6 after infection were monitored and is presented as a summary of two independent experiments with 3–5 mice per group. (c) (Top) Total cell numbers were counted from large intestine at day 6 after infection. Frequencies of monocytes (Ly6G⁻Ly6C^{high}) and granulocytes (Ly6G^{high}Ly6C^{low}) were measured by flow cytometry and cell numbers were calculated according to the respective total cell number. Cell numbers are presented as averages and SD from 3 mice per group from 2 independent experiments. (Bottom) Analysis of *Ccl3*, *Ccl4* and *Ccl5* mRNA transcripts in the large intestine at day 6 after infection. Data are presented as averages with SD from 3 mice per group from 2 independent experiments. Student's t test, ** p<.01, *** p<.001