DEFECTS IN MACROPHAGE SPECIFIC HOMEOSTATIC PATHWAYS IN THE INFLAMMATORY BOWEL DISEASES

SHEHZAD ZAFAR SHEIKH

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology.

Chapel Hill
2010

Approved by:

Advisor: Scott E. Plevy, MD.

Reader: R. Balfour Sartor, MD.

Reader: Jonathan S. Serody, MD.

Reader: Roland M. Tisch, PhD.

Reader: Stephen H. Clarke, PhD.

ABSTRACT

Shehzad Zafar Sheikh: Defects in macrophage specific homeostatic pathways in inflammatory bowel diseases

"Under the direction of Dr. Scott E. Plevy"

Intestinal macrophages are specialized to carry out their functions in the local antigen- and microbiota-rich environment. They are refractory to the induction of pro-inflammatory cytokines, yet still display potent phagocytic and bactericidal activity. These adaptations allow the intestinal macrophage to eradicate microbes that breach the intestinal epithelial barrier while maintaining local tissue homeostasis.

The inflammatory bowel diseases, Crohn's disease and ulcerative colitis, results from an inappropriately directed inflammatory response to the enteric microbiota in a genetically susceptible host. In contrast to the characteristic anti-inflammatory phenotypic and functional profile of normal intestinal macrophages, these macrophages react to luminal microbes and become potent producers of proinflammatory cytokines such as IL-12 family members. IL-12 and 23 are heterodimeric cytokines produced by macrophages and dendritic cells that are important bridges between innate and adaptive immunity.

Although the molecular events that lead to the expression of IL-12 and Il-23 in macrophages through TLR signaling have been well defined, anti-inflammatory pathways

that lead to inhibition of these cytokines in macrophages have not been fully elucidated. We identify two important homeostatic pathways; IFN-γ and HO-1 that regulate enteric microbiota-induced production of IL-12 and 23 by macrophages. IFN-γ inhibits TLR induced IL-23 expression in macrophages, and these events prevent the initiation and progression of spontaneous IL-23-driven experimental colitis in IL-10 deficient (IL-$10^{-/-}$) mice. Moreover, we demonstrate that enteric microbiota-induced heme-oxygenase 1 (HO-1) is critical in the prevention of experimental colitis, through inhibition of IL-12 family members and augmentation of macrophage microbicidal pathways.

In our first study we demonstrate that IFN-γ has anti-inflammatory properties in murine models of Th1/Th17 mediated experimental colitis through attenuation of TLR-mediated IL-23 expression in macrophages. In the second series of experiments, using germ-free WT and colitis-prone IL-$10^{-/-}$ mice we show that intestinal HO-1 expression induced by the enteric microbiota is an important homeostatic pathway. We also identify signaling pathways (MyDD88, MAPK and Pi3K) essential for HO-1 induction in macrophages. Finally, in our third study, protective effects of the HO-1 pathway were determined in Th2-mediated chronic colonic inflammation in T cell receptor- alpha (TCRα) deficient ($^{-/-}$) mice. TCR$\alpha^{-/-}$ mice exposed to carbon monoxide (CO) or treated with a pharmacologic HO-1 inducer demonstrated significant amelioration of active colitis. We demonstrate that HO-1 regulates mucosal innate immune responses in the TCR$\alpha^{-/-}$ mouse through IL-10 induction in colonic macrophages.

ACKNOWLEDGEMENTS

I would like to thank my research advisor Dr. Scott Plevy for his guidance and unwavering support during my tenure as a graduate student. I could not have achieved this important milestone in my career had it not been for your inspirational mentorship. For your tremendous investment of time and effort into making me a better scientist and a physician I remain forever indebted to you. I am also grateful to my dissertation committee members, Dr. Balfour Sartor, Dr. Jonathan Serody, Dr. Roland Tisch and Dr. Stephen Clarke, for generously giving me your valuable time and advice. I am also thankful to the members of the Plevy lab, especially Dr. Katsuyoshi Matsuoka, Dr. Jennifer Uno and Dr. Fengling Li as I could not have accomplished this if were it not for your help and input.

To the casual observer, a doctoral dissertation may appear to be solitary work. However, to complete a project of this magnitude requires a network of support, and I am indebted to many people. To my wife, Huma Sheikh I could not endure the much adversity and hardship that are part and parcel of an academic career without your enduring love and support. Dr. Zafar Sheikh, my father, for instilling and fostering the dignity of hard and honest work in all his children. My sister Saira for sharing my enthusiasm for science, and brother Abdullah for intensifying the joy associated with my success. My son, Zane for reminding me every day of how truly blessed I am. Most importantly, I dedicate this PhD. and all benefits to society it may have to my mother and best friend, Farzana Sheikh.

For “*Ammi*”

Table of Contents

Table of Figures

LIST OF ABBREVIATIONS

AP-1, activator protein-1

BMMs, bone marrow derived macrophages

BV, biliverdin

CD, Crohn's disease

CNS, conserved nucleotide sequence

CNV, conventionalized

CO, carbon monoxide

CoPP, cobalt protoporphyrin

DSS, dextran sodium sulfate

ELISA, enzyme-linked immunosorbant assay

Foxp3, forkhead box P3

GF, germ free

GWAS, genome wide association studies

HO-1, heme oxygenase-1

IBD, inflammatory bowel diseases

IEC, intestinal epithelial cells

IFN-γ, interferon-γ

IFN-γR1$^{-/-}$, interferon-γ receptor 1 deficient

IL-10$^{-/-}$, IL-10 deficient

IRF, interferon regulatory factor

IRF-1$^{-/-}$, interferon regulatory factor 1 deficient

ISRE, interferon stimulated response element

NK, natural killer

RAG, recombinase activating gene

ROR, retinoic acid receptor-related orphan nuclear receptor

SPF, specific pathogen free.

STAT, signal transducer of activated T cells

Tbet, T-box transcription factor expressed in T cells

TCRα, T cell receptor- alpha (TCRα)

Th, T-helper cell

TNBS, trinitrobenzene sulphonic acid

TNF, tumor necrosis factor-α

Treg, CD4+ regulatory T cells

TSLP, thymic stromal lymphopoietin

UC, ulcerative colitis

Chapter 1

INTRODUCTION

1.1. The Inflammatory bowel diseases

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative coltitis (UC) are chronic inflammatory disorders of the gastrointestinal tract that are defined by clinical and pathological features. As many as 1.4 million persons in the United States and 2.2 million persons in Europe suffer from these diseases. Although the incidence and prevalence of CD and UC are beginning to stabilize in high-incidence areas such as northern Europe and North America, they continue to rise in low-incidence areas such as southern Europe, Asia, and much of the developing world (1). Breakdown of immune tolerance, i.e. loss of attenuated innate and adaptive immune responses against the enteric-microbiota, may initiate IBD in genetically susceptible individuals (2). The onset of IBD typically occurs in the second and third decades of life and a majority of affected individuals progress to relapsing and chronic disease (2). CD is characterized by aggregation of macrophages and T cells that frequently form non-caseating granulomas. Gastrointestinal involvement in CD is patchy and segmental, involving both the small bowel and colon, and inflammation typically transmural (ie, extending through all layers of the bowel wall). Key features of UC include diffuse superficial mucosal inflammation that extends proximally from the rectum to a varying degree but is confined to the colon. Histopathological features include the presence of a significant number of neutrophils within the lamina propria and the crypts, where they form micro-abscesses and depletion of goblet cell (2). Despite a continual evolution of therapy for IBD over the past decade and the development of new classes of immunologic interventions (anti-TNFs, anti-integrin monoclonal antibodies), toxicities and a lack of complete response associated with these therapeutic agents demonstrate an unmet need for identification of new potential targets for safer therapy.

Intestinal inflammation in IBD results from an inappropriately directed inflammatory response to the enteric-microbiota in a genetically susceptible host. Genome wide association searches (GWAS) for IBD susceptibility loci have successfully identified genes that affect barrier function, innate and adaptive immune responses in the intestines. The genetic variants that confer Crohn's disease risk highlight the importance of innate immunity include *NOD2* (also designated *CARD15* and *IBD1*) *IBD5*, *IL23R* and *ATG16L1* (3). Interestingly, some genes associated with CD and UC (*IL23R*) are also associated with other chronic inflammatory disorders (psoriasis, spondyloarthrpathies, autoimmune thyroiditis), suggesting that a subset of IBD patients share common immune response defects with these other conditions (3). Despite identification of these important genetic variants underlying mechanisms, functional and biological correlations that cause disease remain largely unexplored.

1.2. The mucosal innate immune response

The innate immune system contributes to the functional integrity of the intestinal mucosa in health and disease through monitoring of luminal contents, especially the microbiota. Key participants in innate immune mediated defenses in the intestine are macrophages. Distinct classes of receptors i.e. Toll-like receptors (TLRs) recognize microbial molecular patterns and are central to the innate immune response (4). 11 human TLRs have been identified. TLRs are expressed in macrophages in addition to many other cell types, including the intestinal epithelium (4). Engagement of these bacterial receptors stimulate central signaling cascades that include nuclear factor-κB (NF-κB), AKT/phosphatidylinositol-3'-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways (Figure 1) (5). These pathways are regulated through induction of inhibitory

molecules, including IκBα, IL-10, and transforming growth factor (TGF)-β (4). Mutations in TLRs have been found to be associated with IBD suggesting that this innate immune detection system is key for regulating mucosal homeostasis (3).

In health, the vast majority of microbial molecular patterns recognized by TLRs in the intestine are not from pathogens but from the enteric-microbiota. TLR signaling in the intestine therefore acts as a double edge sword. TLR expression by intestinal epithelial cells (IECs) shows a polarized pattern. For example, in the small intestine TLR3, TLR4 and TLR5 expression is found predominantly on basolateral surfaces of villus enterocytes (6, 7). In the healthy colon, TLR3 and TLR5 are abundantly expressed, whereas TLR2 and TLR4 expression is low(8). Apical TLR signaling in IECs has been shown to be important for epithelial cell proliferation, IgA production, maintenance of epithelial tight junctions, and antimicrobial peptide expression(7). Despite these beneficial effects on the intestinal epithelium, breaches of the epithelial barrier can trigger pro-inflammatory responses by engagement of basolateral TLRs as well as underlying lamina propria macrophages and DCs. This was demonstrated by using mice that lack specific TLRs or components of the TLR signaling pathway and subjecting them to acute chemical injury in order to disrupt the epithelial barrier. Dextran sodium sulfate (DSS) causes injury to colonic epithelial cells and allows access of luminal bacteria to the lamina propria. MyD88, TLR2 or TLR4 deficient mice are more susceptible to DSS-induced injury than wild type mice (9-11). This observation was confirmed using antibody neutralization of TLR4 or an LPS antagonist (12, 13). DSS treated MyD88 and TLR4 deficient mice demonstrated marked decrease in epithelial cell proliferation an increased apoptosis (4, 14). Therefore, under physiological conditions, bacteria that are unable to penetrate the epithelial barrier (non-pathogenic enteric-microbiota) engage apical TLRs and elicit a homeostatic, anti-inflammatory response. Pathogenic bacteria that are able to breach the barrier elicit a pro-inflammatory

response. However, IECs from patients with IBD express higher levels of TLR4 compared to IECs from healthy controls (8). Inflammatory cytokines like IFN-γ and TNF have been shown to induce transcription of TLR4 and its co-receptor MD2 (15, 16). Although cytokine-mediated induction of TLRs may allow for their selective expression, a cytokine rich milieu and permeable epithelial barrier in IBD results in a constant engagement of upregualted TLRs both across the intestinal epithelial and lamina propria resulting in a vicious cycle of inflammation.

Unlike TLRs, NOD (nucleotide-binding oligomerization domain) 1 and NOD2 are intracellular microbial recognition molecules of the NLR (NOD-like receptor) family (17). Like the TLRs, these proteins are also implicated in the detection of bacterial products and regulate pro-inflammatory pathways in response to bacteria by inducing signaling pathways such as NF-κB and MAPKs. However, the NOD proteins act independently of the TLR cascade, but potently synergize with the latter to trigger innate immune responses to microbes (17). Most importantly, mutations in NOD2 have been shown to confer susceptibility to several chronic inflammatory disorders, including CD, Blau syndrome and early-onset sarcoidosis, underscoring the role of NOD2 in inflammatory homoeostasis (18, 19).

The gastrointestinal innate immune system is in a perpetual state of hyporesponsiveness. Intestinal macrophages show attenuated proliferation and chemotactic activity in response to either microbial ligands or host cytokines/chemokines despite possessing the molecular mechanisms to elaborate strong phagocytic and bactericidal responses(20). However, in IBD, following an inflammatory signal, circulating monocytes migrate to the intestinal mucosa and these cells, unlike resident macrophages, express

TREM1, TLRs (TLR 2,4 and CD14) and are capable of a rapid response to potential luminal triggers e.g. microbiota and microbiota associated products (21).

1.3. The enteric-microbiota in health and disease.

The bacterial component of the enteric-microbiota represents diverse bacterial species, with the lowest total concentration of microbes found in the duodenum and steadily increasing until the peak density of 10^{11}–10^{12} cfu/ml of luminal content in the colon (22, 23). Colonization of the gut with the microbiota occurs immediately after birth followed by a succession of populations until a stable, adult microbiota has been established. However, physiological and environmental conditions eventually determine bacterial density and diversity. Firmicutes and Bacteroidetes dominate the enteric-microbiota in mammals but the bacterial genera and species diversity is huge (24).

Mammals live in a homeostatic symbiosis with their enteric-microbiota. The host provides the microbiota with nutrients and a stable environment; whereas the microbiota helps shaping the host's enteric mucosa and provides nutritional contributions. Wild type mice treated with broad-spectrum antibiotics or antibodies to TLR4 become susceptible to DSS-induced injury akin to mice with TLR-deficiency, suggesting that the enteric-microbiota are required for optimal epithelial expression of genes involved in maintenance of the mucosal barrier (10).

Recently, the enteric-microbiota has been implicated in human obesity, diabetes and cardiovascular diseases (25). When GF mice were conventionalized with a microbiota harvested from conventionally raised mice the body fat content increased 60% compared with GF animals. GF mice also had a higher metabolic rate, increased leptin levels and

increased insulin (26). Similarly, GF mice colonized with microbiota from a mouse with an obese phenotype (ob/ob) gained significantly more body fat than mice receiving microbiota from lean mice, suggesting a contributory effect of the microbiota composition on development of obesity (27). The obese microbiota (Bacteroidetes) is enriched for genes encoding enzymes that break down otherwise indigestible dietary polysaccharides and may have an increased capacity to harvest energy from the diet (27).

Escherichia coli (E.coli) were one of the first bacteria to be linked with IBD. Enterobacterial counts have been found to be up to four-fold higher in IBD patient tissues compared to controls, with increased numbers of *E. coli* belonging to the B2+D group, which is associated with increased pathogenic potential (28). CD patients with active disease have been observed to have increased numbers of fecal enterobacteria (28), while *E. coli* has been reported to account for between 50 to 100% of the bacteria in chronic lesions of the ileal mucosa (29). Thirty-six percent of Crohn's patients with ileal involvement were shown to be colonized by adherent and invasive *E. coli*. The isolates were observed to be able to replicate in macrophages without causing cell death (30),and release large amounts of TNF.

However, the importance of the enteric-microbiota in IBD pathogenesis is supported by numerous lines of evidence from experimental models. IBD is not observed when colitis-prone mouse strains are maintained GF, but emerges when mice are colonized with normal enteric bacterial constituents (31). Moreover, colitis can be induced in a susceptible murine strain with a single species of non-pathogenic bacteria, for example, *Enterococcus faecalis* in the IL-$10^{-/-}$ mouse (31). The enteric-microbiota has been shown to shape the development, distribution, activation level, differentiation status, and inflammatory profile of dendritic cells (DCs), macrophages, natural killer (NK) cells, B cells, $CD4^{+}$ T cells, and $CD8^{+}$ T cells in these susceptible murine strains of colitis (29, 32).

Genetically engineered mice deficient in IL-10 (IL-$10^{-/-}$) display an intestinal phenotype of diarrhea, rectal prolapse, and inflammation. Recent mechanistic studies in IL-$10^{-/-}$ mice have associated uncontrolled IL-23 production by activated macrophages that drive CD4+ Th17 cells with the development of enterocolitis (33). No colitis is observed in mice lacking IL-10 and IL-23. However, mice deficient in both IL-12 and IL-10 developed colitis of similar severity to IL-$10^{-/-}$ mice. Recombinant IL-23 also exacerbated experimental colitis induced in $RAG^{-/-}$ mice by memory T cells ($CD4^{+}CD45RB^{low}$) transferred from diseased IL-$10^{-/-}$ mice (33). Our studies employ this IL-23 driven model of colitis, the IL-$10^{-/-}$ mouse to demonstrate two important *in vivo* homeostatic pathways in the pathogenesis of colitis, IFN-γ and heme oxygenase-1 (HO-1).

