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IL-10 regulates *Il12b* expression via histone deacetylation: Implications for intestinal macrophage homeostasis

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Abstract

To prevent excessive inflammatory responses to commensal microbes, intestinal macrophages unlike their systemic counterparts do not produce inflammatory cytokines in response to enteric bacteria. Consequently, loss of macrophage tolerance to the enteric microbiota plays a central role in the pathogenesis of the inflammatory bowel diseases. Therefore, we examined whether the hyporesponsive phenotype of intestinal macrophages is programmed by prior exposure to the microbiota. IL-10, but not in vivo exposure to the microbiota, programs intestinal macrophage tolerance, as wild-type (WT) colonic macrophages from germ free and specific-pathogen free (SPF) derived mice produce IL-10 but not IL-12 p40 when activated with enteric bacteria. Basal and activated IL-10 expression is mediated through a MyD88 dependent pathway. Conversely, colonic macrophages from germ free and SPF derived colitis-prone *Il10*$^{-/-}$ mice demonstrated robust production of IL-12 p40. Next, mechanisms through which IL-10 inhibits *Il12b* expression were investigated. While *Il12b* mRNA was transiently induced in LPS-activated WT bone marrow derived macrophages (BMDMs), expression persisted in *Il10*$^{-/-}$ BMDMs. There were no differences in nucleosome remodeling, mRNA stability, NF-κB activation or MAPK signaling to explain prolonged transcription of *Il12b* in *Il10*$^{-/-}$ BMDMs. However, acetylated histone H4 (AcH4) transiently associated with the *Il12b* promoter in WT BMDMs, whereas association of these factors was prolonged in *Il10*$^{-/-}$ BMDMs. Experiments utilizing histone deacetylase (HDAC) inhibitors and HDAC3 shRNA indicate that HDAC3 is involved in histone deacetylation of the *Il12b* promoter by IL-10. These results suggest that histone deacetylation on the *Il12b* promoter by HDAC3 mediates homeostatic effects of IL-10 in macrophages.

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INTRODUCTION

The gastrointestinal tract represents a complex interface between the enteric microbiota and immune cell populations. A multitude of diverse microorganisms reside in the intestinal lumen separated from the body's largest reservoir of macrophages by a single layer of epithelial cells. These macrophages serve as the first line of defense against the external environment. To prevent excessive inflammatory responses to commensal microbes, intestinal macrophages have acquired a unique phenotype. Intestinal macrophages, unlike their systemic counterparts, do not produce inflammatory cytokines in response to enteric bacteria (1). Consequently, loss of macrophage tolerance to the enteric microbiota plays a central role in the pathogenesis of the inflammatory bowel diseases (IBD) (2, 3).

The anti-inflammatory cytokine IL-10 is implicated in the maintenance of intestinal homeostasis. Mutations in genes encoding the IL-10 receptor subunit proteins *IL10RA* and *IL10RB* were reported in patients with early onset enterocolitis (4). Moreover, mice deficient in IL-10 or IL-10 receptors develop spontaneously occurring intestinal inflammation dependent on the presence of the enteric microbiota (5, 6). IL-10 is secreted by many cell types, including T cells, mast cells, epithelial cells, macrophages, and dendritic cells; however, a major source of IL-10 involved in the maintenance of intestinal homeostasis is lamina propria or mesenteric lymph node macrophages (7–9).

The IL-12 family members IL-12 and IL-23 expressed by macrophages are important inhibitory targets of IL-10 and are central mediators of chronic intestinal inflammation (10–13). IL-12/IL-23 p40 (encoded by the *Il12b* gene) is the common subunit of IL-12 and IL-23. Despite extensive investigation, molecular mechanisms through which IL-10 inhibits *Il12b* expression have not been fully elucidated (14–16).

Gene transcription is regulated at the chromatin level. DNA-binding factors cannot access DNA in closed chromatin. Therefore, chromatin structure needs to be altered to facilitate gene transcription (17). Histone acetylation induces an open chromatin conformation that allows the transcription machinery to access promoters, whereas histone deacetylation correlates with gene silencing. Inducible chromatin modifications serve as important restriction points in TLR-regulated gene expression. Recruitment of histone acetyltransferases (HATs) such as p300 and CREB-binding protein (CBP) to the *Il12b* promoter has been implicated in its transcriptional activation (18). TLR stimulation of macrophages results in rapid changes in chromatin remodeling at the *Il12b* locus via histone acetylation, enabling transcription factor recruitment (17). Accordingly, histone deacetylation on the *Il12b* promoter by histone deacetylase (HDAC) negatively regulates *Il12b* transcription (19). Therefore, epigenetic changes that inhibit and induce *Il12b* expression in macrophages are likely to be central determinants of intestinal homeostasis and inflammation, respectively.

Here, we report that the anti-inflammatory phenotype of resident colonic macrophages is programmed by IL-10 without requirement for exposure to the microbiota in vivo. In bone marrow derived macrophages (BMDM), IL-10 inhibits IL-12/IL-23 p40 expression through altered kinetics of histone acetylation on the *Il12b* promoter. Inhibition of HDAC3 results in decreased inhibition of *Il12b* by IL-10. These experiments suggest that histone deacetylation on the *Il12b* promoter by HDACs mediates homeostatic effects of IL-10 in macrophages. Consequently, the absence of IL-10 leads to prolonged histone acetylation with persistent transcription of *Il12b*.

MATERIALS AND METHODS

Mice

Wild-type (WT) and *Il10*$^{-/-}$ mice on 129/SvEv background were used to isolate colonic CD11b^{+} lamina propria mononuclear cells (LPMCs). IL-10–IRES-EGFP reporter (Vert-X) mice were created by insertion of a floxed neomycin–IRES-EGFP cassette between the endogenous stop site and the polyadenosine site of IL-10 (20). Germ-free (GF) mice were maintained in the Gnotobiotic Core Facility at the University of North Carolina at Chapel Hill. WT and *Il10*$^{-/-}$ mice on C57BL/6 background maintained in specific-pathogen free (SPF) condition were used for bone marrow derived macrophage (BMDM) derivation. All animal experiments were in accordance with protocols approved by the International Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Reagents

LPS was purchased from InvivoGen (San Diego, CA). M-CSF and IL-10 were obtained from PeproTech Inc (Rocky Hill, NJ). Heat-killed bacteria was prepared as described previously (1). Briefly, *E. coli* and *E. faecalis* in log-phase growth were harvested and washed twice with ice-cold PBS. Bacterial suspensions were heated at 80°C for 30 minutes, washed, resuspended in PBS, and stored at −80°C. Non-viability was confirmed by 72-hour incubation at 37°C on plate medium. Heat-killed bacteria were added at multiplicity of infection (MOI) 10 or 100 for cell stimulation. HDAC inhibitors, trichostatin A (TSA) and MS275 were obtained from Sigma (St. Louis, Mo) and Selleck Chemicals (Houston, TX), respectively.

