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IL-10 Regulates \( \text{II12b} \) Expression via Histone Deacetylation: Implications for Intestinal Macrophage Homeostasis

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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 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, because wild-type (WT) colon 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, colon macrophages from germ-free and SPF-derived colitis-prone \( \text{II10}^{-/-} \) mice demonstrated robust production of IL-12 p40. Next, mechanisms through which IL-10 inhibits \( \text{II12b} \) expression were investigated. Although \( \text{II12b} \) mRNA was transiently induced in LPS-activated WT bone marrow-derived macrophages (BMDMs), expression persisted in \( \text{II10}^{-/-} \) BMDMs. There were no differences in nucleosome remodeling, mRNA stability, NF-κB activation, or MAPK signaling to explain prolonged transcription of \( \text{II12b} \) in \( \text{II10}^{-/-} \) BMDMs. However, acetylated histone H4 transiently associated with the \( \text{II12b} \) promoter in WT BMDMs, whereas association of these factors was prolonged in \( \text{II10}^{-/-} \) BMDMs. Experiments using histone deacetylase (HDAC) inhibitors and HDAC3 short hairpin RNA indicate that HDAC3 is involved in histone deacetylation of the \( \text{II12b} \) promoter by IL-10. These results suggest that histone deacetylation on the \( \text{II12b} \) promoter by HDAC3 mediates homeostatic effects of IL-10 in macrophages. The Journal of Immunology, 2012, 189: 000–000.

The gastrointestinal tract represents a complex interface between the enteric microbiota and immune cell populations. A multitude of diverse microorganisms resides 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 inflammatory bowel diseases (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 \( \text{IL10RA} \) and \( \text{IL10RB} \) were reported in patients with early-onset enterocolitis (4). Moreover, mice deficient in IL-10 or IL-10 receptors develop spontaneously occurring intestinal inflammation that is dependent on the presence of enteric microbiota (5, 6). IL-10 is secreted by many cell types, including T cells, mast cells, epithelial cells, macrophages, and dendritic cells; however, lamina propria or mesenteric lymph node macrophages are a major source of IL-10 involved in the maintenance of intestinal homeostasis (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 \( \text{II12b} \) gene) is the common subunit of IL-12 and IL-23. Despite extensive investigation, the molecular mechanisms through which IL-10 inhibits \( \text{II12b} \) 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, the 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, such as p300 and CREB-binding protein, 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.

In this article, 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 (BMDMs), 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 propia 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) conditions, 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 (Rocky Hill, NJ). Heat-killed bacteria were prepared as described previously (1). Briefly, Escherichia coli and Enterococcus faecalis in log-phase growth were harvested and washed twice with ice-cold PBS. Bacterial suspensions were heated at 80°C for 30 min, washed, resuspended in PBS, and stored at −80°C. Nonviability was confirmed by a 72 h incubation at 37°C on plate medium. Heat-killed bacteria were added at a multiplicity of infection (MOI) 10 or 100 for cell stimulation. HDAC inhibitors, trichostatin A (TSA) and MS275, were confirmed by a 72 h incubation on plate medium. Heat-killed Escherichia coli or Enterococcus faecalis did not express IL-12/23 p40 (Fig. 1A), but expressed as mean ± SEM.

Results

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

GF Il10−/− mice, but not 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). Because 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+ LPMCs were isolated from GF- and SPF-colonized WT mice (Fig. 1A, 1B). 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 they secreted 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 those of CD11b+ LPMCs from GF WT mice (Fig. 1A, 1B). 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 the expression of surface markers (F4/80, CD11c, CD40, MHC class II, CD80, CD86) between WT and Il10−/− CD11b+ LPMCs (from GF or SPF colons). CD40, CD86, and MHC class II expression appeared to be higher in GF CD11b+ LPMCs compared with SPF CD11b+ LPMCs, although these
transcriptional reporter, Vert-X mice, were analyzed by flow cytometry. CA (EF) (MOI = 10) for 24 h. IL-12/23 p40 (Il10) IL-10, but not the enteric microbiota, programs 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+ LPMCs 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 with CD11b+CD11c+ dendritic cells, as reported previously (30) (Fig. 1E). We confirmed that the presence and abundance of IL-10–producing CD11b+CD11c+ macrophages were independent of colonization status by the enteric microbiota: In Vert-X mice raised GF, SPF, or transferred from GF to SPF microbiota, no significant differences in the numbers of IL-10-expressing cells were demonstrated (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 BMDMs produce more IL-10 and less IL-12 p40 compared with GM-CSF–derived BMDMs (1). Therefore, we used 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 were similar between LPS-stimulated BMDMs and heat-killed bacteria-stimulated colonic macrophages from WT and Il10−/− mice (Fig. 2A, 2B).