1.4. Classification of macrophages

Macrophages are primary responders to many endogenous and exogenous danger signals. Innate responses are rapid, initiated within minutes, and directed toward conserved patterns of carbohydrate and lipid structures on infectious agents (PAMPs) recognized by the germ line-encoded macrophage pattern recognition, TLRs (34). A common myeloid progenitor cell gives rise to monocytes which are released from the bone marrow into the bloodstream. These peripheral blood mononuclear cells (PBMCs) migrate to almost all the tissues in the body under a steady state or in response to inflammation. Innate and adaptive signals can then influence macrophage physiology, and these alterations allow macrophages to participate in homeostatic processes, such as tissue remodeling, wound healing, and host defense (35).

Macrophages have long been classified as M1 (high IL-12 and low IL-10 producing) macrophages and M2 macrophages (low IL-12 and high IL-10 producing). The M1 designation is usually reserved for *classically* activated macrophages and the M2 designation for *alternatively* activated macrophages (36). However, recent work has identified a wide spectrum of macrophage phenotypes. M1 or *classically* activated macrophages are produced during cell-mediated immune responses requiring both IFN-γ and TNF. However, this two-signal requirement can be bypassed by certain TLR agonists that induce both TNF and IFN-β (37). Classically activated macrophages form an integral component of host defense through production of pro-inflammatory cytokines like IL-1, IL-6, IL-12 and IL-23 (37). Additionally, these effectors are essential for eradication of intracellular microorganisms (20). Indeed, mice lacking IFN-γ expression are more susceptible to various bacterial, protozoan or viral infections, as are humans with genetic mutations in these signaling pathways (38). Although these classically activated macrophages are vital components of host defense their activation must be strictly controlled. These macrophages are key mediators of the pathology that occurs during several chronic inflammatory disorders, including rheumatoid arthritis and inflammatory bowel disease.

The M2, or *alternatively* activated macrophages represent a wide array of macrophage phenotypes (36). Part of this spectrum includes macrophages with wound-healing and regulatory properties (39). In response to injury, early increase in IL-4 rapidly converts resident macrophages into a population of cells programmed to promote wound healing through production of extracellular matrix (40). Th2 type adaptive immune responses can also lead to production of IL-4 and IL-13 (41). Despite decreased antigen presentation capacity and reduced pro-inflammatory cytokine secretion, these macrophages can secrete components of the extracellular matrix and primarily function to augment wound healing (41).

Similarly to M1 and wound healing macrophages, regulatory macrophages arise following innate or adaptive immune responses and have been shown to dampen the immune response and limit inflammation (39). Despite subtle differences between various regulatory macrophage subpopulations most require two stimuli to induce anti-inflammatory activity. The first signal (immune complexes, prostaglandins, and adenosine or apoptotic cells) generally has little or no stimulatory function on its own. However, when combined with a second stimulus, such as a TLR ligand, the two signals lead to generation of IL-10 producing macrophages (42). Production of IL-10 and the ability to suppress IL-12 production is a hallmark of most regulatory macrophages (43).

1.5. Resident intestinal macrophages

The local intestinal microenvironment substantially affects the functional and phenotypic differentiation of macrophages. The mucosa of the small and large intestine represents the largest reservoir of tissue macrophages in humans and mice (44) (45). Intestinal lamina propria macrophages are separated from the epithelial cells by the basement membrane. However, subepithelial macrophages may directly interact with intestinal epithelial cells by sending cellular extensions through pores in the basement membrane (46). In contrast to their progenitor cells, the resident monocytes, intestinal macrophages do not serve as professional antigen-presenting cells (APC) due to their low cell surface expression of CD40, CD80, and CD86 (47). Intestinal macrophages are also deficient in several innate immune recognition mechanisms and activating receptors (TLR2, TLR4 and CD14) that make them refractory to LPS and other PAMPs (heat-killed *S. aureus* muramyl dipeptides), which are present abundantly in the intestinal microflora (48, 49). They also lack the Fc receptors for IgA (CD89) and for IgG (CD16, CD32, and CD64) and the

complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18). Similarly, most intestinal macrophages also lack the integrin α2β1 (LFA-1 and CD11a/CD18) (49, 50). Intestinal macrophages also mostly lack the triggering receptor expressed on myeloid cells-1 (TREM-1), an efficient amplifier of acute and chronic inflammatory reactions that is generally expressed on most monocytes and macrophages of secondary lymphoid organs (22).

Intestinal lamina propria macrophages under physiological conditions are generally not only refractory to the induction of proinflammatory cytokine production by PAMPs but also by cytokines (e.g., TNF, IFN-γ), or upon phagocytosis of necrotic cells (20). This is in sharp contrast to most other tissue macrophages and blood monocytes. Intestinal epithelial cells, subepithelial myofibroblasts, fibroblasts, lamina propria lymphocytes, and intraepithelial lymphocytes (IELs) are all able to produce soluble factors, particularly, TGF-β and IL-10, which may affect the phenotypic and functional properties of intestinal macrophages (51, 52).

However, these adaptations made by intestinal macrophages do not impair the phagocytic activity which is exceptionally potent. Compared to circulating monocytes, intestinal macrophages are highly effective at killing phagocytosed microorganisms like *S. typhimurium* and *E. coli* (50). Phagocytosis of apoptotic cells by intestinal macrophages also induces secretion of immunosuppressive cytokines such as IL-10, whereas the secretion of proinflammatory cytokines such as TNF, IL-12, IL-6 and IL-1β is significantly reduced (53). Therefore, intestinal macrophages are globally downregulated for pro-inflammatory cytokine production, regardless of whether the stimulus is a soluble factor or a phagocytic event.

1.6. The intestinal macrophage in the IBDs

In patients with active IBD, the numbers of macrophages are increased in the inflamed intestinal mucosa (2). Many of these macrophages display a different phenotypic and functional profile than under healthy conditions. Macrophages in the inflamed mucosa express relevant levels of T cell co-stimulatory molecules such as CD40, CD80, and CD86 (47). Furthermore, subsets of intestinal macrophages express TLR2, TLR4, CD89 and TREM1 at the site of intestinal inflammation (20) (48). Similarly, a series of studies (18–22) have shown that a high proportion of the macrophages in the inflamed mucosa of patients with IBD express CD14. The absence of these receptors on intestinal macrophages has important functional implications. CD14 expressing macrophages (CD14+) infiltrating the mucosa in IBD produce larger amounts of IL-12, IL-23 and TNF-α compared with those in normal controls. These CD14+ macrophages produce IFN-γ that further triggers abnormal macrophage differentiation with an IL-23-hyperproducing phenotype. In these inflammatory conditions, the close proximity of subepithelial macrophages to the intestinal microbiota thus results in a constant, excessive activation of the mucosal innate immune system (54). CD14+ intestinal macrophages from CD patients respond vigorously to *in vitro* microbial stimulation with production of even more TNF, IL-12 and IL-23, compared with CD14- intestinal macrophages and PBMCs (55). CD14+ macrophages also express TREM1. TREM-1 triggers the synthesis and secretion of proinflammatory cytokines such as IL-1β, MCP-1, IL-6 and IL-8, and consequently local tissue destruction (21). Macrophages are also the main source of TNF during the pathogenesis of IBD. The striking effects of this TNF-targeted therapy may in IBD also be attributed to the cell-depleting effect of some of these therapies, including the removal of TNF membrane bound expressing monocytes/macrophages (56). The absence of CD14, the receptor for complexes of LPS and TLR4 is also consistent with the inability of intestinal macrophages to effectively perform

phagocytosis, a feature well suited to macrophages that reside in an environment rich in LPS. Phagocytosis of bovine serum albumin (BSA) beads is potent in CD14 expressing macrophages, but not in CD14–/CD89– intestinal macrophages (50).

An impaired ability to eradicate intracellular pathogens by macrophages in IBD may also be secondary to their inability to secrete proinflammatory cytokines in response to bacteria or specific TLR agonists. It has been provocatively speculated that despite normal levels and stability of cytokine messenger RNA, intracellular levels of TNF were abnormally low in CD macrophages. Coupled with reduced secretion, these findings indicate accelerated intracellular defects in genes notably those encoding proteins involved in vesicle trafficking may result in an abnormal proportion of cytokines being routed to lysosomes and degraded rather than being released through the normal secretory pathway (57).

Other important features that characterize intestinal macrophages include CD80 expression (23) and respiratory burst activity (24) present in macrophages in the inflamed mucosa of patients with CD. These studies highlight the distinct functional characteristics of intestinal resident macrophages in patients with IBD.

Murine models of experimental colitis have also demonstrated the importance of appropriate macrophage regulation for maintaining local tissue homeostasis in the intestines. Selective disruption of Stat3 leads to impaired IL-10signaling in macrophages consequently leads to the development of colitis (58). Similarly, IL-$10^{-/-}$ mice spontaneously develop colitis as a consequence of the preferential macrophage differentiation into proinflammatory subsets that produce large amounts of IL-12 and IL-23 (59). The depletion of macrophages in these IL-$10^{-/-}$ mice prevents the development of colitis (54). The importance of intestinal macrophages in maintaining adaptive immune homeostasis was recently highlighted in studies showing that that IL-10 secreted from resident intestinal

macrophages acts in a paracrine manner on regulatory T cell (Treg) cells to maintain Foxp3 expression (60). These Foxp3 expressing *Treg* cells have been shown to suppress the activity of Th1 and Th17 cells in inflamed tissues (2). The notion that resident macrophages in patients with IBD display distinct phenotypic characteristics compared with resident intestinal macrophages under physiological conditions was further supported by studies demonstrating mutations in genes encoding the IL10R subunit proteins *IIL10RA* and *IL10RB*) in patients with early-onset enterocolitis (61). Consistent with this observation was the increased secretion of TNF, MIP1α, MIP1β, MCP1 and RANTES in PBMCs from patients who were deficient in IL10R subunit proteins suggesting exaggerated proinflammatory immune responses in the intestine (61).

1.7. Adaptive mucosal immune responses in the IBDs.

Recent GWAS in patients with the IBDs reveal an important role for mucosal innate immune responses to the enteric-microbiota. Ultimately the innate immune response activates specific effector T cell populations that perpetuate chronic intestinal inflammation. Effector CD4+T cells have been subdivided into functional subsets, Th1, Th2, and Th17 based on the cytokines they secrete. Each of these T cell subsets can be detected in CD and UC and may play pathogenic or even protective roles. Cytokines derived from macrophages and DCs initiate the transcriptional programs that specify effector T cell differentiation. These transcription factors and secreted cytokines are crucial for lineage self-preservation through positive feedback and cross-regulatory inhibition of other lineages. Until recently it was widely believed that mature CD4+ effector T cells are inherently stable and non-plastic. However, emerging evidence now demonstrates T regulatory (see below)-to-Th17 and Th17-to-Th1 transitions.

Th1 cells develop in response to antigenic engagement of the T cell receptor through sequential activation of signal transducer of activated T cells (STAT)-1 and STAT4 by IFN-γ and IL-12 (62). This promotes induction of T-box transcription factor expressed in T cells (T-bet) and IFN-γ, its signature transcription factor and cytokine, respectively. In the intestine IL-27, another member of the IL-12 cytokine family can also induce expression of T-bet in a STAT1-dependent manner (63, 64), and regulate Th1 differentiation. Human CD and most experimental models of colitis were originally attributed to Th1-mediated pathogenesis. For example, studies in the CD45RBhi T cell transfer and IL-$10^{-/-}$ mice revealed that colitis was ameliorated with the administration of IFN-γ blocking antibody (65, 66). Similarly, adoptive transfer of T cells deficient in T-bet failed to induce colitis. However, showing that pathogenesis in the adoptive transfer model of colitis isn't strictly Th1-mediated, the transfer of IFN-$\gamma^{-/-}$ CD4+CD45RBhi T cells induces colitis (67). Consistent with this observation, chronic colitis induced by the intestinal commensal organism *Helicobacter hepaticus*, also developed in mice deficient for both IL-10 and IFN-γ, suggesting IFN-γ independent effector mechanisms (68). Human CD was initially postulated to result from an exaggerated Th1 response, based on elevated frequencies of IFN-γ and T-bet-expressing CD4+ T cells in inflamed intestines (67, 69, 70).

Th2 cells develop primarily in response to helminthes and allergens. Th2 cells differentiate from naïve T cells under the influence of IL-4, which activates STAT6 and induces upregulation of the Th2 lineage-specifying transcription factor GATA3 (71). IL-4, IL-5 and IL-13 are hallmark cytokines produced by Th2 cells (72). The oxazolone challenge model of experimental colitis is characterized by IL-4-, IL-5-, and IL-13-secreting CD4+ T cells in the inflamed colon. Importantly, administration of anti- IL-4 suppresses disease activity (73). In a chronic variant of the oxazalone model, intestinal inflammation is also dependent on NK T cell produced IL-13, especially in late stages of inflammation. Th2 cell

responses are rapidly induced in the intestines of mice infected with intestinal helminithic parasites. Parasite infection results in the activation of intestinal epithelial cells (IECs) to produce thymic stromal lymphopoietin (TSLP). TSLP-activated DCs have been shown to drive Th2 responses as well as negatively regulate IL-12 and IL-23 dependent responses in the intestine (74). Similarly, constitutively expressed IL-25 in the intestine heightens expression of Th2 effector cytokines IL-5 and IL-13, both major players in driving chronic colonic inflammation in UC. IL-25 has also been shown to inhibit IL-23 dependent mucosal immune responses in the colon (75). Finally, CD1d-restricted natural killer T (NKT) cells that produce IL-13 have also been shown to be important in the induction and amplification of Th2-cytokine-driven intestinal inflammation in ulcerative colitis (76).

In contrast to CD, early descriptive studies in human UC patients showed the presence of Th2 cyokines in the colon (77). However, human T cell biology is complex, and when characterizing intestinal T cell responses in heterogeneous disease populations, the Th1/Th2 classification that may be relevant to mice is overly simplistic. Indeed, Th17 populations have now been identified in inflamed mucosa from CD and UC patients.

Th17 cells are specific effectors that play a role in host defense against extracellular bacteria and fungi. Their differentiation is induced by combined actions of TGF-β and IL-6 that results in the sequential recruitment of STAT3 and retinoic acid receptor-related orphan nuclear receptor (ROR)γt (78). Additionally, cooperation with other transcription factors like IRF-4, aryl hydrocarbon receptor, AP-1, and RORα also affects Th17 lineage differentiation (79) (80, 81) (82) (83). IL-17A and IL-17F are signature Th17 cell cytokines that promote neutrophil development and recruitment, and enhance epithelial barrier function. IL-22, another Th17 cytokine, is important in intestinal epithelial barrier function (84). IL-22 also acts as a growth factor for Th17 cells in an autocrine manner. Developing Th17 cells acquire

responsiveness to IL-23 through upregulation of the inducible component of the IL-23 receptor (IL-23R) (85). IL-23 is critical for late developmental functions of Th17 cells, essential for their immune-effector activity and pathogenicity (86) (87). Adoptive transfer of intestinal bacteria-reactive Th17 cells into $RAG^{-/-}$ recipient mice induces more severe colitis than comparable transfers of Th1 cells (32). Treatment with an IL-23 monoclonal antibody inhibits colitis development when administered at the time of transfer and also suppresses ongoing disease. This is associated with the depletion of the transferred Th17 effectors (88). Furthermore, deletion of *Il23a*, but, not *Il12a* inhibits spontaneous colitis in IL-$10^{-/-}$ mice implicating IL-23, and not IL-12, in the spontaneous colitis that develops in this model (33). Similarly, treatment with IL-23 accelerates disease onset in $RAG^{-/-}$ recipients of memory T cells harvested from IL-$10^{-/-}$ mice (33). Although Th1 effector pathways cannot be completely disregarded in CD, emerging studies with the discovery of Th17 cells have implicated a dominant role for Th17 in CD and UC pathogenesis.

Interestingly, a sizeable fraction of CD4+ T cells recovered from mucosal compartments of colitic mice following CD45RBhi T cell transfer and IL-$10^{-/-}$ mice reveal a distinct subset of ‘‘double producers’’ that express both IL-17 and IFN-γ (89) (33). Collectively, these results implicate IL-23-dependent Th17 effector mechanisms in the development of chronic intestinal inflammation. IL-17A producing lymphocytes are readily detectable in both CD and UC lesions, the frequencies are higher in CD and correlate with disease activity. Ongoing studies will further elucidate the contribution of IL-17 in the human IBDs.