Cell isolation

BMDMs were cultured as described previously (21). LPMCs were isolated from mouse colons by an enzymatic method as previously described (1). LPMCs were further separated into CD11b^{+} cells using anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA).

Quantitative RT-PCR

Quantitative real-time RT-PCR was performed as described previously (22). Primer sequences are available upon request.

Chromatin immunoprecipitation assays (ChIP)

ChIP was performed with ChIP-IT Express kit (Active Motif, Carlsbad, CA) according to manufacturer's instruction and as previously reported (23, 24). Briefly, 2×10^6 (for AcH4, me3H3K4) or 5×10^6 (for RNA polymerase II (RNA Pol II)) BMDMs were stimulated, washed with PBS, and fixed with 1% formaldehyde for 10 minutes at room temperature. Fixed cells were harvested, lysed, and sonicated for 10 cycles of 20-second on/20-second off with Sonic Dismembrator 60 (Thermo Fisher Scientific, Waltham, MA). For AcH4 ChIP, sodium butyrate (20 mM) was added to all the solutions to preserve histone acetylation. Antibodies for AcH4 and RNA pol II were obtained from Millipore (Billerica, MA), and me3H3K4 was from Abcam (Cambridge, MA). Primer pairs for monitoring binding to *Il12b* promoter were as follows: acetylated histone H4 (AcH4), forward 5′-ATGCACTCAGGGAGGCAAG-3′, reverse 5′-TCTGATGGAAACCCAAAGTAGAAAC-3′, RNA polymerase II (RNA Pol II), Forward 5′-GAAGGAACAGTGGGTGTCCAG-3′, Reverse 5′-AGGGAGTTAGCGACAGGGAAG-3′.

Restriction enzyme accessibility assay

To monitor nucleosome remodeling, chromatin accessibility was measured by real-time PCR as previously described (25, 26). Purified DNA was amplified by three sets of primers. PCR-based analysis was validated by Southern blot, as described (25) (Supplemental Fig. 1).

Lentivirus-mediated gene transduction

Lentiviral transduction was optimized based on manufacturer's instructions for FuGENE Transfection Reagent (Roche, Indianapolis, IN) and as previously described (23). Lentivirus for HDAC3-specific shRNA was obtained from Open Biosystems (Huntsville, AL) and transduced to BMDMs from *Il10*$^{-/-}$ mice in 12-well plates. Transduced cells were selected by puromycin, and transduction efficiency was confirmed by Western blot and RT-PCR.

Enzyme-linked immunosorbent assay (ELISA)

IL-12 p40 and IL-10 concentrations were determined by sandwich ELISA according to the manufacturer's instructions (BD Biosciences, San Jose, CA).

Western immunoblots

Western blot analyses were performed on whole cell extracts as described previously (21). NF-κB p65, IκBα, phospho-p38, and phospho-ERK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), phospho-NF-κB p65, phospho-IκBα, and phospho-JNK antibodies were obtained from Cell Signaling (Danvers, MA).

Statistical analysis

Statistical significance for data subsets were assessed by the two-tailed Student's *t* test. p values < 0.05 were considered to be significant. All data are expressed as mean ± standard error (SEM).

RESULTS

IL-10 but not the enteric microbiota programs the anti-inflammatory phenotype of colonic macrophages

Germ free (GF) *Il10*$^{-/-}$ mice, but not wild type (WT) mice, develop colitis when colonized with the enteric microbiota (27). Colitis in *Il10*$^{-/-}$ mice is associated with increased colonic production of IL-12/23 p40 (24). As peripheral macrophages become tolerized to activation of inflammatory pathways upon prolonged or repeated exposure to pathogen associated molecular patterns (28, 29), we reasoned that colonic macrophages may require exposure to the enteric microbiota in vivo to develop a tolerant, anti-inflammatory phenotype. To address this question, colonic CD11b^{+} lamina propria mononuclear cells (LPMCs) were isolated from GF and specific pathogen free (SPF) colonized WT mice (Fig. 1A and B). Surprisingly, CD11b^{+} LPMCs from GF WT mice activated with heat killed *E. coli* or *E. faecalis* did not express IL-12/23 p40 (Fig. 1A), but secrete basal and activated IL-10 (Fig. 1B). This suggests that the anti-inflammatory phenotype is programmed by factors in the local microenvironment and does not require in vivo exposure to the enteric microbiota. CD11b^{+} LPMCs isolated from SPF colonized WT mice demonstrated patterns of IL-12/23 p40 and IL-10 expression identical to that of CD11b^{+} LPMCs from GF WT mice (Fig. 1A and B). Interestingly, CD11b^{+} LPMCs from GF *Il10*$^{-/-}$ mice demonstrate IL-12/23 p40 production upon activation with *E. coli* and *E. faecalis* (Fig. 1A). Indeed, IL-12 p40 activation was as robust in GF CD11b^{+} LPMCs as in CD11b^{+} LPMCs from SPF colonized *Il10*$^{-/-}$ mice (Fig. 1A). No difference was observed in expression of surface markers (F4/80, CD11c, CD40, MHC II, CD80, CD86) between WT and *Il10*$^{-/-}$ CD11b^{+} LPMCs (from GF or SPF colons). CD40, CD86 and MHC II expression appeared to be higher in GF compared