LPS-induced Il12b mRNA was transient in WT BMDMs, with peak expression at 3 h. However, Il12b expression from Il10−/−BMDMs was still increasing at 12 h (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 (IkB 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 h. Interestingly, when exogenous IL-10 was added to Il10−/− BMDMs 3 h post-LPS stimulation,

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 h. IL-12/23 p40 (A) and IL-10 (B) secretion was determined by ELISA. (C) Colonic CD11b+ LPMCs from WT GF mice were stimulated with heat-killed EC or EF (MOI = 10) with or without neutralizing anti–IL-10 Ab (10 μg/ml), IL-10R Ab (anti–IL-10R; 10 μg/ml), or isotype control Abs for 24 h. IL-12/IL-23 p40 secretion was determined by ELISA. Results represent mean ± SEM from three independent experiments. (D) Colonic CD11b+ LPMCs from WT, Mdx88−/−, and Trif−/− SPF mice were stimulated with heat-killed E. coli (MOI = 10). IL-10 secretion was determined by ELISA. A representative result from two independent experiments is shown. (E) Colonic LPMCs from IL-10 transcriptional reporter, Vert-X mice, were analyzed by flow cytometry.

A representative graph from three 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) GF mice were colonized with the SPF microbiota, and cells were isolated 3 and 7 d postcolonization. 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. **p < 0.01, versus isotype-stimulated WT CD11b+ LPMCs. N.D., Not detected.
II10 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) ∼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, 3B, confirmation by Southern blot shown in Supplemental Fig. 1). Il10−/− BMDMs demonstrated identical kinetics of nucleosome remodeling, despite the marked difference in Il10 expression kinetics between WT and Il10−/− BMDMs. Therefore, we next examined histone modifications on the Il12b promoter by ChIP using AcH4 as an indicator of open chromatin. Histone H4 on the Il12b promoter was transiently acetylated in WT BMDMs, peaking at 1.5 h after LPS stimulation and persisting to 6 h in both WT and Il10−/− BMDMs. Therefore, we next examined histone modifications on the Il12b promoter by ChIP using AcH4 as an indicator of open chromatin. Histone H4 on the Il12b promoter was transiently acetylated in WT BMDMs, peaking at 1.5 h after LPS stimulation and persisting to 6 h in both WT and Il10−/− BMDMs. These results suggest that IL-10 specifically induces histone deacetylation on the 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 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 h following LPS stimulation (Fig. 3E). rIL-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, 3C), 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.

**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 the indicated time points post-E. coli stimulation, and Il12b and Il10 expression was 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 the indicated time points post-LPS stimulation, and Il12b and Il10 expression was 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) with or without anti-IL-10 or isotype control (iso) Abs for 2, 4, and 8 h. Kinetics of Il12b mRNA expression were analyzed by real-time RT-PCR. 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). *p < 0.01, versus LPS-stimulated WT BMDMs incubated with isotype control Ab. (D) WT and Il10−/− BMDMs were stimulated with LPS (10 ng/ml) for 2 h plus actinomycin D (ActD; 5 μg/ml). Cells were harvested at the indicated time points, and Il12b expression was analyzed. Results are representative of three independent experiments. (E) Kinetics of NF-κB and MAPK pathway activation was analyzed by Western immunoblot for the 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 h, and rIL-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).
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 a marked decrease in Il12b inhibition by IL-10 in the presence of TSA (Fig. 4C), suggesting that IL-10–mediated inhibition of LPS-induced IL-12 p40 is partially dependent on class I or II HDACs.

Because HDAC1 is reported to be associated with histone deacetylation on the Il12b and Il6 promoters in macrophages (19, 33), we next used 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 that HDAC3 is more important to this process (Fig. 4D, 4E). 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 with 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 in LPS and HDAC3 shRNA-treated cells compared with 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−/− mice but not WT mice upon exposure to SPF microbiota, correlating inversely 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 they 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-10R Abs produced abundant IL-12 p40 upon activation by enteric bacteria; this 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 the precise mechanisms by which intestinal macrophages

**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 TSA, an inhibitor of class I and II HDACs. Treatment of BMDMs with TSA prior to LPS stimulation significantly inhibited Il12b transcription, 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 1 h 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 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, or 6 h, and kinetics of 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) with or without rIL-10. AcH4 on the Il12b promoter was analyzed by ChIP 3 h 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 h, and recruitment of RNA pol II to the Il12b promoter was assessed by ChIP. (F) WT and Il10−/− BMDMs were stimulated with LPS (10 ng/ml) with or without rIL-10 for 3 h, 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.
Results are presented as percentage of input DNA) of AcH4 associated with the proximal promoter and prolonged occupancy by RNA pol II. 