Regulatory T cells (Tregs) have also emerged as important mediators of intestinal homeostasis (90). In addition to thymic-derived Treg cells, induced Treg cells may be generated in the periphery from activated effector memory CD41 CD25+ cells (91). TGF-β

promotes the development of Foxp3+ Tregs that are associated with suppression of the inflammatory response (92). In contrast, in the presence of proinflammatory cytokines such as IL-6, TGF-β induces the differentiation of Th17 cells. The complex interactions between IL-17 and TGF-β influence intestinal homeostasis and may affect initiation, persistence and relapses in human IBD. In the lamina propria, Treg cells may have evolved to suppress immune responses to enteric microbiota. CD8+ regulatory cells (CD81+, CD282+) have been described in IBD (93). These cells are reduced or absent in lamina propria of patients with IBD. B cells that are produce IL-10 and are CD1d restricted also have a regulatory role in intestinal inflammation, on the basis of observations in a number of murine models (94). Unlike CD4+ Treg cells, these cells are seen only in states of inflammation and suppress progression rather than initiation of murine colitis.

A unique and previously unappreciated feature of CD4+ T cell biology has recently been described: Plasticity among CD4+ effector and regulatory T cell lineages. CD4+ T cells expressing both Foxp3 and RORγt have been detected in the normal intestines of both mice and humans, residing alongside subsets that express one or the other of these factors (95) (96) (97). Additionally, CD4+ T cells isolated from the intestinal mucosa of CD patients demonstrate distinct subsets of Th1 and Th17 cells, as well as IL-17 and IFN-γ double-expressing CD4+ T cells (98). Importantly, a feature common to mice and humans is that when Th17 precursors are adoptively transferred into immunodeficient recipients a subset of these Th17 cells transitions to Th1-type cells. However, memory Th17 cells isolated from the mesenteric lymph nodes of WT mice are resistant to transition to Th1 cells (99), implying that under certain conditions, the Th17 program becomes fixed. Mechanisms involved in programming CD4+ T cell plasticity remain poorly understood.

.

1.8. IL-12 family members and the IBDs

Of the pro-inflammatory genes that are induced in macrophages through interactions with microbes, IL-12 family members play a central role in intestinal inflammation in IBD (100). IL-12 and 23 are heterodimeric cytokines produced by macrophages and DCs. They share a common p40 subunit covalently linked to distinct p35 and p19 chains, respectively (101). The discovery of the IL-12 family member IL-23 has lead to a major paradigm shift in our understanding of inflammatory immune responses. In patients with IBD, there is increased expression of IL-23 in inflamed intestinal mucosa (102, 103). IL-23 plays the major pathogenic role in chronic intestinal inflammation as spontaneous colitis does not develop in IL-$10^{-/-}$ mice crossed with IL-23 $p19^{-/-}$ mice(33). Both IL-12 and IL-23 are involved in inducing and maintaining a Th1 and Th17 responses, respectively (104, 105). Inappropriate regulation of IL-23 may lead to immune-mediated inflammatory disorders such as IBD and multiple sclerosis(100, 106). A variety of rodents that harbor mutations in immune response genes develop IBD. Most of the models manifest Th1/Th17-mediated intestinal inflammation. IL-10 deficient mice develop chronic enterocolitis that is dependent on the presence of the enteric-microbiota(107). Direct evidence for the role of IL-12 and IL-23 in chronic mucosal inflammation has emerged from this and other murine models where treatment with anti-*IL-12 p40* and anti-IL-23 antibodies results in histopathologic improvement (108, 109). Th1/Th17-mediated mouse models of chronic intestinal inflammation share several important immunologic features with the human IBD. Increased production of Th1/Th17 cytokines and IL-12 and IL-23 has been detected in lamina propria cells isolated from CD patients (110, 111). Thus, therapies that inhibit mucosal Th1/Th17 responses and IL-23 may be clinically effective (112, 113), and are being studied in human CD.

1.9. IL-23 in Th17 Immune responses

Early work emphasized redundant functions for IL-12 and IL-23. For example, IL-12 p40$^{-/-}$ (*Il12b*$^{-/-}$) mice which lack biologically active IL-12 and IL-23, and IL-12 p35$^{-/-}$ (*Il12a*$^{-/-}$) mice which only lack IL-12, both demonstrate aberrant immune responses to pathogens. However, significant phenotypic differences between *Il12b*$^{-/-}$ and *Il12a*$^{-/-}$ mice ultimately led to the identification and the description of non-redundant functions for IL-23. Importantly, *Il12a*$^{-/-}$ mice developed increased severity of inflammatory diseases such as experimental allergic encephalomyelitis (EAE) and collagen induced arthritis (CIA), while *Il12b*$^{-/-}$ mice were protected from inflammation (101). Similar to *Il12b*$^{-/-}$ mice, *Il23a*$^{-/-}$ mice were resistant to development of EAE and CIA (101, 106). IL-23, unlike IL-12, promotes a distinct CD4+ T cell phenotype characterized by the production of the inflammatory cytokine IL-17. These so-called Th17 cells develop distinct from the Th1 (IFN-γ producing) and Th2 (IL-4, IL-5, IL-13 producing) lineages under the influence of IL-6 and TGF-β (114). IL-23 enhances Th17 function and survival by acting on differentiated Th17 cells which express the IL-23 receptor. Development of Th1, Th2, and Th17 cells are mutually exclusive as differentiation of one subset is inhibited by the presence of another (86, 114-116). Indeed, IFN-γ, the signature Th1 cytokine induced by IL-12, inhibits Th17 development (117). Enterocolitis in IL-10$^{-/-}$ mice was initially thought to be perpetuated by over-expression of Th1 cytokines driven by IL-12. However, IL-10$^{-/-}$ mice crossed with *Il23a*$^{-/-}$ mice did not develop colitis. Importantly, IL-10$^{-/-}$ mice crossed with *Il12a*$^{-/-}$ mice developed colitis of similar severity to the IL-10$^{-/-}$ founders (33). These studies demonstrate that IL-23, not IL-12, is the major driving force in many forms of chronic intestinal inflammation.

1.10. Molecular regulation of the *Il23a* promoter

Recent work revealed the important requirement for NF-κB in LPS-mediated activation of *Il23a* transcription. NF-κB comprises a group of structurally related proteins that includes five members in mammals: p65 (RelA), c-Rel, Rel-B, p50, and p52; which can homo- and heterodimerize. NF-κB, an inducible transcription factor, plays an essential role in the inflammatory response through the regulation of genes encoding pro-inflammatory cytokines, chemokines, and adhesion molecules(118, 119). The *Il23a* promoter contains three NF-κB binding sites. Mutation of either of two binding sites resulted in complete loss of *Il23a* promoter activity induced by TLR ligands. Additionally, *Il23a* mRNA levels were dramatically reduced in c-Rel- and RelA-deficient macrophages (120, 121).

Inducible chromatin modifications serve as an important restriction point in TLR-regulated gene expression. Acetylation of key amino acids on histone proteins decreases their affinity for DNA, and results in a DNA structure that is more permissive to subsequent recruitment of transcription factors. Important co-activators such as p300 and CBP have been implicated in the positive regulation of *Il12b*. Conversely, histone deacetylases (HDACs) catalyze the deacytelation of histone proteins and are classically associated with the inactivation of gene expression (122). LPS induced TLR stimulation resulted in the rapid and dramatic changes in *Il12b* in murine macrophages by histone acetylation at the *Il12b* locus, enabling transcription factor recruitment (123). With the recent shift in focus from IL-12 to IL-23 in immune mediated pathology, it is important to characterize the epigenetic regulation of the *Il23a* gene locus.

1.11. IFN-γ and IL-12 family members.

IFN-γ strongly synergizes with bacterial products to activate and sustain production of IL-12 by DCs and macrophages (100, 110, 124). How IFN-γ regulates IL-23 has yet to be demonstrated. Our lab and others have extensively characterized the regulation of the *Il12b* promoter by IFN-γ (125-127). Several IFN-γ induced interferon regulatory factors (IRFs) participate in the expression of IL-12. IRF-1 is required for activation of the IL-12 p35 (*Il12a*) gene but does not affect *Il12b* gene expression (128, 129). IRF-8 activates both p35 and p40 gene expression, and synergizes with TLR stimulation (130). The protective role of IRF-1 in mucosal inflammation was recently demonstrated in chemically induced murine colitis by the administration of dextran sodium sulfate (DSS) (131). IRF-$1^{-/-}$ mice demonstrated a dramatic increase in lethality and colitis severity compared with wild type mice. Interestingly, a 250 kb risk haplotype within the *IBD5* locus on chromosome 5q31 that is significantly associated with CD contains the gene for *IRF1 (132-134)*.

Although IFN-γ potently augments Th1-mediated immune responses (25), it also has protective properties in inflammatory disease models. For example, IFN-γ gene deletion or administration of anti-IFN-γ antibodies leads to increased severity of EAE and CIA (106) (135) (136) (137). A number of mechanisms have been proposed to explain the protective effect of IFN-γ in autoimmunity. IFN-γ directly inhibits Th17 differentiation (138). Furthermore, conversion of CD4+CD25− T cells into CD4+ CD25+ T regulatory cells requires IFN-γ, and limits severity of inflammation in EAE (139). Beyond these effects on T cell responses, little is known about homeostatic effects of IFN-γ on innate immunity. IFN-γ was previously shown to abrogate the ability of mycobacteria-infected murine DCs to induce Th17 cells by increasing IL-12 and decreasing IL-23 (117). Our work will reveal that IFN-γ,

long proposed to be pro-inflammatory, plays a protective role in the early initiation stages of murine experimental colitis, predominantly through inhibition of IL-23 in macrophages.

1.12 Environmental factors in the pathogenesis of the IBDs.

Recent worldwide trends in IBD epidemiology support a strong role for the environment. In addition to the enteric-microbiota, factors such as non-steroidal anti-inflammatory drug (NSAIDs) use, oral contraceptive use, appendectomy, dietary factors (e.g. refined sugar, fat, and fast food), perinatal events, and childhood infections have been associated with IBD (140) (141) (142) (143). Economic development, leading to improved hygiene and other changes in lifestyle ('westernized lifestyle') have also been implicated in the increase in IBD (144).

The role of the environment in the pathogenesis of IBD is perhaps most clearly demonstrated by the epidemiological observation that cigarette smoking is protective against the development of UC (145). However, the etiology of this protective effect remains unclear. Cigarette smoke is a complex mix of over 500 characterized compounds, each of which may exert independent immunologic effects. Therapeutic trial experience in UC patients treated with nicotine gum and transdermal nicotine has been inconclusive (146, 147). Based on recent findings, the immunomodulatory effects of cigarette smoke may in part be explained by CO (148). Blood carboxyhemoglobin levels, a measure of systemic exposure to CO, range from 1% to 18% in active smokers (149).

The idea of a gaseous molecule exerting biological function has been well known for greater than one hundred years. Ironically CO was originally used to assist in the description of how hemoglobin carries oxygen. CO has been classified as a toxic entity, lethal to aerobic

life and one of the primary pollutants in industrial society. Surprisingly, CO has recently emerged, akin to nitric oxide, to possess potent cytoprotective and immunologic functions. CO exerts key physiological function in various models of tissue inflammation and injury, including endotoxic shock, hepatic injury, and organ xenotransplantation(150, 151). In each instance CO-mediated protection was associated with inhibition of the inflammatory response. Macrophages exposed to LPS in the presence of CO produced significantly less TNF, and this effect was concentration dependent (from 10–500 ppm CO) (152). In a sepsis model where a sublethal dose of LPS (1 mg/kg) was administered, mice also produced significantly less TNF when exposed to CO *in vivo*. Furthermore, serum IL-10 increased in response to LPS in CO exposed mice. In our current studies, we demonstrate that CO may ameliorate murine experimental colitis. In normal physiology, heme oxygenase-1 (HO-1) enzymatically degrades heme in the body generating low levels of CO (153). We have previously demonstrated that CO ameliorates colitis in colitis prone IL-$10^{-/-}$ mice through a HO-1 dependent pathway in macrophages. In our current study we show that HO-1 and CO protect against mucosal immune responses to the enteric-microbiota through increase in macrophage IL-10 production and by enhancing the ability of macrophages to eradicate intracellular bacteria.

1.13. The anti-inflammatory molecule: Heme oxygenase-1 (HO-1).

HO catalyzes the first and rate-limiting step in the degradation of heme to yield equimolar quantities of biliverdin, CO, and iron (154). Three isoforms of HO exist; HO-1 is highly inducible while HO-2 and HO-3 are constitutively expressed (155). In addition to heme degradation, HO-1 plays a vital function in maintaining cellular homeostasis. HO-1 is highly induced by a variety of agents causing oxidative stress and inflammation (156).

Indeed, the induction of endogenous HO-1 provides protection against LPS-induced tissue injury (157). Furthermore, recent analysis of HO-1 deficient (*hmox1-/-*) mice has strengthened the emerging paradigm that HO-1 is an important molecule in host defense against stress. HO-1-/- mice exhibited increased susceptibility to oxidative stress such as LPS. These mice also demonstrate an exaggerated Th1 response (158). Our group was the first to demonstrate that CO ameliorates active intestinal inflammation in IL-$10^{-/-}$ mouse. CO exerted its anti-inflammatory effects specifically through induction of HO-1 which in turn inhibited IL-12 expression in macrophages. Our current studies reveal that the enteric-microbiota regulates HO-1 in the murine colon. In colitis-prone IL-$10^{-/-}$ mice, enteric-microbiota induced HO-1 is defective resulting in chronic inflammation. Mechanistically, HO-1 derived CO enhances the ability of macrophages to eradicate intracellular bacteria.

1.14 . Defects in macrophage specific homeostatic pathways in the IBDs.

In summary, altered immune responses to the enteric-microbiota and other environmental triggers in a genetically susceptible host lead to chronic intestinal inflammation. This dissertation focuses on macrophage specific regulatory pathways that are protective against the initiation and perpetuation of chronic intestinal inflammation. We identify IL-23 as a central mediator of experimental colitis. We reveal an anti-inflammatory role for IFN-γ in IBD through regulation of IL-12 family members. Furthermore, we identify HO-1 and its byproduct, CO as important '*molecular brakes*' on the pro-inflammatory immune response to enteric- microbiota in IBD. In essence our studies further elucidate the key complex genetic, environmental and immune elements implicated in IBD pathogenesis.

1.15. References

1. Loftus, E.V., Jr. 2004. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 126:1504-1517.

2. Xavier, R.J., and D.K. Podolsky. 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448:427-434.

3. Cho, J.H. 2008. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 8:458-466.

4. Rakoff-Nahoum, S., and R. Medzhitov. 2008. Innate immune recognition of the indigenous microbial flora. *Mucosal Immunol* 1 Suppl 1:S10-14.

5. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat Rev Immunol* 4:499-511.

6. Fusunyan, R.D., N.N. Nanthakumar, M.E. Baldeon, and W.A. Walker. 2001. Evidence for an innate immune response in the immature human intestine: toll-like receptors on fetal enterocytes. *Pediatr Res* 49:589-593.

7. Abreu, M.T. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol* 10:131-144.

8. Cario, E., and D.K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* 68:7010-7017.

9. Fukata, M., K.S. Michelsen, R. Eri, L.S. Thomas, B. Hu, K. Lukasek, C.C. Nast, J. Lechago, R. Xu, Y. Naiki, A. Soliman, M. Arditi, and M.T. Abreu. 2005. Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am J Physiol Gastrointest Liver Physiol* 288:G1055-1065.

10. Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118:229-241.

11. Araki, A., T. Kanai, T. Ishikura, S. Makita, K. Uraushihara, R. Iiyama, T. Totsuka, K. Takeda, S. Akira, and M. Watanabe. 2005. MyD88-deficient mice develop severe intestinal inflammation in dextran sodium sulfate colitis. *J Gastroenterol* 40:16-23.

12. Ungaro, R., M. Fukata, D. Hsu, Y. Hernandez, K. Breglio, A. Chen, R. Xu, J. Sotolongo, C. Espana, J. Zaias, G. Elson, L. Mayer, M. Kosco-Vilbois, and M.T. Abreu. 2009. A novel Toll-like receptor 4 antagonist antibody ameliorates inflammation but impairs mucosal healing in murine colitis. *Am J Physiol Gastrointest Liver Physiol* 296:G1167-1179.

13. Fort, M.M., A. Mozaffarian, A.G. Stover, S. Correia Jda, D.A. Johnson, R.T. Crane, R.J. Ulevitch, D.H. Persing, H. Bielefeldt-Ohmann, P. Probst, E. Jeffery, S.P. Fling, and R.M. Hershberg. 2005. A synthetic TLR4 antagonist has anti-inflammatory effects in two murine models of inflammatory bowel disease. *J Immunol* 174:6416-6423.

14. Abreu, M.T., M. Fukata, and M. Arditi. 2005. TLR signaling in the gut in health and disease. *J Immunol* 174:4453-4460.

15. Abreu, M.T., E.T. Arnold, L.S. Thomas, R. Gonsky, Y. Zhou, B. Hu, and M. Arditi. 2002. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J Biol Chem* 277:20431-20437.

16. Suzuki, M., T. Hisamatsu, and D.K. Podolsky. 2003. Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex. *Infect Immun* 71:3503-3511.