to SPF CD11b$^+$ LPMCs, although these differences did not impact *Il10* or *Il12b* expression (Supplemental Fig. 2). To substantiate the role of IL-10 in determining colonic macrophage phenotype, CD11b$^+$ LPMCs from GF WT colons were cultured with blocking antibodies to IL-10 and IL-10 receptor prior to activation with heat-killed *E. coli* and *E. faecalis*. By blocking IL-10 signaling, activated WT colonic CD11b$^+$ LPMCs demonstrate robust production of IL-12/23 p40 compared to CD11b$^+$ LPMCs cultured with isotype control antibodies (Fig. 1C). These results indicate that endogenous IL-10 production is essential for the regulation of IL-12 p40 and consequently, the anti-inflammatory phenotype of colonic macrophages. Interestingly, IL-10 production by colonic CD11b$^+$ LPMCs was MyD88-dependent (Fig. 1D): Both basal and enteric bacteria-activated IL-10 expression is absent in colonic CD11b$^+$ LPMC from SPF colonized *Myd88*$^{-/-}$ but not *Trif*$^{-/-}$ mice, suggesting that endogenous signals through MyD88 control homeostatic macrophage function. The in vivo expression of IL-10 in different subsets of CD11b$^+$ cells was further elucidated using IL-10 transcriptional GFP reporter mice, Vert-X mice (20). First, IL-10 expressing cells were quantitated and compared in colonic CD11b$^+$CD11c$^-$ macrophage and CD11b$^+$ CD11c$^+$ dendritic cell populations from SPF-raised Vert-X mice. CD11b$^+$CD11c$^-$ macrophages demonstrated greater numbers of IL-10 producing cells compared to CD11b$^+$CD11c$^+$ dendritic cells, as reported previously (30) (Fig. 1E). We confirmed that the presence and abundance of IL-10 producing CD11b$^+$CD11c$^-$ macrophages was independent of colonization status by the enteric microbiota in Vert-X mice raised in GF, SPF or transferred from GF to SPF microbiota (Fig. 1F). Overall, these findings implicate locally produced IL-10, not exposure to the microbiota, as a requisite factor determining colonic macrophage phenotype through attenuated expression of IL-12/23 p40 upon subsequent exposure to enteric microbial products.

IL-10 regulates *Il12b* in macrophages through epigenetic mechanisms

M-CSF-derived bone marrow derived macrophages (BMDMs) produce more IL-10 and less IL-12 p40 compared to GM-CSF-derived BMDM (1). Therefore, we utilized M-CSF-derived BMDMs as a model to explore molecular mechanisms through which IL-10 attenuates *Il12b* activation. Indeed, kinetics of *Il12b* and *Il10* expression was similar between LPS-stimulated BMDMs and heat-killed bacteria-stimulated colonic macrophages from WT and *Il10*$^{-/-}$ mice (Fig. 2A, B). LPS-induced *Il12b* mRNA was transient in WT BMDMs with peak expression at 3 hours. However, *Il12b* expression from *Il10*$^{-/-}$ BMDMs was still increasing at 12 hours (Fig. 2A). In the presence of anti-IL-10, the kinetics of *Il12b* expression in WT BMDMs was identical to *Il10*$^{-/-}$ BMDMs (Fig. 2C). There was no detectable difference in *Il12b* mRNA stability between WT and *Il10*$^{-/-}$ BMDMs (Fig. 2D). NF-κB (IκBα phosphorylation and degradation, RelA phosphorylation) and MAPK kinase activation kinetics were also identical in WT and *Il10*$^{-/-}$ BMDMs (Fig. 2E), with peak activation between 0.5 and 3 hours. Interestingly, when exogenous IL-10 was added to *Il10*$^{-/-}$ BMDMs 3 hours post-LPS stimulation, *Il12b* expression was still attenuated at later time points (Fig. 2F). These results suggest that IL-10 inhibits IL-12 p40 not through altered induction of downstream signal transduction pathways, but through other mechanisms that affect gene transcription.

IL-10 alters histone H4 acetylation kinetics on the *Il12b* proximal promoter

Gene expression is regulated at the chromatin level through nucleosome remodeling and covalent histone modifications. Histone acetylation is associated with transcriptionally active chromatin, whereas deacetylation correlates with gene repression. Upon LPS stimulation, *Il12b* promoter activation is accompanied by selective remodeling of a nucleosome (referred to as Nuc1) in the proximal promoter and a DNase I hypersensitive site (HSS1) approximately 10 kb upstream of the transcription start site (16). As previously described (25), restriction enzyme accessibility assays revealed that Nuc1 and HSS1 were

remodeled upon LPS stimulation of WT BMDMs (Fig. 3A and B; Confirmation by Southern blot shown in Supplemental Fig. 1). *Il10*$^{-/-}$ BMDMs demonstrated identical kinetics of nucleosome remodeling, despite the marked difference in *Il12b* expression kinetics between WT and *Il10*$^{-/-}$ BMDMs. Therefore, we next examined histone modifications on the *Il12b* promoter by chromatin immunoprecipitation (ChIP) using acetylated histone H4 as an indicator of open chromatin. Histone H4 on the *Il12b* promoter was transiently acetylated in WT BMDMs, peaking at 1.5 hours after LPS stimulation and decreasing to baseline by 3 hours, whereas histone H4 acetylation on the *Il12b* promoter persisted for 6 hours in *Il10*$^{-/-}$ BMDMs (Fig. 3C). Recombinant IL-10 inhibited LPS-induced histone acetylation on the *Il12b* promoter in *Il10*$^{-/-}$ BMDMs (Fig. 3D). In contrast, trimethylation of histone H3 at lysine 4 (H3K4me3), another marker of transcriptionally active promoters, was induced upon LPS stimulation and persisted at 6 hours in both WT and *Il10*$^{-/-}$ BMDMs (Supplemental Fig. 3A), whereas *Il12b* mRNA is decreasing at 6 hours post-LPS in WT BMDMs (Fig. 2A). This suggests that IL-10 specifically induces histone deacetylation on *Il12b* promoter.

