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IL-10 Regulates Il12b 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) 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. Although 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 transiently associated with the Il12b promoter in WT BMDMs, whereas association of these factors was prolonged in Il10−/− BMDMs. Experiments using histone deacetylase (HDAC) inhibitors and HDAC3 short hairpin RNA 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.

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 microbiota, gene expression of inflammatory cytokines is limited. Basal expression of cytokines is regulated through histone modifications, and several studies have shown that histone acetylation mediates inflammatory cytokine expression (17). Histone acetylation induces an open chromatin conformation that allows the transcription machinery to access transcription factors. However, the chromatin structure needs to be altered to facilitate gene expression (14–16). 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 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 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 Il12b gene) is the common subunit of IL-12 and IL-23. Despite extensive investigation, the 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, 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

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Abbreviations used in this article: AcH4, acetylated histone H4; BMDM, bone marrow-derived macrophage; ChIP, chromatin immunoprecipitation; GF, germ free; HDAC, histone deacetylase; H3K4me3, histone H3 trimethylated at lysine 4; HSS1, DNase I hypersensitive site; LPMC, lamina propria mononuclear cell; M01, multiplicity of infection; RNA pol II, RNA polymerase II; siRNA, short hairpin RNA; SPF, specific pathogen free; TSA, trichostatin A; WT, wild-type.

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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). Consequently, 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 propria mononuclear cells (LPMCs). IL-10–IRES–EGFP reporter (Vert-X) mice were created by insertion of the GFP gene under the Il10 intron (20). Lysates 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). 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 used at a 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

Chromatin immunoprecipitation (ChIP) was performed with a ChIP-IT Express kit (Active Motif, Carlsbad, CA), according to the manufacturer’s instructions and as previously reported (23, 24). Briefly, 2 × 10^6 (for acetylated histone H4 [AcH4], histone H3 trimethylated at lysine 4 [H3K4me3] or 5 × 10^6 [for RNA polymerase II [RNA pol II] BMDMs) were stimulated, washed with PBS, and fixed with 1% formaldehyde for 10 min at room temperature. Fixed cells were harvested, lysed, and sonicated for 10 cycles of 20 s on/20 s off with Sonic Dismembrator 60 (Thermo Fisher Scientific, Waltham, MA). For AcH4 ChIP, sodium butyrate (20 mM) was added to all of the solutions to preserve histone acetylation. Abs for AcH4 and RNA pol II were obtained from Millipore (Billerica, MA), and Ab for H3K4me3 was from Abcam (Cambridge, MA). Primer pairs for monitoring binding to Il12b promoter were as follows: AcH4, forward 5’-ATGCATCTCAGG-GAGGCAG-3’, reverse 5’-TCTGATGGAAAACCCAATGAAAC-3’, and RNA pol II, forward 5’-GAAGGAACAGTGCGGTTCACCAG-3’, reverse 5’-AGGGTTAGCGACAGGAA-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 the manufacturer’s instructions for PuGene Transfection Reagent (Roche, Indianapolis, IN) and as previously described (23). Lentivirus for HDAC3-specific short hairpin RNA (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.

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, p-p38, and p-ERK Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); p-NF-κB p65, IκBα, and p-JNK Abs were obtained from Cell Signaling (Danvers, MA).

Statistical analysis

Statistical significance for data subsets was assessed by the two-tailed Student t test; p values < 0.05 were considered significant. All data are 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). Interestingly, CD11b+ LPMCs from GF WT mice (Fig. 1A, 1B). No difference was observed in the expression of surface markers (F4/80, CD11c, CD40, MHC class II, CD80, CD86) between WT and Il10−/− mice (Fig. 1C, 1D). Consequently, the absence of IL-10 leads to prolonged histone acetylation with persistent transcription of Il12b.
transcriptional reporter, Vert-X mice, were analyzed by flow cytometry. Cdc determined by ELISA. (A) Colonic CD11b+ LPMCs from GF (MOI = 10) for 24 h. IL-12/23 p40 (Il10) secretion was determined by ELISA. Results represent mean ± SEM from three independent experiments. (B) Colonic LPMCs from IL-10+/− mice were stimulated with heat-killed E. coli or E. faecalis (EF) (MOI = 10) for 24 h. IL-12/23 p40 (Il10) secretion was determined by ELISA. Results represent mean ± SEM from three independent experiments. (C) Colonic CD11b+ LPMCs from GF were stimulated with 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-12/23 p40 secretion was determined by ELISA. Results represent mean ± SEM from three independent experiments. (D) Colonic CD11b+ LPMCs from WT, Myd88−/−, 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. 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 Abs to IL-10 and IL-10R prior to activation with heat-killed E. coli and E. faecalis. By blocking IL-10 signaling, activated WT colonic CD11b+ LPMCs demonstrated robust production of IL-12/23 p40 compared with CD11b+ LPMCs cultured with isotype control Abs (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+ 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 transcripational 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, 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 days 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.
IL-10–mediated epigenetic regulation of Il12b