17. Ting, J.P., R.C. Lovering, E.S. Alnemri, J. Bertin, J.M. Boss, B.K. Davis, R.A. Flavell, S.E. Girardin, A. Godzik, J.A. Harton, H.M. Hoffman, J.P. Hugot, N. Inohara, A. Mackenzie, L.J. Maltais, G. Nunez, Y. Ogura, L.A. Otten, D. Philpott, J.C. Reed, W. Reith, S. Schreiber, V. Steimle, and P.A. Ward. 2008. The NLR gene family: a standard nomenclature. *Immunity* 28:285-287.

18. Ogura, Y., D.K. Bonen, N. Inohara, D.L. Nicolae, F.F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R.H. Duerr, J.P. Achkar, S.R. Brant, T.M. Bayless, B.S. Kirschner, S.B. Hanauer, G. Nunez, and J.H. Cho. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411:603-606.

19. Okafuji, I., R. Nishikomori, N. Kanazawa, N. Kambe, A. Fujisawa, S. Yamazaki, M. Saito, T. Yoshioka, T. Kawai, H. Sakai, H. Tanizaki, T. Heike, Y. Miyachi, and T.

Nakahata. 2009. Role of the NOD2 genotype in the clinical phenotype of Blau syndrome and early-onset sarcoidosis. *Arthritis Rheum* 60:242-250.

20. Smith, P.D., C. Ochsenbauer-Jambor, and L.E. Smythies. 2005. Intestinal macrophages: unique effector cells of the innate immune system. *Immunol Rev* 206:149-159.

21. Schenk, M., A. Bouchon, F. Seibold, and C. Mueller. 2007. TREM-1--expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *J Clin Invest* 117:3097-3106.

22. Schenk, M., A. Bouchon, S. Birrer, M. Colonna, and C. Mueller. 2005. Macrophages expressing triggering receptor expressed on myeloid cells-1 are underrepresented in the human intestine. *J Immunol* 174:517-524.

23. Sartor, R.B. 2008. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 134:577-594.

24. Abt, M.C., and D. Artis. 2009. The intestinal microbiota in health and disease: the influence of microbial products on immune cell homeostasis. *Curr Opin Gastroenterol* 25:496-502.

25. Wen, L., R.E. Ley, P.Y. Volchkov, P.B. Stranges, L. Avanesyan, A.C. Stonebraker, C. Hu, F.S. Wong, G.L. Szot, J.A. Bluestone, J.I. Gordon, and A.V. Chervonsky. 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 455:1109-1113.

26. Backhed, F., H. Ding, T. Wang, L.V. Hooper, G.Y. Koh, A. Nagy, C.F. Semenkovich, and J.I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A* 101:15718-15723.

27. Turnbaugh, P.J., R.E. Ley, M.A. Mahowald, V. Magrini, E.R. Mardis, and J.I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444:1027-1031.

28. Kotlowski, R., C.N. Bernstein, S. Sepehri, and D.O. Krause. 2007. High prevalence of Escherichia coli belonging to the B2+D phylogenetic group in inflammatory bowel disease. *Gut* 56:669-675.

29. Darfeuille-Michaud, A., C. Neut, N. Barnich, E. Lederman, P. Di Martino, P. Desreumaux, L. Gambiez, B. Joly, A. Cortot, and J.F. Colombel. 1998. Presence of

adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology* 115:1405-1413.

30. Rolhion, N., and A. Darfeuille-Michaud. 2007. Adherent-invasive Escherichia coli in inflammatory bowel disease. *Inflamm Bowel Dis* 13:1277-1283.

31. Sellon, R.K., S. Tonkonogy, M. Schultz, L.A. Dieleman, W. Grenther, E. Balish, D.M. Rennick, and R.B. Sartor. 1998. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* 66:5224-5231.

32. Elson, C.O., Y. Cong, V.J. McCracken, R.A. Dimmitt, R.G. Lorenz, and C.T. Weaver. 2005. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol Rev* 206:260-276.

33. Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M.A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D.J. Cua, R.A. Kastelein, and D. Rennick. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 116:1310-1316.

34. Gordon, S. 2007. The macrophage: past, present and future. *Eur J Immunol* 37 Suppl 1:S9-17.

35. Gordon, S., and P.R. Taylor. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 5:953-964.

36. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.

37. O'Shea, J.J., and P.J. Murray. 2008. Cytokine signaling modules in inflammatory responses. *Immunity* 28:477-487.

38. Filipe-Santos, O., J. Bustamante, A. Chapgier, G. Vogt, L. de Beaucoudrey, J. Feinberg, E. Jouanguy, S. Boisson-Dupuis, C. Fieschi, C. Picard, and J.L. Casanova. 2006. Inborn errors of IL-12/23- and IFN-gamma-mediated immunity: molecular, cellular, and clinical features. *Semin Immunol* 18:347-361.

39. Mosser, D.M., and J.P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958-969.

40. Kreider, T., R.M. Anthony, J.F. Urban, Jr., and W.C. Gause. 2007. Alternatively activated macrophages in helminth infections. *Curr Opin Immunol* 19:448-453.

41. Loke, P., I. Gallagher, M.G. Nair, X. Zang, F. Brombacher, M. Mohrs, J.P. Allison, and J.E. Allen. 2007. Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. *J Immunol* 179:3926-3936.

42. Edwards, J.P., X. Zhang, K.A. Frauwirth, and D.M. Mosser. 2006. Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol* 80:1298-1307.

43. Gerber, J.S., and D.M. Mosser. 2001. Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J Immunol* 166:6861-6868.

44. Bull, D.M., and M.A. Bookman. 1977. Isolation and functional characterization of human intestinal mucosal lymphoid cells. *J Clin Invest* 59:966-974.

45. Lee, S.H., P.M. Starkey, and S. Gordon. 1985. Quantitative analysis of total macrophage content in adult mouse tissues. Immunochemical studies with monoclonal antibody F4/80. *J Exp Med* 161:475-489.

46. Schenk, M., and C. Mueller. 2007. Adaptations of intestinal macrophages to an antigen-rich environment. *Semin Immunol* 19:84-93.

47. Rugtveit, J., A. Bakka, and P. Brandtzaeg. 1997. Differential distribution of B7.1 (CD80) and B7.2 (CD86) costimulatory molecules on mucosal macrophage subsets in human inflammatory bowel disease (IBD). *Clin Exp Immunol* 110:104-113.

48. Hausmann, M., S. Kiessling, S. Mestermann, G. Webb, T. Spottl, T. Andus, J. Scholmerich, H. Herfarth, K. Ray, W. Falk, and G. Rogler. 2002. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology* 122:1987-2000.

49. Smith, P.D., L.E. Smythies, M. Mosteller-Barnum, D.A. Sibley, M.W. Russell, M. Merger, M.T. Sellers, J.M. Orenstein, T. Shimada, M.F. Graham, and H. Kubagawa. 2001. Intestinal macrophages lack CD14 and CD89 and consequently are down-regulated for LPS- and IgA-mediated activities. *J Immunol* 167:2651-2656.

50. Smythies, L.E., M. Sellers, R.H. Clements, M. Mosteller-Barnum, G. Meng, W.H. Benjamin, J.M. Orenstein, and P.D. Smith. 2005. Human intestinal macrophages

display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* 115:66-75.

51. Khoo, U.Y., I.E. Proctor, and A.J. Macpherson. 1997. CD4+ T cell down-regulation in human intestinal mucosa: evidence for intestinal tolerance to luminal bacterial antigens. *J Immunol* 158:3626-3634.

52. Maloy, K.J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2:816-822.

53. Zhang, W.J., and S.S. Zheng. 2005. In vitro study of immunosuppressive effect of apoptotic cells. *J Zhejiang Univ Sci B* 6:919-925.

54. Kamada, N., T. Hisamatsu, S. Okamoto, T. Sato, K. Matsuoka, K. Arai, T. Nakai, A. Hasegawa, N. Inoue, N. Watanabe, K.S. Akagawa, and T. Hibi. 2005. Abnormally differentiated subsets of intestinal macrophage play a key role in Th1-dominant chronic colitis through excess production of IL-12 and IL-23 in response to bacteria. *J Immunol* 175:6900-6908.

55. Kamada, N., T. Hisamatsu, S. Okamoto, H. Chinen, T. Kobayashi, T. Sato, A. Sakuraba, M.T. Kitazume, A. Sugita, K. Koganei, K.S. Akagawa, and T. Hibi. 2008. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest* 118:2269-2280.

56. van Deventer, S.J. 2001. Transmembrane TNF-alpha, induction of apoptosis, and the efficacy of TNF-targeting therapies in Crohn's disease. *Gastroenterology* 121:1242-1246.

57. Smith, A.M., F.Z. Rahman, B. Hayee, S.J. Graham, D.J. Marks, G.W. Sewell, C.D. Palmer, J. Wilde, B.M. Foxwell, I.S. Gloger, T. Sweeting, M. Marsh, A.P. Walker, S.L. Bloom, and A.W. Segal. 2009. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med* 206:1883-1897.

58. Takeda, K., B.E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster, and S. Akira. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 10:39-49.

59. Sartor, R.B. 2006. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 3:390-407.

60. Murai, M., O. Turovskaya, G. Kim, R. Madan, C.L. Karp, H. Cheroutre, and M. Kronenberg. 2009. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol* 10:1178-1184.

61. Glocker, E.O., D. Kotlarz, K. Boztug, E.M. Gertz, A.A. Schaffer, F. Noyan, M. Perro, J. Diestelhorst, A. Allroth, D. Murugan, N. Hatscher, D. Pfeifer, K.W. Sykora, M. Sauer, H. Kreipe, M. Lacher, R. Nustede, C. Woellner, U. Baumann, U. Salzer, S. Koletzko, N. Shah, A.W. Segal, A. Sauerbrey, S. Buderus, S.B. Snapper, B. Grimbacher, and C. Klein. 2009. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* 361:2033-2045.

62. Murphy, K.M., W. Ouyang, J.D. Farrar, J. Yang, S. Ranganath, H. Asnagli, M. Afkarian, and T.L. Murphy. 2000. Signaling and transcription in T helper development. *Annu Rev Immunol* 18:451-494.

63. Kamiya, S., T. Owaki, N. Morishima, F. Fukai, J. Mizuguchi, and T. Yoshimoto. 2004. An indispensable role for STAT1 in IL-27-induced T-bet expression but not proliferation of naive CD4+ T cells. *J Immunol* 173:3871-3877.

64. Takeda, A., S. Hamano, A. Yamanaka, T. Hanada, T. Ishibashi, T.W. Mak, A. Yoshimura, and H. Yoshida. 2003. Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J Immunol* 170:4886-4890.

65. Berg, D.J., N. Davidson, R. Kuhn, W. Muller, S. Menon, G. Holland, L. Thompson-Snipes, M.W. Leach, and D. Rennick. 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* 98:1010-1020.

66. Powrie, F., M.W. Leach, S. Mauze, S. Menon, L.B. Caddle, and R.L. Coffman. 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* 1:553-562.

67. Simpson, S.J., S. Shah, M. Comiskey, Y.P. de Jong, B. Wang, E. Mizoguchi, A.K. Bhan, and C. Terhorst. 1998. T cell-mediated pathology in two models of experimental colitis depends predominantly on the interleukin 12/Signal transducer and activator of transcription (Stat)-4 pathway, but is not conditional on interferon gamma expression by T cells. *J Exp Med* 187:1225-1234.

68. Kullberg, M.C., A.G. Rothfuchs, D. Jankovic, P. Caspar, T.A. Wynn, P.L. Gorelick, A.W. Cheever, and A. Sher. 2001. Helicobacter hepaticus-induced colitis in

interleukin-10-deficient mice: cytokine requirements for the induction and maintenance of intestinal inflammation. *Infect Immun* 69:4232-4241.

69. Fuss, I.J., M. Neurath, M. Boirivant, J.S. Klein, C. de la Motte, S.A. Strong, C. Fiocchi, and W. Strober. 1996. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 157:1261-1270.

70. Matsuoka, K., N. Inoue, T. Sato, S. Okamoto, T. Hisamatsu, Y. Kishi, A. Sakuraba, O. Hitotsumatsu, H. Ogata, K. Koganei, T. Fukushima, T. Kanai, M. Watanabe, H. Ishii, and T. Hibi. 2004. T-bet upregulation and subsequent interleukin 12 stimulation are essential for induction of Th1 mediated immunopathology in Crohn's disease. *Gut* 53:1303-1308.

71. Ansel, K.M., I. Djuretic, B. Tanasa, and A. Rao. 2006. Regulation of Th2 differentiation and Il4 locus accessibility. *Annu Rev Immunol* 24:607-656.

72. O'Garra, A., L.M. McEvoy, and A. Zlotnik. 1998. T-cell subsets: chemokine receptors guide the way. *Curr Biol* 8:R646-649.

73. Boirivant, M., I.J. Fuss, A. Chu, and W. Strober. 1998. Oxazolone colitis: A murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. *J Exp Med* 188:1929-1939.

74. Artis, D., C.M. Kane, J. Fiore, C. Zaph, S. Shapira, K. Joyce, A. Macdonald, C. Hunter, P. Scott, and E.J. Pearce. 2005. Dendritic cell-intrinsic expression of NF-kappa B1 is required to promote optimal Th2 cell differentiation. *J Immunol* 174:7154-7159.

75. Zaph, C., Y. Du, S.A. Saenz, M.G. Nair, J.G. Perrigoue, B.C. Taylor, A.E. Troy, D.E. Kobuley, R.A. Kastelein, D.J. Cua, Y. Yu, and D. Artis. 2008. Commensal-dependent expression of IL-25 regulates the IL-23-IL-17 axis in the intestine. *J Exp Med* 205:2191-2198.

76. Fuss, I.J., F. Heller, M. Boirivant, F. Leon, M. Yoshida, S. Fichtner-Feigl, Z. Yang, M. Exley, A. Kitani, R.S. Blumberg, P. Mannon, and W. Strober. 2004. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest* 113:1490-1497.

77. Niessner, M., and B.A. Volk. 1995. Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). *Clin Exp Immunol* 101:428-435.

78. Weaver, C.T., R.D. Hatton, P.R. Mangan, and L.E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25:821-852.

79. Brustle, A., S. Heink, M. Huber, C. Rosenplanter, C. Stadelmann, P. Yu, E. Arpaia, T.W. Mak, T. Kamradt, and M. Lohoff. 2007. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* 8:958-966.

80. Quintana, F.J., A.S. Basso, A.H. Iglesias, T. Korn, M.F. Farez, E. Bettelli, M. Caccamo, M. Oukka, and H.L. Weiner. 2008. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453:65-71.

81. Schraml, B.U., K. Hildner, W. Ise, W.L. Lee, W.A. Smith, B. Solomon, G. Sahota, J. Sim, R. Mukasa, S. Cemerski, R.D. Hatton, G.D. Stormo, C.T. Weaver, J.H. Russell, T.L. Murphy, and K.M. Murphy. 2009. The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature* 460:405-409.

82. Veldhoen, M., K. Hirota, A.M. Westendorf, J. Buer, L. Dumoutier, J.C. Renauld, and B. Stockinger. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453:106-109.

83. Yang, X.O., B.P. Pappu, R. Nurieva, A. Akimzhanov, H.S. Kang, Y. Chung, L. Ma, B. Shah, A.D. Panopoulos, K.S. Schluns, S.S. Watowich, Q. Tian, A.M. Jetten, and C. Dong. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28:29-39.

84. Zheng, Y., D.M. Danilenko, P. Valdez, I. Kasman, J. Eastham-Anderson, J. Wu, and W. Ouyang. 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 445:648-651.

85. Weaver, C.T., L.E. Harrington, P.R. Mangan, M. Gavrieli, and K.M. Murphy. 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 24:677-688.

86. McGeachy, M.J., K.S. Bak-Jensen, Y. Chen, C.M. Tato, W. Blumenschein, T. McClanahan, and D.J. Cua. 2007. TGF-beta and IL-6 drive the production of IL-17

and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* 8:1390-1397.

87. McGeachy, M.J., Y. Chen, C.M. Tato, A. Laurence, B. Joyce-Shaikh, W.M. Blumenschein, T.K. McClanahan, J.J. O'Shea, and D.J. Cua. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol* 10:314-324.

88. Elson, C.O., Y. Cong, C.T. Weaver, T.R. Schoeb, T.K. McClanahan, R.B. Fick, and R.A. Kastelein. 2007. Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology* 132:2359-2370.

89. Izcue, A., S. Hue, S. Buonocore, C.V. Arancibia-Carcamo, P.P. Ahern, Y. Iwakura, K.J. Maloy, and F. Powrie. 2008. Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* 28:559-570.

90. Izcue, A., J.L. Coombes, and F. Powrie. 2006. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev* 212:256-271.

91. Akbar, A.N., M. Vukmanovic-Stejic, L.S. Taams, and D.C. Macallan. 2007. The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. *Nat Rev Immunol* 7:231-237.