Histone deacetylation decreases the accessibility of chromatin to the basal transcriptional machinery. Therefore, we next determined whether IL-10-mediated histone deacetylation correlates with decreased occupancy of RNA polymerase II (RNA pol II) on the *Il12b* promoter. LPS-stimulated WT BMDMs demonstrated transient RNA pol II occupancy on the *Il12b* promoter. In *Il10*$^{-/-}$ BMDMs, RNA pol II occupancy persisted for 6 hours following LPS stimulation (Fig. 3E). Recombinant IL-10 inhibited RNA pol II binding in *Il10*$^{-/-}$ BMDMs (Fig. 3F). Kinetics of NF-κB p65 recruitment to the *Il12b* promoter was also similar to RNA pol II in WT and *Il10*$^{-/-}$ BMDMs and binding of p65 was also inhibited by exogenous IL-10 (Supplemental Fig. 3B and C), similar to findings in bone marrow derived dendritic cells described previously (31). Taken together, these results demonstrate that IL-10 limits transcriptional activity of the *Il12b* promoter, likely through alterations in histone acetylation kinetics.

Inhibition of LPS-induced IL-12 p40 by IL-10 involves histone deacetylation by HDAC3 in bone marrow derived and colonic macrophages

The altered kinetics of histone acetylation led us to study the role of HDACs in IL-10-mediated *Il12b* inhibition. We first used an inhibitor of class I and II HDACs, trichostatin A (TSA). Pretreatment of BMDMs with TSA prior to LPS stimulation significantly inhibit *Il12b* transcription induction, as previously described (32) (Supplemental Fig. 4A), greatly affecting our ability to detect IL-10 mediated inhibition of *Il12b* transcription. Therefore, *Il12b* transcription was first induced with LPS for one hour before treating the BMDMs with TSA. Interestingly, TSA prolonged the expression of *Il12b* in WT BMDMs resulting in kinetics similar to that in *Il10*$^{-/-}$ BMDMs (Fig. 4A). However, TSA also affected the kinetics of other cytokines, including IL-10 (Supplemental Fig. 4B). Next, exogenous IL-10 was added back to *Il10*$^{-/-}$ BMDMs in the presence or absence of TSA to compare IL-10-induced deacetylation on the *Il12b* promoter without potential confounding effects mediated by endogenous IL-10 production (Fig. 4B). As predicted, inhibition of IL-12 p40 by IL-10 was significantly impaired by TSA(Fig. 4C). Quantitative PCR also showed marked decrease of *Il12b* inhibition by IL-10 in the presence of TSA (Fig. 4C), suggesting IL-10 mediated inhibition of LPS-induced IL-12 p40 is partially dependent on class I or II HDACs.

Since HDAC1 is reported to be associated with histone deacetylation on the *Il12b* and *Il6* promoters in macrophages (19, 33), we next utilized the HDAC1- and 3- specific inhibitor MS275 (IC50 0.3 μM for HDAC1 and 8 μM for HDAC3). Unexpectedly, loss of IL-10 mediated inhibition of *Il12b* was observed only at the highest dose of MS275 (10 μM), suggesting HDAC3 is more important to this process (Fig. 4D and E). HDAC3-specific

lentiviral shRNA(Fig. 4F) was used to confirm this role of HDAC3 in the inhibitory effect of IL-10 on LPS-induced IL-12 p40. IL-12 p40 inhibition by IL-10 was significantly diminished in LPS and HDAC3 shRNA-treated cells compared to control cells (scrambled HDAC3 shRNA) (Fig. 4G). Furthermore, inhibition of HDAC3 prevented histone H4 deacetylation on the *Il12b* promoter by IL-10 (Fig. 4H).

HDAC3 is a homeostatic factor in IL-10-mediated intestinal immunity

To determine whether our findings in BMDMs are relevant for colonic macrophage function, IL-12 p40 production by colonic $CD11b^+$ LPMCs from $Il10^{-/-}$ mice was determined in the presence of HDAC inhibitors. $Il10^{-/-}$ colonic $CD11b^+$ LPMCs were activated with heat killed *E.coli* prior to the addition of MS275 or TSA and IL-10. As demonstrated in BMDMs, IL-12 p40 inhibition by IL-10 was significantly diminished by blocking HDAC3 compared to the control (Fig. 5A). Next, colonic *Hdac3* expression was characterized before and after transition of GF mice to an SPF microbiota (34). Interestingly, colonic *Hdac3* expression was significantly induced after colonization of WT mice, but not colitis-prone $Il10^{-/-}$ mice (Fig. 5B). Colonic *Il12b* expression was significantly induced in $Il10^{-/-}$ but not WT mice upon exposure to SPF microbiota, inversely correlating with *Hdac3* induction. As a control, no significant induction of colonic *Hdac1* was observed (Fig. 5B).

DISCUSSION

Mechanisms operative in programming an anti-inflammatory phenotype in intestinal macrophages are incompletely understood. We initially speculated that a tolerant colonic macrophage phenotype might be acquired upon exposure to the enteric microbiota. Given this unique intestinal environment where macrophages intimately coexist with the enteric microbiota, we hypothesized that a phenomenon similar to the induction of endotoxin tolerance in peripheral macrophages may occur (28, 29). However, colonic macrophages isolated from GF WT mice were phenotypically identical to macrophages derived from colonized mice, and failed to produce *Il12b* upon stimulation with enteric bacteria. Moreover, both GF and SPF WT colonic macrophages produced abundant IL-10. Colonic macrophages from GF $Il10^{-/-}$ mice and WT macrophages treated with anti-IL-10/IL-10 receptor antibodies produced abundant IL-12 p40 upon activation by enteric bacteria, demonstrates that locally produced IL-10 is a requisite factor for maintaining anti-inflammatory responses in colonic macrophages. It is also intriguing that MyD88-deficient intestinal macrophages lack both basal and inducible IL-10. Further studies are needed to unravel precise mechanisms by which intestinal macrophages acquire the anti-inflammatory phenotype to produce IL-10 through MyD88. Speculatively, endogenous factors that activate the MyD88 signaling pathway in the colonic microenvironment may shape the colonic macrophage phenotype and mediate tolerance to the enteric microbiota. However, given inherent limitations of the germ free mouse model system, we also cannot exclude exogenous microbial products and other exogenous substance present in small amounts in the mouse diet as contributing to this process. Nonetheless, these results further define the unique immune environment in the gastrointestinal tract, focusing on IL-12 p40 regulation by endogenous IL-10 production as a well established prototype for a mucosal innate inflammatory response.