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 Il12b 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 decreasing to baseline by 3 h, whereas histone H4 acetylation on the Il12b promoter persisted for 6 h in Il10−/− BMDMs (Fig. 3C). rIL-10–induced histone acetylation on the Il12b promoter in Il10−/− BMDMs (Fig. 3D). In contrast, H3K4me3, another marker of transcriptionally active promoters, was induced upon LPS stimulation and persisted at 6 h in both WT and Il10−/− BMDMs (Supplemental Fig. 3A), whereas Il12b mRNA was decreasing at 6 h post-LPS stimulation in WT BMDMs (Fig. 2A). This suggests that IL-10 specifically induces histone deacylation on the Il12b promoter.

Histone deacylation decreases the accessibility of chromatin to the basal transcriptional machinery. Therefore, we next determined whether IL-10–mediated histone deacylation 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.
IL-10 was added back to II10−/− BMDMs in the presence or absence of TSA to compare IL-10–induced deacetylation on the II12b 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 II12b 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 II12b proximal promoter. Effects of IL-10 on nucleosome remodeling at nucleosome 1 (Nuc1) position (A), and HSS1 was monitored by restriction enzyme-accessibility assays (B). Quantification of SpeI and PstI cleavage products was analyzed by real-time RT-PCR using primers spanning the Nuc1 and HSS1 regions in the II12b promoter. Results are expressed as a percentage of input DNA of AcH4 associated with the II12b promoter. (C) WT and II10−/− BMDMs were stimulated with LPS (10 ng/ml) with or without rIL-10. AcH4 on the II12b promoter was analyzed by ChIP. Results are presented as enrichment (percentage of input DNA) of AcH4 associated with the II12b promoter. (D) II10−/− BMDMs were stimulated with LPS (10 ng/ml) and HDAC inhibitor of class I and II HDACs. Treatment of BMDMs with TSA. Interestingly, TSA prolonged the expression of II12b with or without rIL-10 for 3 h, and recruitment of RNA pol II to the II12b 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.

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 II12b upon stimulation with enteric bacteria. Moreover, both GF and SPF WT colonic macrophages produced abundant IL-10. Colonic macrophages from GF II10−/− mice and II6−/− mice were determined in the presence of HDAC inhibitors. II10−/− 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 control cells (scrambled HDAC3 shRNA) (Fig. 4G). Furthermore, inhibition of HDAC3 prevented histone H4 deacetylation on the II12b 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 II10−/− mice was determined in the presence of HDAC inhibitors. II10−/− 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 control (Fig. 5A). Next, colonic Hdc3 expression was characterized before and after transition of GF mice to an SPF microbiota (34). Interestingly, colonic Hdc3 expression was significantly induced after colonization of WT mice but not colitis-prone II10−/− mice (Fig. 5B). Colonic II12b expression was significantly induced in II10−/− mice but not WT mice upon exposure to SPF microbiota, correlating inversely with Hdc3 induction. As a control, no significant induction of colonic Hdc1 was observed (Fig. 5B).

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 II12b inhibition. We first used TSA, an inhibitor of class I and II HDACs. Treatment of BMDMs with TSA prior to LPS stimulation significantly inhibited II12b transcription induction, as previously described (32) (Supplemental Fig. 4A), greatly affecting our ability to detect IL-10–mediated inhibition of II12b transcription. Therefore, II12b transcription was first induced with LPS for 1 h before treating the BMDMs with TSA. Interestingly, TSA prolonged the expression of II12b in WT BMDMs, resulting in kinetics similar to that in II10−/− 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 II10−/− BMDMs in the presence or absence of TSA to compare IL-10–induced deacetylation on the II12b 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 II12b 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 II12b and II6 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 II12b 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 II12b promoter by IL-10 (Fig. 4H).
Results are presented as percentage of input DNA) of AcH4 associated with the
Il12b promoter. *p < 0.05, versus DMSO. (F) HDAC3 is a homeostatic factor in IL-10–mediated intestinal immunity. (A) Il10−/− conical 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. IL-10 (1 ng/ml) was added 2 h post-E. coli. Results are presented as percent inhibition of IL-12 p40 by IL-10. All results represent mean ± SEM from three independent experiments. (B) WT GF 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...
remodeling of the Il12b promoter. Although 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 Zhou et al. (16) 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 rIL-10. Indeed, the investigators 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 (Figs. 1C, 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 ~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 the loss of IL-10 regulation are so significant biologically. Indeed, IL-10 induces many genes in macrophages at the same time that it inhibits others (35), and it 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 Straw-berry 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 inhibition. Accordingly, multiple redundant mechanisms for IL-10–mediated IL-12 p40 regulation have been described, including NF, 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. Furthermore, we also demonstrated that IL-10, 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