92. Kim, J.M., and A. Rudensky. 2006. The role of the transcription factor Foxp3 in the development of regulatory T cells. *Immunol Rev* 212:86-98.

93. Allez, M., J. Brimnes, I. Dotan, and L. Mayer. 2002. Expansion of CD8+ T cells with regulatory function after interaction with intestinal epithelial cells. *Gastroenterology* 123:1516-1526.

94. Mizoguchi, A., and A.K. Bhan. 2006. A case for regulatory B cells. *J Immunol* 176:705-710.

95. Miyara, M., Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, A. Mathian, T. Nakahata, T. Yamaguchi, T. Nomura, M. Ono, Z. Amoura, G. Gorochov, and S. Sakaguchi. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 30:899-911.

96. Zhou, L., J.E. Lopes, M.M. Chong, Ivanov, II, R. Min, G.D. Victora, Y. Shen, J. Du, Y.P. Rubtsov, A.Y. Rudensky, S.F. Ziegler, and D.R. Littman. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 453:236-240.

97. Yang, X.O., R. Nurieva, G.J. Martinez, H.S. Kang, Y. Chung, B.P. Pappu, B. Shah, S.H. Chang, K.S. Schluns, S.S. Watowich, X.H. Feng, A.M. Jetten, and C. Dong. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29:44-56.

98. Annunziato, F., L. Cosmi, V. Santarlasci, L. Maggi, F. Liotta, B. Mazzinghi, E. Parente, L. Fili, S. Ferri, F. Frosali, F. Giudici, P. Romagnani, P. Parronchi, F. Tonelli, E. Maggi, and S. Romagnani. 2007. Phenotypic and functional features of human Th17 cells. *J Exp Med* 204:1849-1861.

99. Lexberg, M.H., A. Taubner, A. Forster, I. Albrecht, A. Richter, T. Kamradt, A. Radbruch, and H.D. Chang. 2008. Th memory for interleukin-17 expression is stable in vivo. *Eur J Immunol* 38:2654-2664.

100. Adorini, L. 1999. Interleukin-12, a key cytokine in Th1-mediated autoimmune diseases. *Cell Mol Life Sci* 55:1610-1625.

101. Hunter, C.A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nature reviews* 5:521-531.

102. Fuss, I.J., C. Becker, Z. Yang, C. Groden, R.L. Hornung, F. Heller, M.F. Neurath, W. Strober, and P.J. Mannon. 2006. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. *Inflamm Bowel Dis* 12:9-15.

103. Schmidt, C., T. Giese, B. Ludwig, I. Mueller-Molaian, T. Marth, S. Zeuzem, S.C. Meuer, and A. Stallmach. 2005. Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. *Inflamm Bowel Dis* 11:16-23.

104. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 3:133-146.

105. Brombacher, F., R.A. Kastelein, and G. Alber. 2003. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol* 24:207-212.

106. Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S.A. Lira, D. Gorman, R.A. Kastelein, and J.D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.

107. Madsen, K.L., J.S. Doyle, L.D. Jewell, M.M. Tavernini, and R.N. Fedorak. 1999. Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice [see comments]. *Gastroenterology* 116:1107-1114.

108. Neurath, M.F., I. Fuss, B.L. Kelsall, E. Stuber, and W. Strober. 1995. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med* 182:1281-1290.

109. Davidson, N.J., S.A. Hudak, R.E. Lesley, S. Menon, M.W. Leach, and D.M. Rennick. 1998. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J Immunol* 161:3143-3149.

110. Pallone, F., and G. Monteleone. 1998. Interleukin 12 and Th1 responses in inflammatory bowel disease. *Gut* 43:735-736.

111. Monteleone, G., L. Biancone, R. Marasco, G. Morrone, O. Marasco, F. Luzza, and F. Pallone. 1997. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* 112:1169-1178.

112. Plevy, S. 2002. The immunology of inflammatory bowel disease. *Gastroenterol Clin North Am* 31:77-92.

113. Mannon, P.J., I.J. Fuss, L. Mayer, C.O. Elson, W.J. Sandborn, D. Present, B. Dolin, N. Goodman, C. Groden, R.L. Hornung, M. Quezado, M.F. Neurath, J. Salfeld, G.M. Veldman, U. Schwertschlag, W. Strober, and Z. Yang. 2004. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 351:2069-2079.

114. Harrington, L.E., R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, and C.T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132.

115. Zhou, L., Ivanov, II, R. Spolski, R. Min, K. Shenderov, T. Egawa, D.E. Levy, W.J. Leonard, and D.R. Littman. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 8:967-974.

116. Zhou, L., A.A. Nazarian, J. Xu, D. Tantin, L.M. Corcoran, and S.T. Smale. 2007. An inducible enhancer required for Il12b promoter activity in an insulated chromatin environment. *Mol Cell Biol* 27:2698-2712.

117. Cruz, A., S.A. Khader, E. Torrado, A. Fraga, J.E. Pearl, J. Pedrosa, A.M. Cooper, and A.G. Castro. 2006. Cutting edge: IFN-gamma regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection. *J Immunol* 177:1416-1420.

118. Natoli, G., S. Saccani, D. Bosisio, and I. Marazzi. 2005. Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time. *Nat Immunol* 6:439-445.

119. Karin, M., and A. Lin. 2002. NF-kappaB at the crossroads of life and death. *Nat Immunol* 3:221-227.

120. Carmody, R.J., Q. Ruan, H.C. Liou, and Y.H. Chen. 2007. Essential roles of c-Rel in TLR-induced IL-23 p19 gene expression in dendritic cells. *J Immunol* 178:186-191.

121. Carmody, R.J., Q. Ruan, S. Palmer, B. Hilliard, and Y.H. Chen. 2007. Negative regulation of toll-like receptor signaling by NF-kappaB p50 ubiquitination blockade. *Science* 317:675-678.

122. Smale, S.T., and A.G. Fisher. 2002. Chromatin structure and gene regulation in the immune system. *Annu Rev Immunol* 20:427-462.

123. Weinmann, A.S., S.E. Plevy, and S.T. Smale. 1999. Rapid and selective remodeling of a positioned nucleosome during the induction of IL-12 p40 transcription. *Immunity* 11:665-675.

124. Albrecht, I., T. Tapmeier, S. Zimmermann, M. Frey, K. Heeg, and A. Dalpke. 2004. Toll-like receptors differentially induce nucleosome remodelling at the IL-12p40 promoter. *EMBO Rep* 5:172-177.

125. Zhu, C., K. Gagnidze, J.H. Gemberling, and S.E. Plevy. 2001. Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach. *J Biol Chem* 276:18519-18528.

126. Xiong, H., C. Zhu, H. Li, F. Chen, L. Mayer, K. Ozato, J.C. Unkeless, and S.E. Plevy. 2003. Complex formation of the interferon (IFN) consensus sequence-binding protein

with IRF-1 is essential for murine macrophage IFN-gamma-induced iNOS gene expression. *J Biol Chem* 278:2271-2277.

127. Zhu, C., K. Rao, H. Xiong, K. Gagnidze, F. Li, C. Horvath, and S. Plevy. 2003. Activation of the murine interleukin-12 p40 promoter by functional interactions between NFAT and ICSBP. *J Biol Chem* 278:39372-39382.

128. Brant, S.R., M.F. Picco, J.P. Achkar, T.M. Bayless, S.V. Kane, A. Brzezinski, F.J. Nouvet, D. Bonen, A. Karban, T. Dassopoulos, R. Karaliukas, T.H. Beaty, S.B. Hanauer, R.H. Duerr, and J.H. Cho. 2003. Defining complex contributions of NOD2/CARD15 gene mutations, age at onset, and tobacco use on Crohn's disease phenotypes. *Inflamm Bowel Dis* 9:281-289.

129. Liu, J., S. Cao, L.M. Herman, and X. Ma. 2003. Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. *J Exp Med* 198:1265-1276.

130. Zhao, J., H.J. Kong, H. Li, B. Huang, M. Yang, C. Zhu, M. Bogunovic, F. Zheng, L. Mayer, K. Ozato, J. Unkeless, and H. Xiong. 2006. IRF-8/interferon (IFN) consensus sequence-binding protein is involved in Toll-like receptor (TLR) signaling and contributes to the cross-talk between TLR and IFN-gamma signaling pathways. *J Biol Chem* 281:10073-10080.

131. Siegmund, B., J.A. Sennello, H.A. Lehr, G. Senaldi, C.A. Dinarello, and G. Fantuzzi. 2004. Frontline: interferon regulatory factor-1 as a protective gene in intestinal inflammation: role of TCR gamma delta T cells and interleukin-18-binding protein. *Eur J Immunol* 34:2356-2364.

132. Reinhard, C., and J.D. Rioux. 2006. Role of the IBD5 susceptibility locus in the inflammatory bowel diseases. *Inflamm Bowel Dis* 12:227-238.

133. Rioux, J.D., M.J. Daly, M.S. Silverberg, K. Lindblad, H. Steinhart, Z. Cohen, T. Delmonte, K. Kocher, K. Miller, S. Guschwan, E.J. Kulbokas, S. O'Leary, E. Winchester, K. Dewar, T. Green, V. Stone, C. Chow, A. Cohen, D. Langelier, G. Lapointe, D. Gaudet, J. Faith, N. Branco, S.B. Bull, R.S. McLeod, A.M. Griffiths, A. Bitton, G.R. Greenberg, E.S. Lander, K.A. Siminovitch, and T.J. Hudson. 2001. Genetic variation in the 5q31 cytokine gene cluster confers susceptibility to Crohn disease. *Nat Genet* 29:223-228.

134. Urcelay, E., J.L. Mendoza, A. Martinez, L. Fernandez, C. Taxonera, M. Diaz-Rubio, and E.G. de la Concha. 2005. IBD5 polymorphisms in inflammatory bowel disease: association with response to infliximab. *World J Gastroenterol* 11:1187-1192.

135. Billiau, A., H. Heremans, F. Vandekerckhove, R. Dijkmans, H. Sobis, E. Meulepas, and H. Carton. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol* 140:1506-1510.

136. Kelchtermans, H., S. Struyf, B. De Klerck, T. Mitera, M. Alen, L. Geboes, M. Van Balen, C. Dillen, W. Put, C. Gysemans, A. Billiau, J. Van Damme, and P. Matthys. 2007. Protective role of IFN-gamma in collagen-induced arthritis conferred by inhibition of mycobacteria-induced granulocyte chemotactic protein-2 production. *J Leukoc Biol* 81:1044-1053.

137. Vermeire, K., H. Heremans, M. Vandeputte, S. Huang, A. Billiau, and P. Matthys. 1997. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *J Immunol* 158:5507-5513.

138. Park, H., Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.

139. Wang, Z., J. Hong, W. Sun, G. Xu, N. Li, X. Chen, A. Liu, L. Xu, B. Sun, and J.Z. Zhang. 2006. Role of IFN-gamma in induction of Foxp3 and conversion of CD4+ CD25- T cells to CD4+ Tregs. *J Clin Invest* 116:2434-2441.

140. Feagins, L.A., and B.L. Cryer. Do non-steroidal anti-inflammatory drugs cause exacerbations of inflammatory bowel disease? *Dig Dis Sci* 55:226-232.

141. Okoro, N.I., and S.V. Kane. 2009. Gender-related issues in the female inflammatory bowel disease patient. *Expert Rev Gastroenterol Hepatol* 3:145-154.

142. Radford-Smith, G.L. 2008. What is the importance of appendectomy in the natural history of IBD? *Inflamm Bowel Dis* 14 Suppl 2:S72-74.

143. Lakatos, P.L. 2009. Environmental factors affecting inflammatory bowel disease: have we made progress? *Dig Dis* 27:215-225.

144. Asakura, H., K. Suzuki, T. Kitahora, and T. Morizane. 2008. Is there a link between food and intestinal microbes and the occurrence of Crohn's disease and ulcerative colitis? *J Gastroenterol Hepatol* 23:1794-1801.

145. Regueiro, M., K.E. Kip, O. Cheung, R.A. Hegazi, and S. Plevy. 2005. Cigarette smoking and age at diagnosis of inflammatory bowel disease. *Inflamm Bowel Dis* 11:42-47.

146. Pullan, R.D., J. Rhodes, S. Ganesh, V. Mani, J.S. Morris, G.T. Williams, R.G. Newcombe, M.A. Russell, C. Feyerabend, G.A. Thomas, and et al. 1994. Transdermal nicotine for active ulcerative colitis. *N Engl J Med* 330:811-815.

147. Lashner, B.A., S.B. Hanauer, and M.D. Silverstein. 1990. Testing nicotine gum for ulcerative colitis patients. Experience with single-patient trials. *Dig Dis Sci* 35:827-832.

148. van der Vaart, H., D.S. Postma, W. Timens, and N.H. ten Hacken. 2004. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* 59:713-721.

149. Smith, C.J., T.D. Guy, M.F. Stiles, M.J. Morton, B.B. Collie, B.J. Ingebrethsen, and J.H. Robinson. 1998. A repeatable method for determination of carboxyhemoglobin levels in smokers. *Hum Exp Toxicol* 17:29-34.

150. Otterbein, L.E., P.J. Lee, B.Y. Chin, I. Petrache, S.L. Camhi, J. Alam, and A.M. Choi. 1999. Protective effects of heme oxygenase-1 in acute lung injury. *Chest* 116:61S-63S.

151. Soares, M.P., Y. Lin, J. Anrather, E. Csizmadia, K. Takigami, K. Sato, S.T. Grey, R.B. Colvin, A.M. Choi, K.D. Poss, and F.H. Bach. 1998. Expression of heme oxygenase-1 can determine cardiac xenograft survival. *Nat Med* 4:1073-1077.

152. Otterbein, L.E., F.H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R.J. Davis, R.A. Flavell, and A.M. Choi. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 6:422-428.

153. Otterbein, L.E., M.P. Soares, K. Yamashita, and F.H. Bach. 2003. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 24:449-455.

154. Tenhunen, R., H.S. Marver, and R. Schmid. 1970. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. *J Lab Clin Med* 75:410-421.

155. McCoubrey, W.K., Jr., T.J. Huang, and M.D. Maines. 1997. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem* 247:725-732.

156. Applegate, L.A., P. Luscher, and R.M. Tyrrell. 1991. Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. *Cancer Res* 51:974-978.

157. Otterbein, L.E., J.K. Kolls, L.L. Mantell, J.L. Cook, J. Alam, and A.M. Choi. 1999. Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. *J Clin Invest* 103:1047-1054.

158. Kapturczak, M.H., C. Wasserfall, T. Brusko, M. Campbell-Thompson, T.M. Ellis, M.A. Atkinson, and A. Agarwal. 2004. Heme oxygenase-1 modulates early inflammatory responses: evidence from the heme oxygenase-1-deficient mouse. *Am J Pathol* 165:1045-1053.

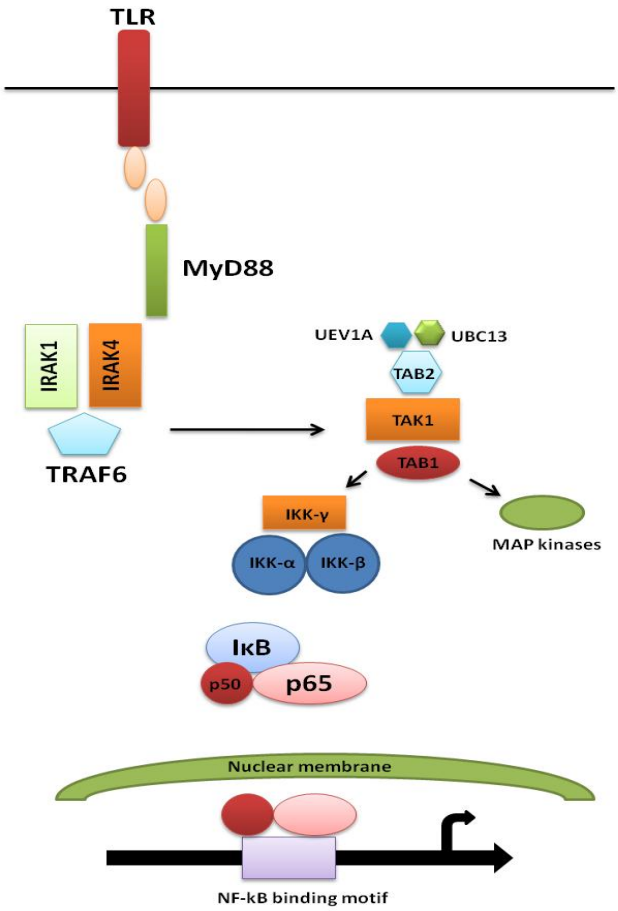


Figure 1. TLR signaling pathways. Stimulation of TLRs triggers the association of MyD88 (myeloid differentiation primary-response protein 88), which in turn recruits IRAK4 (IL-1R-associated kinase 4), thereby allowing the association of IRAK1. IRAK4 then induces the phosphorylation of IRAK1. TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) is also recruited to the receptor complex, by associating with phosphorylated IRAK1. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TAK1 (transforming-growth-factor-β-activated kinase), TAB1 (TAK1-binding protein 1), which induces the phosphorylation of TAB2 and TAK1. IRAK1 is degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1 and TAB2) translocates to the cytosol, where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. TAK1, in turn, phosphorylates both mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex), which consists of IKK-α, IKK-β and IKKγ (nuclear factor-κB (NF-κB) essential modulator, NEMO). The IKK complex then phosphorylates IκB, which leads to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes.