Utilizing BMDMs as a model to understand molecular mechanisms through which IL-10 inhibits *Il12b* expression, IL-10 was found to mediate histone deactelyation of the *Il12b* promoter with the consequence of attenuated transcription of *Il12b*. In $Il10^{-/-}$ BMDMs, prolonged kinetics of *Il12b* mRNA and protein expression correlated with prolonged histone H4 acetylation on the proximal promoter and prolonged occupancy by RNA pol II. Zhou et al. previously demonstrated that exogenous IL-10 abolishes RNA pol II binding to the *Il12b* promoter partly through inhibition of nucleosome remodeling of the *Il12b* promoter (16).

While our results also demonstrated altered RNA pol II recruitment, we did not observe differences in nucleosome remodeling between WT and *Il10*$^{-/-}$ BMDMs. An important difference between our studies is that they used peritoneal macrophages. Additionally, alterations of nucleosome remodeling demonstrated in the prior study were relatively small in magnitude compared with profound IL-12 p40 inhibition by exogenous addition of recombinant IL-10. Indeed, the authors speculated that alterations in nucleosome remodeling induced by IL-10 were likely to be a consequence of transcription inhibition rather than the cause. Furthermore, comparisons between WT and *Il10*$^{-/-}$ BMDMs may be more relevant to dissect molecular differences given that we demonstrate the importance of autocrine regulation of IL-12 p40 by endogenous IL-10 (Fig. 1C and 2C).

Although our studies clearly implicate HDAC3 in the inhibitory effect of IL-10 on *Il12b*, it is important to note that the inhibitory effect was not complete. Indeed, HDAC inhibitors reverse approximately 30% of the inhibitory effect of IL-10. Based on many other studies looking at mechanisms of IL-10 inhibition, this is not surprising. IL-10 is such an important homeostatic factor that multiple independent mechanisms must mediate its inhibitory effect even on a single gene such as IL-12 p40, because the consequences of loss of IL-10 regulation are so significant biologically. Indeed, IL-10 induces many genes in macrophages at the same time as it inhibits others (35) and exerts its potent anti-inflammatory function in innate immunity through multiple mechanisms. Transcription elongation (36), miR-155 (37), and induction of transcriptional repressors such as tristetraprolin (38), ETV3, and Strawberry notch homologue 2 (SBNO2) (39) have been suggested as mechanisms for IL-10-mediated innate immune regulation, although they have not been explicitly implicated in IL-12 p40 inhibition. Accordingly, multiple redundant mechanisms for IL-10-mediated IL-12 p40 regulation have been described, including nuclear factor, interleukin-3 regulated (NFIL3) (40), interferon regulatory factor-8 (IRF-8) (41), nucleosome remodeling (16) all of which seem to have incremental but biologically significant effects.

We have utilized CD11b^{+} LPMCs as representative of colonic macrophages since these cells are the main sources of IL-12 p40 and IL-10 in the intestinal lamina propria (23, 24). IL-10 and IL-12 p40 production in response to heat-killed enteric bacteria from these cells are identical in both GF and SPF-raised WT mice. Although this population also includes CD11b^{+}CD11c^{+} dendritic cells, CD11b^{+} CD11c^{-} macrophages are more abundant than CD11b^{+}CD11c^{+} dendritic cells in number. Furthermore, we also showed that vast majority of IL-10 producing cells belongs to CD11b^{+} CD11c^{-} macrophages, as reported previously (30).

Histone acetylation and deacetylation are regulated by HATs and HDACs, respectively. We demonstrated altered histone acetylation on *Il12b* promoter by IL-10, suggesting that IL-10 represses *Il12b* transcription by this mechanism. Currently, eighteen HDACs have been identified in mammalian cells and are classified into 4 classes, of which class I and II are the major and best characterized groups. Class I HDACs are widely expressed in most cell types, whereas class II HDACs demonstrate more restricted expression and have roles in cell differentiation (42). Accordingly, HDAC inhibition has many overarching consequences in immune cells, including apoptosis (43), differentiation (42, 44), signal transduction (45, 46), and cytokine production (47–49). Indeed, the class I and II HDAC inhibitor, TSA was previously described to inhibit LPS-induced pro-inflammatory cytokine expression in macrophages, including IL-12 p40, when cells were treated with TSA prior to LPS activation (32). In contrast, treatment with TSA following activation of macrophages leads to an increased pro-inflammatory response (42, 50). Therefore, to specifically address the role of HDACs in IL-10 inhibition of *Il12b*, we treated macrophages with HDAC inhibitors post-LPS stimulation. Although the magnitude of LPS-induced IL-12 p40 production varied, both TSA and MS275 significantly diminished IL-12 p40 inhibition by IL-10, indicating that

inhibition is HDAC dependent. Among class I and II HDACs, we have shown that HDAC3 is likely to be involved in this process by using the class I specific HDAC inhibitor, MS275. Moreover, HDAC3-specific knockdown resulted in impaired inhibition of IL-12 p40 by IL-10, and IL-10-mediated histone deacetylation of *Il12b* promoter was blocked by MS275. Furthermore, GF mice colonized with SPF microbiota were used as an in vivo model for characterizing colonic HDAC induction upon the exposure to commensal bacteria. *Hdac3* expression was induced in WT mice but not in $Il10^{-/-}$ mice after colonization. *Hdac3* expression inversely correlated with *Il12b* expression. This finding implicates HDAC3 in IL-10 mediated *Il12b* regulation, with colonic *Hdac3* induction requiring IL-10 and the enteric microbiota in vivo. Hence, we provide multiple lines of evidence that HDAC3 contributes to IL-10-mediated *Il12b* inhibition. However, precise mechanisms for IL-10 control of HDAC3 function on the *Il12b* promoter await clarification. HDAC3 may function directly on the *Il12b* promoter or indirectly through expression of other genes involved in *Il12b* regulation. There are multiple factors controlling HDAC activity including its expression, nuclear translocation and binding to DNA. Additionally, co-repressors such as SMRT (silencing mediator for retinoid and thyroid receptors), and N-CoR (nuclear receptor co-repressor) (51) may participate in an HDAC3 regulation. Involvement of STAT3 in this process is also of interest. STAT3 is both necessary and sufficient for inhibitory effect of IL-10 on many target pro-inflammatory genes. In fact, $Stat3^{-/-}$ cells and mice show a phenotype similar to those of $Il10^{-/-}$ (52). Furthermore, Hoentjen et al. demonstrated that IL-10 regulates p65 recruitment to the *Il12b* promoter through STAT3 phosphorylation in bone marrow derived dendritic cells (31). Therefore, it is likely that control of HDAC3 function by IL-10 also involves STAT3.