**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 (100 MOI) and treated with MS275 (1 or 10 μM), TSA (100 nM), or DMSO 1 h post-E. coli. (B) WT and Il10−/− mice were transitioned to an SPF enteric microbiota. Colonic mucosal Hdac1, Hdac3, and Il12b expression was analyzed before and 3 and 14 d after colonization. Results are expressed as fold induction versus WT GF colons normalized to β-actin (mean ± SEM from four mice/time point). *p < 0.05, ***p < 0.005, versus Il10−/−.

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 GF mouse model system, we also cannot exclude that exogenous microbial products and other exogenous substances present in small amounts in the mouse diet contribute 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.

Using BMDMs as a model to understand molecular mechanisms through which IL-10 inhibits Il12b expression, IL-10 was found to mediate histone deacetylation 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. Demonstrated that exogenous IL-10 abolishes RNA pol II binding to the Il12b promoter, in part through inhibition of nucleosome

**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 TSA (100 nM) 1 h after LPS. Cells were harvested at 3, 6, or 12 h post-LPS stimulation, and Il12b expression was analyzed by real-time RT-PCR. (B and C) Il10−/− BMDMs were stimulated with LPS (10 ng/ml) and treated with TSA (100 nM) or DMSO 1 h post-LPS. IL-10 (1 ng/ml) was added 2 h post-LPS. Il12b expression was determined by real-time RT-PCR 12 h post-LPS, and IL-12 p40 protein was determined by ELISA after 24 h. Results are presented as percentage Il12b (C, left panel) and IL-12 p40 (C, right panel) expression by LPS in the presence of IL-10 relative to LPS alone. **p < 0.05, versus DMSO. (D and 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 2 h post-LPS. Inhibition of Il12b expression (D) and IL-12 p40 (E) was examined as above. *p < 0.05, versus DMSO. (F) Il10−/− BMDMs transduced with 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. **p < 0.05, versus control shRNA. (G) Il10−/− BMDMs transduced with HDAC3 shRNA or control scrambled shRNA were stimulated with LPS (10 ng/ml), treated with IL-10 2 h post-LPS, and harvested 24 h post-LPS. Inhibition of IL-12 p40 is presented as above. *p < 0.05, versus control shRNA. (H) Il10−/− BMDMs were stimulated with LPS (10 ng/ml) with or without IL-10 in the presence or absence of MS275 (10 μM). AcH4 on the Il12b promoter was analyzed by ChIP 4 h 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.
showed that the vast majority of IL-10–producing cells belong to the CD11b+CD11c+ cells, and IL-10 in the intestinal lamina propria (23, 24). IL-10 and IL-12 p40 regulation have been described, including NFκB (36), miR-155 (37), and induction of transcription elongation (36), miR-155 (37), and induction of transcriptional repressors, such as tristetraprolin (38), ETV3, and Strawberry notch homolog 2 (39), have been suggested as mechanisms for IL-10–mediated innate immune regulation, although they have not been explicitly implicated in IL-12 p40 production. Accordingly, multiple redundant mechanisms for IL-10–mediated IL-12 p40 regulation have been described, including NFκB, IL-3 regulated (40), IRF-8 (41), and nucleosome remodeling (16), all of which seem to have incremental, but biologically significant, effects.

We used CD11b+ LPMCs as representatives of colonic macrophages because 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 in number than are CD11b+CD11c− dendritic cells. Furthermore, we also showed that the vast majority of IL-10–producing cells belong to the CD11b+CD11c+ macrophage population, as reported previously (30).

Histone acetylation and deacetylation are regulated by histone acetyltransferases and HDACs, respectively. We demonstrated altered histone acetylation on the Il12b promoter by IL-10, suggesting that IL-10 represses Il12b transcription by this mechanism. Currently, 18 HDACs have been identified in mammalian cells and are divided into four 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 described to inhibit LPS-induced proinflammatory 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 proinflammatory 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, and it correlated inversely 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, corepressors, such as silencing mediator for retinoid and thyroid receptors and nuclear receptor corepressor (51), may participate in HDAC3 regulation. Involvement of STAT3 in this process is also of interest. STAT3 is both necessary and sufficient for the inhibitory effect of IL-10 on many target proinflammatory genes. In fact, Stat3−/− cells and mice show a phenotype similar to those of Il10−/− cells (52). Furthermore, Hoentjen et al. (31) demonstrated that IL-10 regulates p65 recruitment to the Il12b promoter through STAT3 phosphorylation in bone marrow-derived dendritic cells. 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 the 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.

Disclosures
The authors have no financial conflicts of interest.

References