CHAPTER 2

IFN-γ IS A NEGATIVE REGULATOR OF IL-23 IN MURINE MACROPHAGES AND EXPERIMENTAL COLITIS

SHEHZAD Z. SHEIKH, KATSUYOSHI MATSUOKA, TAKU KOBAYASHI, FENGLING LI, TARA RUBINAS, SCOTT E. PLEVY

2.1 Abstract

Rationale: IL-12 and IL-23 are heterodimeric cytokines expressed in macrophages composed of a common p40 subunit (*Il12b*) and a p35 (*Il12a*) and p19 subunit (*Il23a*), respectively. IL-23 regulation is established as a central event in the pathogenesis of the inflammatory bowel diseases (IBD). Objectives: Here, we determine molecular mechanisms of *Il23a* regulation in murine macrophages and elucidate novel *Il23a* regulatory pathways in experimental colitis. Results: We demonstrate that IFN-γ attenuates LPS mediated IL-23 expression in murine macrophages. Mechanistically, IFN-γ inhibits *Il23a* promoter activation through interferon regulatory factor (IRF)-1, NF-κB interactions, and histone modifications. Moreover, intestinal inflammation is inhibited by IFN-γ signaling through attenuation of *Il23a* gene expression. The enteric-microbiota induce colonic IL-23 in colitis-prone IL-10$^{-/-}$ mice but not wild type mice when transitioned from germ-free to conventionalized microbiota. Importantly, IFN-γ receptor 1/IL-10 and IRF-1/IL-10 double deficient mice demonstrate increased colonic inflammation and *IL23a* expression compared to IL-10$^{-/-}$ mice. Colonic CD11b^{+} macrophages are a source of IL-23 and a target for IFN-γ. Conclusions: This study describes an important anti-inflammatory role for IFN-γ through inhibition of IL-23 in macrophages. Converging human genetic and functional findings suggest that IL-23, IFN-γ and IRF-1 may be important pathogenic molecules in human IBD.

2.2 Introduction

The inflammatory bowel diseases (IBD) result from inappropriately directed inflammatory responses to the enteric-microbiota in a genetically susceptible host (1). Key participants in the innate immune response to the enteric-microbiota are macrophages and dendritic cells (DCs) (2). These cells recognize microbial products through pattern recognition receptors and elaborate inflammatory cytokines that recruit other inflammatory cells and activate T cell responses (3). Of the inflammatory genes that are induced in macrophages through interactions with microbes, IL-12 family members play a central role in mediating intestinal inflammation (4). Recently the IL-12 family member IL-23 has been implicated in the pathogenesis of human IBD (5, 6). Anti-IL-12/23 antibodies ameliorate colitis in experimental models such as IL-10$^{-/-}$ mice (5, 7) and show promise in early clinical trials in human Crohn's disease (CD) (8, 9). However, the molecular regulation and the biological significance of *Il23a* expression in chronic intestinal inflammation are incompletely understood.

IL-12 and IL-23 are heterodimeric cytokines composed of a common p40 subunit and a p35 and p19 subunit, respectively (10). The IL-12 p40 (*Il12b*) and IL-23 p19 (*Il23a*) subunits are expressed in macrophages and DCs and are induced by microbial stimuli such as LPS (11). IL-23, unlike IL-12, promotes a distinct CD4+ T cell phenotype characterized by the production of the cytokine IL-17, denoted T_H17 cells. IL-23 enhances T_H17 function and survival by acting on differentiated T_H17 cells which express the IL-23 receptor. Development of T_H1, T_H2, and T_H17 cells are mutually exclusive as differentiation of one subset is inhibited by the presence of another (12). Indeed, IFN-γ, the signature T_H1 cytokine induced by IL-12, inhibits T_H17 development (13). Moreover, IFN-γ strongly synergizes with bacterial products to activate and sustain production of IL-12 by DCs and macrophages (14).

Although the molecular events leading to T cell subset differentiation are well studied in T cells, less is known about the coordinate regulation of IL-12 family members in macrophages. Here, we demonstrate that IFN-γ inhibits LPS-mediated *Il23a* expression in murine macrophages through recruitment of interferon regulatory factor (IRF)-1 to an interferon stimulated response element (ISRE), and through inhibition of NF-κB recruitment to the *IL23a* promoter. In contrast, IFN-γ augments LPS induced *Il12b* and *Il12a* expression. Our experiments also suggest that intestinal inflammation is inhibited by IFN-γ through attenuation of *Il23a* gene expression. Importantly, the enteric-microbiota induce colonic IL-23 in colitis-prone IL-10$^{-/-}$ mice but not wild type mice when transitioned from germ-free (GF) to conventionalized (CNV) microbiota. IL-10/IRF-1 (IL-10/IRF-1$^{-/-}$) and IL-10/IFN-γR1 (IL-10/IFN-γR1$^{-/-}$) double deficient mice reveal increased colonic *Il23a* gene expression and more severe inflammation compared to IL-10$^{-/-}$ mice. We implicate colonic CD11b^{+} macrophages as a primary source of IL-23 and a target for IFN-γ during the initiation of spontaneously occurring colonic inflammation.

2.3 Results

2.3.1 IFN-γ inhibits LPS-induced IL-23 in murine macrophages. IL-23, IL-12 p40 and IL-12 p70 expression was studied in bone marrow-derived macrophages (BMMs) from C57BL/6 mice. LPS was a potent inducer of IL-12 p40, IL-12 p70, and IL-23 protein expression. IFN-γ significantly inhibited LPS-induced IL-23. However, IFN-γ and LPS synergistically induced IL-12 p40 and IL-12 p70 in BMMs, as previously reported (4, 15). Consequently, in LPS-activated BMMs from interferon-γ receptor 1 (IFN-γR1)$^{-/-}$ mice, a loss of IFN-γ mediated IL-23 inhibition (Figure 2.1A), as well as IL-12 p40 (Figure 2.1B) and p70 (Figure 2.1C) synergistic induction was observed.

The kinetics of *Il23a* and *Il12b* mRNA expression was next determined in LPS-stimulated BMMs, with or without IFN-γ. *Il23a* was rapidly induced by LPS one hour after stimulation. Notably, IFN-γ inhibited LPS induced *Il23a* and augmented *Il12b* mRNA expression. *Il23a* expression levels returned to baseline by 6 hours, while LPS plus IFN-γ induced *Il12b* continued to rise (Figure 2.1D). These results reveal important differences between the regulation of *Il23a*; which is rapidly induced, rapidly diminished, and strongly inhibited by IFN-γ; compared to *Il12b*.

IL-10 inhibits LPS-induced IL-23, IL-12 p40 and IL-12 p70 in macrophages (Supplemental Figure 2.1A-C) (16, 17). Whether the inhibitory effects of IFN-γ on IL-23 expression were dependent on the production of IL-10 in BMMs was next determined. IL-10$^{-/-}$ macrophages demonstrate significantly enhanced LPS-induced IL-23 secretion compared to wild type (WT) BMMs. IFN-γ inhibited IL-23 expression in IL-10$^{-/-}$ BMMs, and inhibition was abrogated in IL-10$^{-/-}$/IFN-γR1$^{-/-}$ double deficient (IL-10/IFN-γR1$^{-/-}$) BMMs (Figure 2.2A). IFN-γ-mediated synergistic induction of IL-12 p40 (Figure 2.2B) and IL-12 p70 (Figure 2.2C)

was also abrogated in IL-10/IFN-γR1$^{-/-}$ BMMs. These results suggest that IFN-γ inhibits IL-23 in macrophages through IL-10 independent mechanisms.

2.3.2 *Il23a* is a primary response gene. The inflammatory response against microbes requires rapid and selective activation of numerous genes in macrophages. Primary response genes have promoters that either exist in an open chromatin structure and/or undergo rapid nucleosome remodeling. In contrast, secondary response genes with delayed induction kinetics require selective ATP dependent nucleosome remodeling at their promoters and new protein synthesis prior to transcription initiation (18). In the presence of the protein synthesis inhibitor cyclohexamide, *Il23a* was rapidly induced by LPS in BMMs (Figure 2.3; Supplemental Figure 2.2, upper panel). The preservation of *Il23a* induction in the absence of new protein synthesis is consistent with other described primary response genes such as *tnf* and *Cxcl2* (Supplemental Figure 2.2, upper panel), as recently reported (18). Unlike *Il23a*, *Il12b* induction is dramatically reduced in the absence of new protein synthesis, similar to other secondary response genes such as *inos* and *Il6* (Figure 2.3; Supplemental Figure 2.2, lower panel) (18). Interestingly, in the absence of new protein synthesis, IFN-γ mediated inhibition of LPS induced *Il23a* is preserved at 1 hour but lost at 3 hours, suggesting multiple mechanisms through which IFN-γ inhibits *Il23a* gene expression. In contrast, *Il12b* induction by LPS and IFN-γ remains inhibited at 1 and 3 hours in the absence of new protein synthesis (Figure 2.3). These results further characterize *Il23a* as a primary response gene and provide insight into diverse mechanisms through which IFN-γ may negatively regulate its transcription.

2.3.3 Characterization of an ISRE in the *Il23a* promoter. To characterize potential IFN-γ responsive regulatory regions within the *Il23a* locus, conserved nucleotide sequences (CNS)

were identified in multiple species around the *Il23a* gene (Supplemental Figure 2.3A). Within a murine proximal promoter CNS, a putative interferon stimulated response element (ISRE) was located at -378 to -384 with respect to the transcription start site (Supplemental Figure 2.3A). A 1.8 kb fragment of the promoter containing this ISRE was cloned upstream of a luciferase reporter gene (provided by Dr. Y.H. Chen**,** University of Pennsylvania School of Medicine) for functional analyses (Supplemental Figure 2.3B). *AMAXA Nucleofector* technology was optimized to transiently transfect BMMs (transfection efficiencies of 50% are routinely obtained; data not shown). LPS strongly induced *Il23a* promoter activity and IFN-γ inhibited luciferase activity. BMMs transfected with a reporter plasmid containing a site directed mutation within the ISRE demonstrated abrogation of IFN-γ inhibition of LPS-induced luciferase activity (Figure 2.4A). These results suggest that interactions on or around this ISRE are important for regulation of the *Il23a* by LPS and IFN-γ.

2.3.4 Interferon regulatory factors (IRFs) interact with an ISRE in the *Il23a* promoter.

To determine DNA-protein interactions at the *Il23a* promoter ISRE, electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) experiments were performed. Using an EMSA probe that spans the *Il23a* promoter sequence –368 to –388 (368/388, containing the ISRE), nuclear extracts from LPS and IFN-γ activated BMMs demonstrated enhanced protein binding (Figure 2.4B, complexes I and II, Lanes 3-11) compared to extracts from unstimulated cells. An EMSA probe (368/388m) with a mutated sequence from –378 to –384 within the ISRE abrogated DNA binding of complexes I and II (Figure 2.4B, Lane 12). Likewise, competition experiments with unlabelled double stranded oligonucleotides corresponding to probe 368/388 (368/388u, Figure 2.4B, Lane 6) revealed significant loss of complex I and II DNA binding. When nuclear extracts from LPS and IFN-γ stimulated BMMs were pre-incubated with specific antibodies to IRF-1, but not IRF-2 and 8;

inhibition and supershift of complexes I and II were observed, suggesting recruitment of IRF-1 to the ISRE on the *Il23a* promoter (Figure 2.4B Lane 7). As another negative control, antibodies to c-Rel did not inhibit or supershift complexes I or II (Figure 2.4B, Lane 10).

To assess whether IRFs associate with the *Il23a* ISRE *in vivo*, chromatin immunoprecipitation (ChIP) experiments were performed using IRF-1, IRF-2 and IRF-8 antibodies and cross-linked chromatin prepared from unstimulated or LPS ± IFN-γ activated BMMs. Quantitative real-time PCR analysis (using PCR primers spanning the ISRE) of DNA immunoprecipitated with IRF antibodies revealed that IRF-1, 2 and 8 associate with the ISRE of the *Il23a* promoter in LPS-activated BMMs (Figure 2.4C, left panel). However, the PCR signal was markedly increased in ChIPs with the IRF-1 antibody 1 hour post LPS and IFN-γ stimulation compared with IRF-2 and IRF-8 (Figure 2.4C, left panel). The ISRE region was not enriched when a rabbit polyclonal IgG antibody was used as a control (Figure 2.4C, left panel). ChIP experiments at the *Il12b* locus using PCR primers that amplified sequences that include an ISRE (-62 to -71) demonstrate that IRF-8 association is augmented in LPS and IFN-γ-activated BMMs relative to IRF-1 and IRF-2 (Figure 2.4C, right panel), as previously reported (15).

2.3.5 IRF-1 negatively regulates LPS-induced *Il23a* gene expression in BMMs. The functional role of IRF-1 in IL-23 regulation was next studied. LPS-activated IL-23 protein secretion was significantly enhanced in BMMs from IRF-$1^{-/-}$ mice compared to WT BMMs. IFN-γ inhibited LPS-induced IL-23 in both WT and IRF-$1^{-/-}$ BMMs (Figure 2.5A). In the presence of IFN-γ, IL-23 levels were higher in IRF-$1^{-/-}$ BMMs than in LPS-activated WT BMMs, although inhibition of LPS-induced IL-23 was still apparent. LPS and IFN-γ-stimulated IRF-$1^{-/-}$ BMMs revealed significantly reduced levels of IL-12 p70 (Figure 2.5B)

and minimal differences in IL-12 p40 protein (Figure 2.5C) compared to WT BMMs, as previously reported (16). To validate these findings, IRF-1 expression in WT BMMs was inhibited using siRNA. LPS-induced *Il23a* mRNA was significantly increased in WT BMMs transfected with IRF-1 siRNA compared with WT BMMs transfected with control scrambled siRNA (Figure 2.5D, lower panel). Western blot analysis confirmed that IRF-1 siRNA effectively decreased IRF-1 expression (Figure 2.5D, upper panel).These results implicate IRF-1 as a negative regulator of LPS-induced *Il23a*. These experiments also suggest that IRF-1 is involved in IFN-γ mediated inhibition of IL-23, but IFN-γ also has IRF-1 independent effects on IL-23 expression.

2.3.6 IFN-γ prevents RelA binding to the *Il23a* promoter. Two NF-κB sites have been reported to mediate LPS-induced activation of the *Il23a* promoter in murine macrophages (19, 20). BMMs were cultured with LPS plus IFN-γ, and occupancy of RelA on the distal *Il23a* NF-κB binding site (see Supplemental Figure 2.3B) was analyzed by ChIP using PCR primers from -549 to -680. Recruitment of RelA was demonstrated 1 hour after LPS stimulation. IFN-γ inhibited LPS-induced RelA recruitment to the *Il23a* promoter and enhanced the recruitment of NF-κB p50 (Figure 2.6A). Interestingly, LPS-induced RelA occupancy of the *Il23a* proximal promoter was prolonged in IRF-$1^{-/-}$ BMMs compared to WT BMMs (Figure 2.6B).

Histone acetylation is associated with transcriptionally active chromatin (21). The core histone H4 was acetylated (H4Act) one hour after LPS stimulation at the distal *Il23a* NF-κB binding site. IFN-γ inhibited LPS-induced histone H4 acetylation (Figure 2.6C). In contrast, as previously reported (22), LPS plus IFN-γ stimulation was associated with histone H4 acetylation around an NF-κB site in the *Il12b* proximal promoter (Figure 2.6D).

Therefore, IFN-γ may limit RelA access to the *Il23a* promoter by altering the dynamics of NF-κB subunit recruitment and chromatin level nucleosome remodeling by regulating covalent histone modifications. Additionally, IRF-1 may prevent prolonged *Il23a* promoter occupancy by RelA.