In conclusion, we provide evidence that IL-10 is a necessary homeostatic factor for maintaining the anti-inflammatory phenotype of colonic macrophages, without requirement for in vivo exposure to the enteric microbiota. IL-10 altered the kinetics of histone H4 acetylation on the *Il12b* promoter. This finding led to the identification of a novel epigenetic mechanism of IL-10-mediated IL-12 p40 regulation through histone deacetylation by HDAC3 which is operative in colonic macrophages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AcH4	acetylated histone H4
BMDMs	bone marrow derived macrophages
ChIP	chromatin immunoprecipitation
CBP	CREB-binding protein
EF	*Enterococcus feacalis*
EC	*Escherichia coli*

GF	germ free
HAT	histone acetyltransferase
HDAC	histone deacetylase
HSS1	DNase I hypersensitive site
H3K4me3	histone H3 trimethylated at lysine 4
IBD	inflammatory bowel disease
LPMCs	lamina propria mononuclear cells
MOI	multiplicity of infection
NFIL3	nuclear factor, interleukin-3 regulated
N-CoR	nuclear receptor co-repressor
RNA pol II	RNA polymerase II
SBNO2	strawberry notch homologue 2
shRNA	small hairpin ribonucleic acid
SMRT	silencing mediator for retinoid and thyroid receptors
SPF	specific pathogen free
TSA	trichostatin A
WT	wild-type

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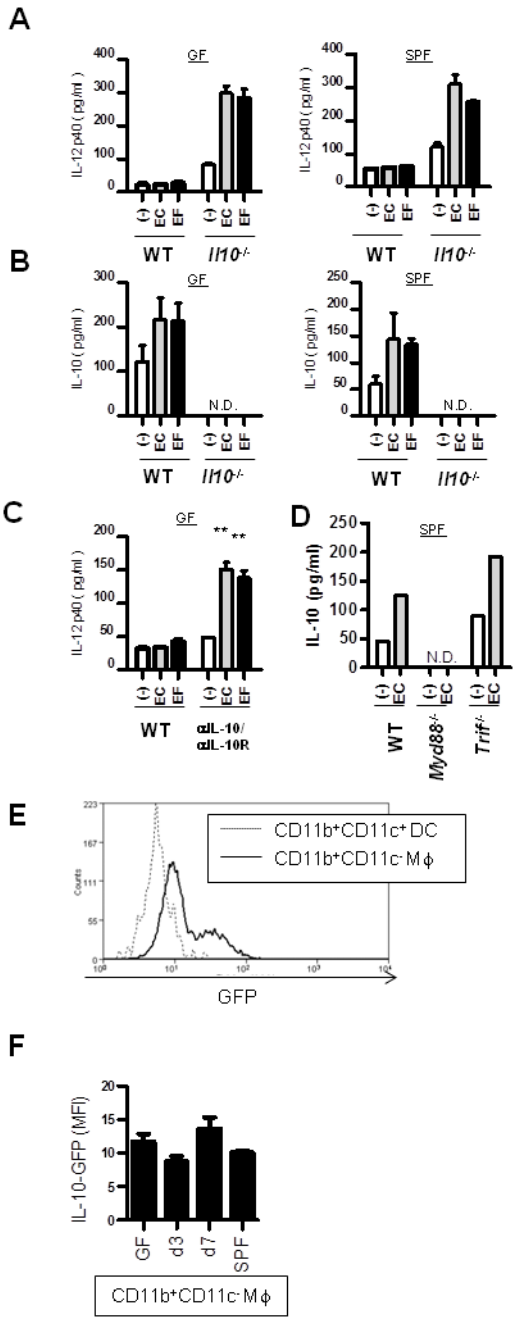

Figure 1. IL-10 but not the enteric microbiota programs the anti-inflammatory phenotype of colonic macrophages

WT and *Il10*$^{-/-}$ colonic CD11b^{+} LPMCs from GF (left panel) and SPF microbiota colonized (right panel) mice were stimulated with heat-killed *E. coli* (EC) or *E. faecalis* (EF) (MOI = 10) for 24 hours. **(A)** IL-12/23 p40 and **(B)** IL-10 secretion was determined by ELISA. **(C)** Colonic CD11b^{+} LPMCs from WT GF mice were stimulated with heat-killed EC or EF (MOI = 10) ± neutralizing anti-IL-10 (10 μg/ml), IL-10 receptor (anti-IL-10R) antibodies (10 μg/ml) or isotype control antibodies for 24 hours. IL12/IL-23 p40 secretion was determined by ELISA. **p < 0.01 relative to isotype stimulated WT CD11b^{+} LPMCs. Results represent mean ± SEM from three independent experiments. N.D., not detected. **(D)**

Colonic CD11b$^+$ LPMCs from WT, *Myd88*$^{-/-}$, and *Trif*$^{-/-}$ SPF mice were stimulated with heat-killed EC (MOI = 10). IL-10 secretion was determined by ELISA. Representative result from 2 independent experiment is shown. N.D., not detected. **(E)** Colonic lamina propria mononuclear cells from IL-10 transcriptional reporter, Vert-X mice, are analyzed by flow cytometry. A representative histogram from 3 independent experiments is shown for GFP, representing IL-10 expression in gated CD11b$^+$CD11c$^+$ dendritic cells and CD11b$^+$CD11c$^-$ macrophages in SPF-raised Vert-X mice. **(F)** Germ-free raised (GF) mice were colonized with the SPF microbiota and cells were isolated 3 and 7 days post-colonization. Results are shown as mean fluorescence intensity (MFI) for GFP representing IL-10 expression in gated CD11b$^+$CD11c$^-$ macrophages, at each time point of colonization. Results represent mean ± SEM from three independent experiments.