2.3.7 The enteric-microbiota induce colonic IL-23 expression in IL-10 deficient (IL-10$^{-/-}$) mice. IL-10$^{-/-}$ mice develop chronic intestinal inflammation mediated by IL-23 (5). We investigated the role of the enteric-microbiota in the regulation of mucosal IL-23 in wild type (WT) and IL-10$^{-/-}$ mice raised germ free (GF) and transitioned to a conventionalized (CNV) specific pathogen free microbiota at 8 weeks of age. Two weeks after transition, colonic explants from CNV IL-10$^{-/-}$ mice secreted significantly more IL-23 (Figure 2.7A) than GF WT, GF IL-10$^{-/-}$ and CNV WT mice. A significant increase in colonic *Il23a* (Figure 2.7B) mRNA was detected in IL-10$^{-/-}$ compared to WT mice as early as 7 days post-colonization with enteric- microbiota. Additionally, secretion of the IL-23 target IL-17 (Figure 2.7C) from colonic explants of CNV IL-10$^{-/-}$ mice was markedly increased compared to GF and CNV WT mice and GF IL-10$^{-/-}$ mice. Increased colonic expression of IL-23 correlated with severity of intestinal inflammation (Figure 2.7D). IL-12 p40 (Supplemental Figure 2.4A) and *Il12b* mRNA (Supplemental Figure 2.4B) were also increased in colons from CNV IL-10$^{-/-}$ mice compared to GF WT, GF IL-10$^{-/-}$ and CNV WT mice. However, IL-12 p70 secretion was undetectable. These results further implicate IL-23 and not IL-12 in the pathogenesis of enteric-microbiota dependent colitis in IL-10$^{-/-}$ mice.

2.3.8 IFN-γ inhibits *Il23a* expression in colonic CD11b^{+} lamina propria cells from IL-10$^{-/-}$ mice. Colonic CD11b^{+} lamina propria mononuclear cells (LPMC) from IL-10$^{-/-}$ mice were

the primary source of *Il12a, Il12b* and *Il23a* (Figure 2.8A). IFN-γ inhibited heat killed *E. coli*-induced expression of *Il23a* in colonic CD11b^{+} IL-10$^{-/-}$ LPMC, while *Il12b* expression was augmented. Heat killed *E. coli*-stimulated WT colonic CD11b^{+} LPMC did not demonstrate *Il23a* or *Il12b* induction (Figure 2.8B). These studies show that IFN-γ and IL-10 are negative regulators of IL-23 in colonic macrophages.

2.3.9 Increased mucosal expression of IL-23 correlates with severity of colonic inflammation in IFN-γR1/IL-10 $^{-/-}$ and IRF-1/IL-10$^{-/-}$ mice. To understand consequences of IFN-γ and IRF-1 deficiency in the development of colitis, colonic inflammation and IL-23 expression was determined in IL-10$^{-/-}$, IFN-γR1/IL-10 $^{-/-}$ and IRF-1/IL-10$^{-/-}$ mice. Eight week old IL-10$^{-/-}$ mice demonstrated minimal or no inflammatory changes. However, age matched littermate IL-10/IFN-γR1 $^{-/-}$ and IL-10/IRF-1$^{-/-}$ mice developed significant colonic inflammation (Figure 2.9A). By 20 weeks of age, 50% (5/10 mice) of IL-10/IRF-1$^{-/-}$ and 30% (3/10 mice) IL-10/IFN-γR1$^{-/-}$ mice developed rectal prolapse, a sign of significant inflammatory disease, whereas no IL-10$^{-/-}$ mice developed rectal prolapse (data not shown). Severity of colonic inflammation correlated with increased colonic *Il23a* expression and IL-23 secretion in supernatants from colon explant cultures (Figure 2.9B and 2.9C, respectively). There were no significant differences in colonic *Il12b* and *Il12a* expression between IL-10$^{-/-}$, IFN-γR1/IL-10 $^{-/-}$ and IRF-1/IL-10$^{-/-}$ mice (Supplemental Figure 2.5). Moreover, IRF-1/IL-10$^{-/-}$ colonic CD11b^{+} LPMCs demonstrated increased basal and heat killed *E. coli*-activated *Il23a* expression compared to IL-10$^{-/-}$ and WT CD11b^{+} LPMCs (Figure 2.9D). These experiments implicate IFN-γ and IRF-1 as negative regulators of IL-23 and colonic inflammation in IL-10$^{-/-}$

mice, and suggest that a primary defect in *Il23a* inhibition in colonic macrophages may underlie the phenotype of severe IBD in IFN-γR1/IL-10 $^{-/-}$ and IRF-1/IL-10$^{-/-}$ mice.

2.4 Discussion

We have identified anti-inflammatory properties of IFN-γ signaling in murine macrophages and experimental colitis. IFN-γ inhibits IL-23 expression in macrophages activated through TLR pathways. Mechanistically, IFN-γ regulates IL-23 through IRF-1, NF-κB interactions, and histone modifications around an ISRE and an NF-κB site in the *Il23a* promoter. We propose that local effects of IFN-γ signaling in the intestine may be essential in maintaining homeostasis through inhibition of IL-23. This hypothesis is supported *in vivo* through several experimental observations. The enteric-microbiota induce colonic IL-23 in colitis-prone IL-10$^{-/-}$ mice but not wild type mice when transitioned from GF to CNV microbiota. Moreover, IFN-γR1/IL-10$^{-/-}$ and IRF-1/IL-10$^{-/-}$ mice demonstrate increased colonic inflammation and mucosal IL-23 expression compared to IL-10$^{-/-}$ mice. Colonic CD11b^{+} macrophages are implicated as a primary source of IL-23 and a target for IFN-γ during the initiation of spontaneously occurring colonic inflammation.

Elucidation of IL-23 biology has led to advances in our understanding of inflammatory immune responses previously ascribed to IL-12. For example, IL-10$^{-/-}$ mice crossed with *Il23a*$^{-/-}$ mice do not develop colitis, while IL-10$^{-/-}$ mice crossed with *Il12a*$^{-/-}$ mice develop colitis of similar severity to the IL-10$^{-/-}$ founders (23). Previous studies in GF IL-10$^{-/-}$ reveal that enteric bacteria are necessary for the development of spontaneous colitis (24). Although we detected abundant colonic IL-23 in conventionalized GF IL-10$^{-/-}$ mice, IL-12 p70 levels were undetectable, despite histological evidence of colitis as early as 2 weeks post-colonization. Consistent with our results in WT mice, the enteric-microbiota was shown to play an inhibitory role in the expression of IL-23 with subsequent effects on expansion and survival of T_H17 cells in the colon (25).

Although IFN-γ potently augments Th1-mediated immune responses (26), it also has protective properties in inflammatory disease models. For example, IFN-γ gene deletion or administration of anti-IFN-γ antibodies leads to increased severity of experimental autoimmune encephalomyelitis (EAE) and collagen induced arthritis (10, 27-30). A number of mechanisms have been proposed to explain the protective effect of IFN-γ in autoimmunity. IFN-γ directly inhibits T_H17 differentiation (31). Furthermore, conversion of $CD4^+CD25^-$ T cells into $CD4^+$ $CD25^+$ T regulatory cells requires IFN-γ, and limits severity of inflammation in EAE (32). Beyond these effects on T cell responses, little is known about homeostatic effects of IFN-γ on innate immunity. IFN-γ was previously shown to abrogate the ability of mycobacteria-infected murine dendritic cells to induce T_H17 cells by increasing IL-12 and decreasing IL-23 (13).

A recent study demonstrated that CD patients have increased numbers of intestinal $CD14^+CD33^+$ macrophages that produce IL-23, TNF, and IL-6. Differentiation of human peripheral blood macrophages in the presence of IFN-γ resulted in a macrophage phenotype similar to CD intestinal macrophages that secrete IL-23 (33). However, prolonged culture with IFN-γ affected macrophage maturation. Direct effects of IFN-γ on IL-23 expression in differentiated human macrophages were not tested in this study. Moreover, mouse models allow description of events during the *initiation* of spontaneous colitis. By utilizing GF mice transitioned to a CNV microbiota, and through studies in IL-$10^{-/-}$, IFN-γR1/IL-$10^{-/-}$ and IRF-1/IL-$10^{-/-}$ mice, we have clarified mechanisms that may be operative at disease onset. It is possible that with longstanding inflammation, as in human IBD, other mechanisms become more relevant. However, there also may be differences in *Il23a* regulation between murine and human cells.

IL-$10^{-/-}$ mice on a C57BL/6 background are relatively resistant to spontaneous colitis (34). IFN-γ and IRF-1 deficiency severely exacerbated IL-23 mediated colitis in IL-$10^{-/-}$ mice

on this resistant background. A limitation of our analysis is that IFN-γ and IRF-1 deficiency affects multiple innate and adaptive immune pathways (35). However, as *in vivo* proof of concept that IFN-γ regulates *Il23a*, we demonstrated increased *Il23a* expression in the colon and in colonic $CD11b^{+}$ cells from IRF-1/IL-$10^{-/-}$ mice compared to IL-$10^{-/-}$ mice.

Our results provide new insights into transcriptional inhibition of *Il23a*. Recent work revealed the involvement of NF-κB in LPS-mediated activation of *Il23a* transcription (19, 20). Additionally, *Il23a* mRNA levels were dramatically reduced in c-Rel and RelA deficient macrophages (19, 20). We demonstrate that *Il23a* expression has markedly different kinetics of induction and is regulated through notably divergent mechanisms compared to another NF-κB dependent gene, IL-12 p40 (*Il12b*). Where IFN-γ potently synergizes with bacterial products for optimal induction of *Il12b* gene expression (36), IFN-γ inhibits LPS-mediated *Il23a* expression, surprisingly, through effects on NF-κB DNA binding. Our results demonstrate recruitment of p50 to the *Il23a* promoter in IFN-γ activated macrophages, consistent with reports of increased *Il23a* expression in p52-deficient macrophages (37). DNA binding factors, including NF-κB, cannot access chromatin that is in a condensed state. Accessibility to chromatin is enhanced through ATP-dependent nucleosome remodeling complexes or histone modifications (38). We demonstrate that in addition to IRF recruitment, IFN-γ prevents acetylation of the core histone H4, limiting access of transcription factors to the *Il23a* promoter.

The IRF family is a group of transcription factors that respond to signals from type I and II interferons. They share significant homology in their N-terminal domains that interact with a consensus DNA sequence, the ISRE (39). Several IRFs participate in the expression of IL-12 family members. IRF-1 is required for activation of the *Il12a* gene but does not affect *Il12b* gene expression (16). IRF-8 activates both *Il23a* and *Il12b* gene expression (36). We identify an ISRE in the *Il23a* promoter critically located between two proximal NF-κB

sites and show that IRF-1 is a negative regulator. IRFs can interact with other transcription factors, including NF-κB, NFAT, STAT and Ets families, to affect target gene transcription (39). In the absence of IRF-1, RelA recruitment to the *Il23a* promoter in macrophages is prolonged, suggesting that IRF-1 functionally interacts with NF-κB. Although LPS-induced IL-23 was increased in IRF-1$^{-/-}$ BMMs, inhibitory effects of IFN-γ were still evident. It is plausible that in the absence of IRF-1 other IRFs play a compensatory inhibitory role (40, 41). Alternatively, IRF independent mechanisms may exist through which IFN-γ regulates *Il23a* expression.

We confirm a recent report showing *Il23a* is a primary response gene, differing from *Il12b,* a secondary response gene (18). Interestingly, in the absence of new protein synthesis, IFN-γ inhibits LPS activated *Il23a* expression at one hour. This finding is consistent with our observation that IFN-γ inhibits RelA recruitment to the *Il23a* promoter, a rapidly occurring event. However, by three hours, the inhibitory effect of IFN-γ on LPS activated *Il23a* expression required new protein synthesis, suggesting multiple mechanisms through which IFN-γ inhibits *Il23a* gene expression. Future studies will identify factors responsible for rapid *Il23a* gene induction and inhibition as well as those recruited to modify long term gene activation.

Differences in regulation of the p19 subunit of IL-23 and the common p40 subunit of IL-12 and IL-23 may represent an important *in vivo* check point to shape the subsequent T cell response. Hypothetically, as T_H1 and T_H17 responses are counter-regulatory, IFN-γ may act directly upon the macrophage to attenuate T_H17 responses through inhibition of IL-23. We also further implicate macrophage-derived IL-23 in the initiation of experimental colitis, highlighting the protective effects of IFN-γ signaling in IL-10$^{-/-}$ mice. Recently, genome wide association studies (GWAS) in human IBD have provided insight into the contributions of single nucleotide polymorphisms (SNPs) located in genomic loci relevant to the IL-12/23

pathway. Chromosome 1p31 harbors the *IL23R* gene, containing SNPs which confer susceptibility to or protection against IBD. Similarly, SNPs within the *Il12b* gene on chromosome 5q33 confer susceptibility to CD and UC (42). A recent GWAS in ulcerative colitis patients revealed that the most significant chromosome 12q15 association signal was located in a region proximal to the *IFNG* (IFN-γ) gene (43). Moreover, a 250 kb risk haplotype within the *IBD5* locus on chromosome 5q31 associated with CD contains the gene for *IRF1* (44, 45). Therefore, converging genetic and functional findings suggest that IL-23, IFN-γ and IRF-1 may be important pathogenic molecules and therapeutic targets in human IBD.

2.5 Materials and Methods

Mice. Wild-type and genetically deficient mice (WT, IL-10$^{-/-}$, IRF-γ R1$^{-/-}$, IRF-1$^{-/-}$, IL-10/ IRF-γ R1$^{-/-,}$ IL-10/IRF-1$^{-/-}$) in specific pathogen free conventionalized housing (CNV) were on the C57BL/6 background (Jackson Laboratories) and matched for age in all experiments. All genetically deficient mice assessed for spontaneous colitis were littermates. 129S6/SvEv GF mice (WT and IL-10 $^{-/-}$) were Caesarian derived and were maintained according to standard techniques (46) in Trexler flexible film isolators at the Gnotobiotic Animal Facility of the Center for Gastrointestinal Biology and Disease at the University of North Carolina, Chapel Hill. CNV mice were maintained in a dedicated room at the University of North Carolina Laboratory Animal Resources Facility. GF status was monitored every 2 weeks by aerobic and anaerobic culture and gram stain of stool samples and/or bedding material. Mice were colonized with enteric-microbiota at 10-12 weeks of age with a murine microbiota that was isolated from WT mice raised in SPF conditions (24). Additionally, CNV mice were determined to be negative for *Helicobacter* species (*Helicobacter bilis, Helicobacter hepaticus, Helicobacter rodentium, Helicobacter trogontum and Helicobacter sp.*) using *Helicobacter* PCR profile performed on freshly harvested fecal pellets (RADIL Laboratories, Columbia MO). All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. At the end of the study period, animals were euthanized using excess CO2 inhalation.

Murine bone marrow-derived macrophages. Bone-marrow-derived macrophages (BMMs) were harvested as previously described (47). Briefly, BMMs were grown for 7 days in RPMI 1640 containing 10% FCS, 10mM HEPES, 1% penicillin/streptomycin and supplemented

with 40 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ). BMMs were stimulated with 100 ng/ml of high purity LPS and/or 10 ng/ml of IFN-γ (Invivogen, San Diego, CA).

Cytokine ELISAs. Murine IL-12 p40, IL-12 p70, IFN-γ and IL-17 immunoassay kits (R&D Systems) and IL-23 (eBioscience) were used according to the manufacturers' instructions.

Western immunoblot. Western blot analyses were performed on whole cell extracts as described previously (47). Anti–IRF-1 antibodies were from Santa Cruz Biotechnology, Inc, CA and β-actin antibodies were purchased from Abcam, MA.

RNA extraction and quantitative real-time RT PCR (qRT-PCR) analysis. Total RNA was extracted with RNeasy kit (Qiagen) and reverse-transcribed with ramdom hexamers using Superscript reverse transcriptase II (Invitrogen). Complementary DNA was analyzed by quantitative real-time PCR using SYBR Green Master Mix (Applied Biosystems) on a HT-7900
(Applied Biosystems). Single-product amplification was confirmed by melting-curve analysis. Primer sequences are as follow: *Il23a* Forward 5'-gacccacaaggactcaaggac-3', Reverse 5'-atggggctatcagggagtagag-3', *Il12b* Forward 5'-cgcaagaaagaaaagatgaaggag-3', Reverse 5'-ttgcattggacttcggtagatg-3'; *Il12a* Forward 5'-cattctagacaagggcatgctg-3', Reverse 5'-ttttcactctgtaagggtctgcttc-3', *β-Actin* Forward 5'-agccatgtacgtagccatccag-3', Reverse 5'-tggcgtgagggagagcatag-3'. Expression was normalized to β-Actin and represented as fold induction over unstimulated cells.

Plasmids. The *Il23a* 1.8 kb luciferase construct was kindly provided Dr. Y.H. Chen (Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania) (19). The QuikChange XL system (Stratagene) was used to make mutations in the ISRE in the *Il23a* murine promoter according to manufacturer's instructions. Mutations

were confirmed by sequencing. The *Il23a* promoter coordinates were determined relative to a dominant transcription start site (TIS) previously described (48).

Transient transfections. Bone marrow-derived murine macrophages were transiently transfected using *AMAXA Nucleofector Technology* (AMAXA) by the described protocol for murine macrophages. After incubation for 3 hours at 37^{o}C, the cells were either unactivated or activated with 100 ng/ml LPS, 10 ng/ml IFN-γ, or both. 24 h after activation, the cells were harvested using 1X Reporter Lysis Buffer (Promega). Luciferase activity was determined from 20 µl of cell extract as described previously (15). Cells were co-transfected with a constitutively active HSP promoter that expresses β-galactosidase to monitor transfection efficiency. IRF-1 siRNA and control scrambled siRNA was purchased from Santa Cruz.