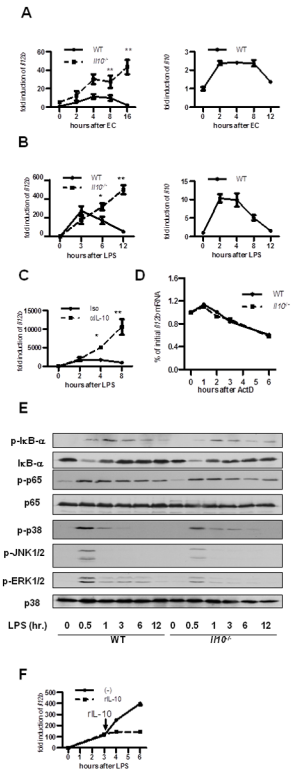

Figure 2. IL-10 inhibition of *Il12b* expression does not involve mRNA stability, NF-κB activation, MAPK signaling, or nucleosome remodeling

(A) WT and *Il10*$^{-/-}$ colonic CD11b^{+} LPMCs were stimulated with heat-killed *E. coli* (EC) (MOI=100). Cells were harvested at indicated time points post-EC stimulation and *Il12b* and *Il10* expression analyzed by real-time RT-PCR. ** $p < 0.01$ versus WT. (B) WT and *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml). Cells were harvested at indicated time points post-LPS stimulation and *Il12b* and *Il10* expression analyzed by real-time RT-PCR. *$p < 0.05$; ** $p < 0.01$ versus LPS stimulated WT BMDMs. (C) WT BMDMs were stimulated with LPS (10 ng/ml) ± anti-IL-10 or isotype control (iso) antibodies for 2, 4, and 8 hours. Kinetics of *Il12b* mRNA expression was analyzed by real-time RT-PCR. ** $p < 0.01$ versus LPS stimulated WT BMDMs incubated with isotype control antibody. For real time RT-PCR experiments, results are expressed as fold induction versus unstimulated control BMDMs normalized to β-actin (mean ± SEM from three independent experiments). (D) WT and *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) for 2 hours + actinomycin D (ActD, 5 μg/ml). Cells were harvested at the indicated time points and *Il12b* expression analyzed. Results are representative of three independent experiments. (E) Kinetics of NF-κB and MAPK pathway activation was analyzed by Western immunoblot for indicated proteins in WT and *Il10*$^{-/-}$ BMDMs stimulated with LPS (10 ng/ml). Representative results from three independent experiments are shown. (F) *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) for 3 hours and recombinant IL-10 (20 ng/ml) was added. Cells were harvested at the indicated time points for mRNA purification and *Il12b* mRNA expression was analyzed by real-time PCR. Results are expressed as fold induction versus unstimulated WT BMDMs normalized to β-actin (mean ± SEM from three independent experiments).

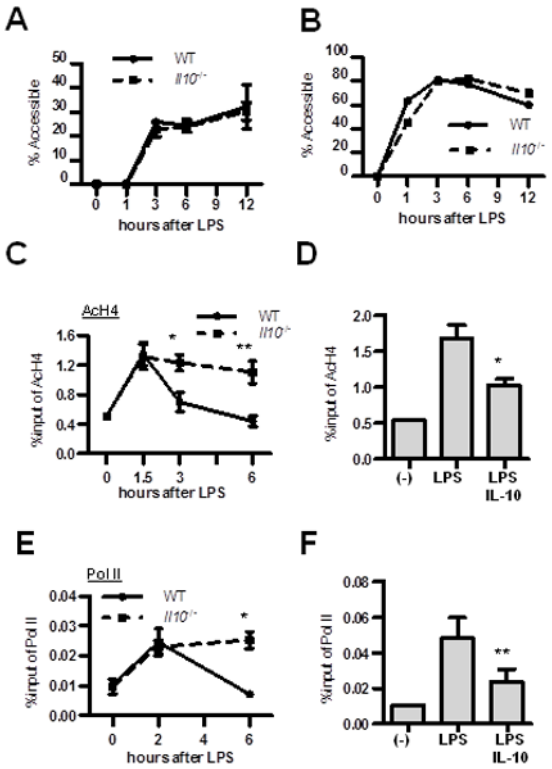

Figure 3. IL-10 alters histone H4 acetylation kinetics on the *Il12b* proximal promoter

(A) Effects of IL-10 on nucleosome remodeling at nucleosome 1 (Nuc1) position and **(B)** an upstream DNase I hypersensitivity site 1 (HSS1) was monitored by restriction enzyme accessibility assays. Quantification of *Spe*I and *Pst*I cleavage products was analyzed by real-time RT-PCR using primers spanning the Nuc1 and HSS1 regions in the *Il12b* promoter. Results are expressed as a percentage of Nuc1 and HSS1 accessibility observed in LPS stimulated BMDMs relative to unstimulated DNA (mean ± SEM from three independent experiments). **(C)** WT and *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) for 1.5, 3, and 6 hours and kinetics of acetylation of histone H4 (AcH4) on the *Il12b* promoter was analyzed by ChIP. Results are presented as enrichment (percentage of input DNA) of AcH4 associated with the *Il12b* promoter. **(D)** *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) ± recombinant IL-10. Acetylation of histone H4 on the *Il12b* promoter was analyzed by ChIP 3 hours following LPS stimulation. Results are presented as enrichment (percentage of input DNA) of AcH4 associated with the *Il12b* promoter. **(E)** WT and *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) for 2 and 6 hours and recruitment of RNA polymerase II (RNA pol II) to the *Il12b* promoter was assessed by ChIP. **(F)** *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) ± recombinant IL-10 for 3 hours and RNA pol II binding on the *Il12b* promoter was assessed by ChIP. Results are presented as enrichment (percentage of input DNA) of RNA pol II promoter occupancy. All ChIP assays are presented as mean ± SEM of chromatin preparations from three independent experiments. *$p < 0.05$, **$p < 0.01$ versus LPS-stimulated BMDMs.

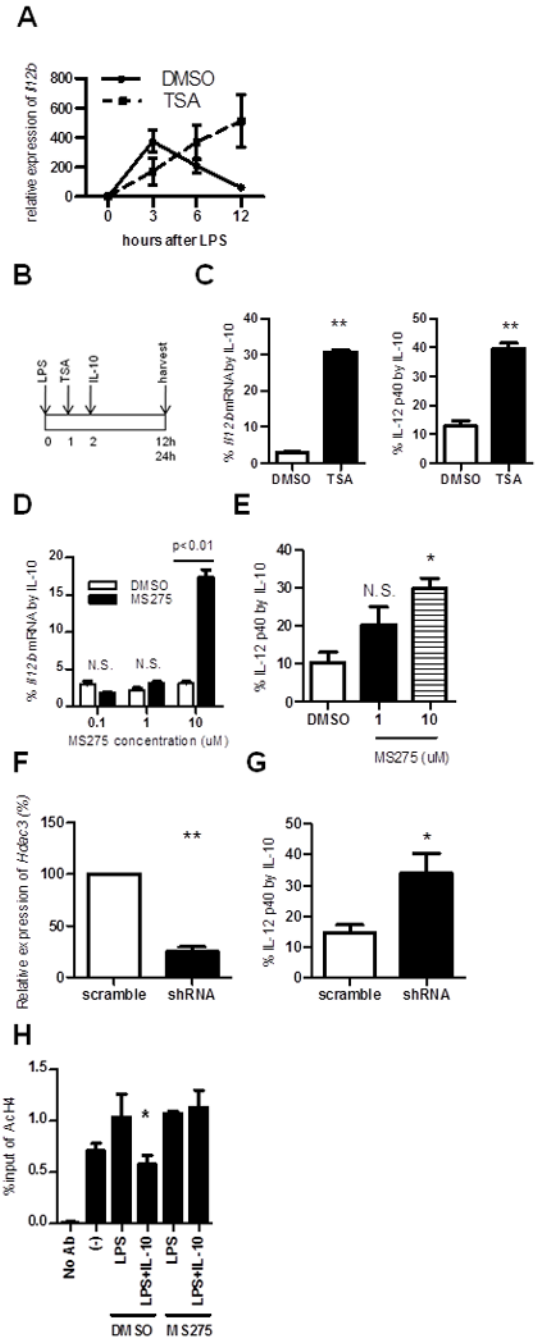

Figure 4. HDACs are involved in inhibition of *Il12b* by IL-10

(A) WT BMDMs were stimulated with LPS (10 ng/ml) with or without trichostatin A (TSA) (100 nM) 1 hour after LPS. Cells were harvested at 3, 6, and 12 hours post-LPS stimulation and *Il12b* expression analyzed by real-time RT-PCR. **(B, C)** *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) and treated with TSA (100 nM) or DMSO 1h post-LPS. IL-10 (1 ng/ml) was added 2h-post LPS. *Il12b* expression was determined by real-time RT-PCR 12 h post-LPS and IL-12 p40 protein by ELISA after 24 hours. Results are presented as percent *Il12b* (4C, left) and IL-12 p40 (4C, right) expression by LPS in the presence of IL-10 relative to LPS alone. **(D, E)** *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) and treated with MS275, an inhibitor of HDAC1 (IC50=0.3 μM) and HDAC3 (IC50=8

μM), or DMSO 1 h post-LPS. IL-10 was added 2h-post LPS. Inhibition of *Il12b* expression (D) and IL-12 p40 (E) was examined as above. **(F)** *Il10*$^{-/-}$ BMDMs transduced HDAC3 shRNA or control scrambled shRNA were analyzed for *Hdac3* mRNA expression. Results are expressed as relative expression (%) versus BMDMs transduced with scrambled shRNA normalized to β-actin. **(G)** *Il10*$^{-/-}$ BMDMs transduced with HDAC3 shRNA or control scrambled shRNA were stimulated with LPS (10 ng/ml), treated with IL-10 2 hours post-LPS, and harvested 24 hours post-LPS. Inhibition of IL-12 p40 is presented as above. **(H)** *Il10*$^{-/-}$ BMDMs were stimulated with LPS (10 ng/ml) ± IL-10 in the presence or absence of MS275 (10 μM). Acetylation of histone H4 on the *Il12b* promoter was analyzed by ChIP four hours following LPS stimulation. Results are presented as enrichment (percentage of input DNA) of AcH4 associated with the *Il12b* promoter. *$p < 0.05$ versus LPS + DMSO.

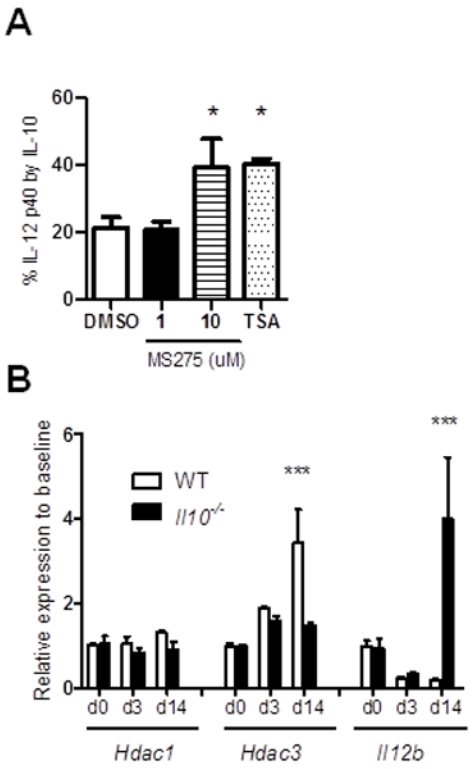

Figure 5. HDAC3 is a homeostatic factor in IL-10-mediated intestinal immunity

(A) *Il10*$^{-/-}$ colonic CD11b^{+} LPMCs were stimulated with heat-killed *E. coli* (EC, 100 MOI) and treated with MS275 (1 or 10 μM), TSA (100 nM) or DMSO 1h post-EC. Recombinant IL-10 (1 ng/ml) was added 2h post-EC. Inhibition of IL-12 p40 was examined as above. All results represent mean ± SEM from three independent experiments. *$p < 0.05$, **$p < 0.01$ versus controls. **(B)** GF WT and *Il10*$^{-/-}$ mice were transitioned to an SPF enteric microbiota. Colonic mucosal *Hdac1, Hdac3, and Il12b* expression were analyzed before, 3 and 14 days post-colonization. Results are expressed as fold induction versus WT GF colons normalized to β-actin (mean ± SEM from four mice per each time point). ***$p < 0.005$ versus *Il10*$^{-/-}$.