Colonic tissue explant cultures. Sections of the transverse colon were processed as previously described (49). Tissue fragments (0.5 g dry weight) were incubated in 1.0 ml RPMI 1640 supplemented with 50 µg/ml gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone (GIBCO BRL), and 5% heat-inactivated fetal calf serum. Tissue fragment supernatants were collected after 24 hours for cytokine ELISAs.

Preparation of heat-killed bacteria. *E. coli* in log-phase growth was harvested and washed twice with ice-cold PBS. Bacterial suspensions were, then, heated at 80°C for 30 minutes, washed, resuspended in PBS, and stored at –80°C. Complete killing was confirmed by 72-hour incubation at 37°C on plate medium. Heat-killed bacteria were added at multiplicity of infection (M.O.I.) = 10.

Isolation of colonic macrophages. Lamina propria cells (LPCs) were isolated from mouse colon by a modified enzymatic method (50). Briefly, colons were dissected into small pieces, and washed three times in Hank's buffered saline solution (HBSS) containing 2.5% FBS. The last wash was done with 1mM DTT to remove mucus. The pieces were, then, incubated

in HBSS containing 1mM EDTA three times for 20 min each at 37°C. The remaining tissue was digested in HBSS containing 1 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO) for 1.5 hours at 37°C. The supernatant was collected, filtered and centrifuged to obtain a cell pellet. LPCs were isolated by density gradient centrifugation using 40 % and 75 % Percoll solution (GE Healthcare, Piscataway, NJ). The intermediate layer containing LPCs was collected. LPCs were further separated into CD11b+ cells using anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA). Purity was more than 90% by flow cytometric analysis (data not shown).

Histology. Colonic tissue sections were fixed in 10% buffered formalin and embedded in paraffin. 4-µm-thick sections were stained with hematoxylin and eosin. Spontaneous colitis scoring was adapted from the criteria reported by Berg et al, as previously described (49). All histological scores were determined by a staff pathologist (T. Rubinas) who was blinded to the experimental protocols.

Chromatin immunoprecipitation assays (ChIP). ChIP was performed with ChIP-IT Express kit (Active Motif, Carlsbad, CA) according to manufacturer's protocol. Briefly, $2X10^6$ BMDMs were stimulated, washed with PBS, and fixed with 0.8% formaldehyde for 10 minutes at room temperature. Formaldehyde fixation was stopped with the addition of 125 mM glycine. Fixed cells were harvested, lysed, and sonicated for 5 cycles of 20-second on/20-second off with Sonic Dismembrator 60 (Thermo Fisher Scientific, Waltham, MA). DNA-protein complexes were immunoprecipitated with specific antibodies (Anti–IRF-1, 2, 8, RelA , p50 and rabbit polyclonal IgG, Santa Cruz Biotechnology Inc, CA.), H4Act (Upstate, N.Y) eluted, and reverse-cross linked. Quantitative real-time PCR primers for the *Il23a* promoter (κ1 : Forward 5'-taggctaagcaggctgagaaatg-3', Reverse 5'- gccctggttttgaaggtgatag-3', a154 bp product; and ISRE: Forward 5'-gagatgaatgagtgctgttttgg-3', Reverse 5'-agaaggggcagggaagtaatg-3', a 198 bp product), real-time PCR primers for the *Il12b* promoter

(ISRE Forward 5'-tgtgaaaagaaaggggaaagtgag-3', Reverse 5'- ctcctggtttgccatcgtttt-3', with a 197 bp product), Real-time PCR primers for Nuc1 region on *Il12b* promoter Forward 5'-gaaggaacagtgggtgtccag-3', Reverse 5'- agggagttagcgacagggaag-3' with a 131 bp product, immunoprecipitated DNA and input DNA (diluted 10-fold) were amplified. ChIP data is represented as % input.

Electrophoretic mobility shift assays (EMSA). BMM nuclear extracts were prepared by modified Dignam protocol, as previously described (15). Briefly, BMMs were untreated or treated with LPS (100 ng/mL) with or without IFN-γ (10 ng/ml). Synthetic, double-stranded oligonucleotides were designed to span the *Il23a* promoter region from –368 bp to –388 bp. The EMSA probe 368/388m contains a mutated sequence from –372 to –378. DNA oligonucleotides were end labeled with [γ-^{32}P] ATP, and 4 μg of nuclear extract was incubated with 1 ng of labeled probe in binding buffer containing 10 mM HEPES (pH 7.5), 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, and 50 μg/ml poly(dI-dC). After incubation at room temperature for 20–30 min, the mixture was electrophoresed on a 6% polyacrylamide gel in 0.5 x Tris-boric acid-EDTA buffer. For competition experiments, 100- fold molar excess of unlabeled oligonucleotides was added to the reaction mixture before adding labeled probes. For supershift assays, 1.5 μg of a rabbit polyclonal antibody raised against specific IRF family members (IRF-1, 2 and 8, Santa Cruz) was added to nuclear extracts from LPS and IFN-γ stimulated BMMs 30 minutes prior to the probe.

Statistical Analysis. Statistical significance for data subsets from experiments performed in cells was assessed by the two-tailed Student's *t*-test. Statistical significance for *in vivo* data subsets was assessed by the Mann-Whitney U test (SPSS, Chicago, IL, USA)) with Bonferroni correction.

2.6 Acknowledgments

This work was supported by grants from the National Institutes of Health (NIH): RO1 DK54452 (SEP), Gastroenterology Research Training Grant T32 DK007737 (SZS), NRSA F32 DK083186 (SZS), P40 RR018603 (National Gnotobiotic Rodent Resource Center), P30 DK034987 (Center for Gastrointestinal Biology and Disease; Immunotechnologies, Gnotobiotic and Histology Cores), and a Crohn's and Colitis Foundation of America Research Fellowship Award (KM).

2.7 References

1. Xavier, R.J., and D.K. Podolsky. 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448:427-434.

2. Sartor, R.B. 2008. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 134:577-594.

3. Sartor, R.B. 2006. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 3:390-407.

4. Adorini, L. 1999. Interleukin-12, a key cytokine in Th1-mediated autoimmune diseases. *Cell Mol Life Sci* 55:1610-1625.

5. Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M.A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D.J. Cua, R.A. Kastelein, and D. Rennick. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 116:1310-1316.

6. Duerr, R.H., K.D. Taylor, S.R. Brant, J.D. Rioux, M.S. Silverberg, M.J. Daly, A.H. Steinhart, C. Abraham, M. Regueiro, A. Griffiths, T. Dassopoulos, A. Bitton, H. Yang, S. Targan, L.W. Datta, E.O. Kistner, L.P. Schumm, A.T. Lee, P.K. Gregersen, M.M. Barmada, J.I. Rotter, D.L. Nicolae, and J.H. Cho. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314:1461-1463.

7. Davidson, N.J., S.A. Hudak, R.E. Lesley, S. Menon, M.W. Leach, and D.M. Rennick. 1998. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J Immunol* 161:3143-3149.

8. Mannon, P.J., I.J. Fuss, L. Mayer, C.O. Elson, W.J. Sandborn, D. Present, B. Dolin, N. Goodman, C. Groden, R.L. Hornung, M. Quezado, M.F. Neurath, J. Salfeld, G.M. Veldman, U. Schwertschlag, W. Strober, and Z. Yang. 2004. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 351:2069-2079.

9. Sandborn, W.J., B.G. Feagan, R.N. Fedorak, E. Scherl, M.R. Fleisher, S. Katz, J. Johanns, M. Blank, and P. Rutgeerts. 2008. A randomized trial of Ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease. *Gastroenterology* 135:1130-1141.

10. Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S.A. Lira, D. Gorman, R.A. Kastelein, and J.D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.

11. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 3:133-146.

12. Harrington, L.E., R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, and C.T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132.

13. Cruz, A., S.A. Khader, E. Torrado, A. Fraga, J.E. Pearl, J. Pedrosa, A.M. Cooper, and A.G. Castro. 2006. Cutting edge: IFN-gamma regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection. *J Immunol* 177:1416-1420.

14. Plevy, S.E., J.H. Gemberling, S. Hsu, A.J. Dorner, and S.T. Smale. 1997. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol Cell Biol* 17:4572-4588.

15. Zhu, C., K. Rao, H. Xiong, K. Gagnidze, F. Li, C. Horvath, and S. Plevy. 2003. Activation of the murine interleukin-12 p40 promoter by functional interactions between NFAT and ICSBP. *J Biol Chem* 278:39372-39382.

16. Liu, J., S. Cao, L.M. Herman, and X. Ma. 2003. Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. *J Exp Med* 198:1265-1276.

17. Muhlbauer, M., P.M. Chilton, T.C. Mitchell, and C. Jobin. 2008. Impaired Bcl3 up-regulation leads to enhanced lipopolysaccharide-induced interleukin (IL)-23P19 gene expression in IL-10(-/-) mice. *J Biol Chem* 283:14182-14189.

18. Ramirez-Carrozzi, V.R., D. Braas, D.M. Bhatt, C.S. Cheng, C. Hong, K.R. Doty, J.C. Black, A. Hoffmann, M. Carey, and S.T. Smale. 2009. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell* 138:114-128.

19. Carmody, R.J., Q. Ruan, H.C. Liou, and Y.H. Chen. 2007. Essential roles of c-Rel in TLR-induced IL-23 p19 gene expression in dendritic cells. *J Immunol* 178:186-191.

20. Carmody, R.J., Q. Ruan, S. Palmer, B. Hilliard, and Y.H. Chen. 2007. Negative regulation of toll-like receptor signaling by NF-kappaB p50 ubiquitination blockade. *Science* 317:675-678.

21. Bhaumik, S.R., E. Smith, and A. Shilatifard. 2007. Covalent modifications of histones during development and disease pathogenesis. *Nat Struct Mol Biol* 14:1008-1016.

22. Weinmann, A.S., S.E. Plevy, and S.T. Smale. 1999. Rapid and selective remodeling of a positioned nucleosome during the induction of IL-12 p40 transcription. *Immunity* 11:665-675.

23. Sellon, R.K., S. Tonkonogy, M. Schultz, L.A. Dieleman, W. Grenther, E. Balish, D.M. Rennick, and R.B. Sartor. 1998. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* 66:5224-5231.

24. Kim, S.C., S.L. Tonkonogy, C.A. Albright, J. Tsang, E.J. Balish, J. Braun, M.M. Huycke, and R.B. Sartor. 2005. Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. *Gastroenterology* 128:891-906.

25. Zaph, C., Y. Du, S.A. Saenz, M.G. Nair, J.G. Perrigoue, B.C. Taylor, A.E. Troy, D.E. Kobuley, R.A. Kastelein, D.J. Cua, Y. Yu, and D. Artis. 2008. Commensal-dependent expression of IL-25 regulates the IL-23-IL-17 axis in the intestine. *J Exp Med* 205:2191-2198.

26. von Herrath, M.G., and M.B. Oldstone. 1997. Interferon-gamma is essential for destruction of beta cells and development of insulin-dependent diabetes mellitus. *J Exp Med* 185:531-539.

27. Billiau, A., H. Heremans, F. Vandekerckhove, R. Dijkmans, H. Sobis, E. Meulepas, and H. Carton. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol* 140:1506-1510.

28. Dalton, D.K., S. Pitts-Meek, S. Keshav, I.S. Figari, A. Bradley, and T.A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 259:1739-1742.

29. Kelchtermans, H., S. Struyf, B. De Klerck, T. Mitera, M. Alen, L. Geboes, M. Van Balen, C. Dillen, W. Put, C. Gysemans, A. Billiau, J. Van Damme, and P. Matthys. 2007. Protective role of IFN-gamma in collagen-induced arthritis conferred by inhibition of mycobacteria-induced granulocyte chemotactic protein-2 production. *J Leukoc Biol* 81:1044-1053.

30. Vermeire, K., H. Heremans, M. Vandeputte, S. Huang, A. Billiau, and P. Matthys. 1997. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *J Immunol* 158:5507-5513.

31. Park, H., Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.

32. Wang, Z., J. Hong, W. Sun, G. Xu, N. Li, X. Chen, A. Liu, L. Xu, B. Sun, and J.Z. Zhang. 2006. Role of IFN-gamma in induction of Foxp3 and conversion of CD4+ CD25- T cells to CD4+ Tregs. *J Clin Invest* 116:2434-2441.

33. Kamada, N., T. Hisamatsu, S. Okamoto, H. Chinen, T. Kobayashi, T. Sato, A. Sakuraba, M.T. Kitazume, A. Sugita, K. Koganei, K.S. Akagawa, and T. Hibi. 2008. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest* 118:2269-2280.

34. Mahler, M., and E.H. Leiter. 2002. Genetic and environmental context determines the course of colitis developing in IL-10-deficient mice. *Inflamm Bowel Dis* 8:347-355.

35. Kano, S., K. Sato, Y. Morishita, S. Vollstedt, S. Kim, K. Bishop, K. Honda, M. Kubo, and T. Taniguchi. 2008. The contribution of transcription factor IRF1 to the interferon-gamma-interleukin 12 signaling axis and TH1 versus TH-17 differentiation of CD4+ T cells. *Nat Immunol* 9:34-41.

36. Zhao, J., H.J. Kong, H. Li, B. Huang, M. Yang, C. Zhu, M. Bogunovic, F. Zheng, L. Mayer, K. Ozato, J. Unkeless, and H. Xiong. 2006. IRF-8/interferon (IFN) consensus sequence-binding protein is involved in Toll-like receptor (TLR) signaling and contributes to the cross-talk between TLR and IFN-gamma signaling pathways. *J Biol Chem* 281:10073-10080.

37. Mise-Omata, S., E. Kuroda, J. Niikura, U. Yamashita, Y. Obata, and T.S. Doi. 2007. A proximal kappaB site in the IL-23 p19 promoter is responsible for RelA- and c-Rel-dependent transcription. *J Immunol* 179:6596-6603.

38. Li, C.C., V.R. Ramirez-Carrozzi, and S.T. Smale. 2006. Pursuing gene regulation 'logic' via RNA interference and chromatin immunoprecipitation. *Nat Immunol* 7:692-697.

39. Ozato, K., P. Tailor, and T. Kubota. 2007. The interferon regulatory factor family in host defense: mechanism of action. *J Biol Chem* 282:20065-20069.

40. Mamane, Y., C. Heylbroeck, P. Genin, M. Algarte, M.J. Servant, C. LePage, C. DeLuca, H. Kwon, R. Lin, and J. Hiscott. 1999. Interferon regulatory factors: the next generation. *Gene* 237:1-14.

41. Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka. 2001. IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol* 19:623-655.

42. Cho, J.H. 2008. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 8:458-466.

43. Silverberg, M.S., J.H. Cho, J.D. Rioux, D.P. McGovern, J. Wu, V. Annese, J.P. Achkar, P. Goyette, R. Scott, W. Xu, M.M. Barmada, L. Klei, M.J. Daly, C. Abraham, T.M. Bayless, F. Bossa, A.M. Griffiths, A.F. Ippoliti, R.G. Lahaie, A. Latiano, P. Pare, D.D. Proctor, M.D. Regueiro, A.H. Steinhart, S.R. Targan, L.P. Schumm, E.O. Kistner, A.T. Lee, P.K. Gregersen, J.I. Rotter, S.R. Brant, K.D. Taylor, K. Roeder, and R.H. Duerr. 2009. Ulcerative colitis-risk loci on chromosomes 1p36 and 12q15 found by genome-wide association study. *Nat Genet* 41:216-220.

44. Urcelay, E., J.L. Mendoza, A. Martinez, L. Fernandez, C. Taxonera, M. Diaz-Rubio, and E.G. de la Concha. 2005. IBD5 polymorphisms in inflammatory bowel disease: association with response to infliximab. *World J Gastroenterol* 11:1187-1192.

45. Reinhard, C., and J.D. Rioux. 2006. Role of the IBD5 susceptibility locus in the inflammatory bowel diseases. *Inflamm Bowel Dis* 12:227-238.

46. MacDonald, T.T., and P.B. Carter. 1978. Contact sensitivity in the germ-free mouse. *J Reticuloendothel Soc* 24:287-293.

47. Xiong, H., C. Zhu, F. Li, R. Hegazi, K. He, M. Babyatsky, A.J. Bauer, and S.E. Plevy. 2004. Inhibition of Interleukin-12 p40 Transcription and NF-{kappa}B Activation by Nitric Oxide in Murine Macrophages and Dendritic Cells. *J Biol Chem* 279:10776-10783.

48. Liu, W., X. Ouyang, J. Yang, J. Liu, Q. Li, Y. Gu, M. Fukata, T. Lin, C. He, M. Abreu, J.C. Unkeless, L. Mayer, and H. Xiong. 2009. AP-1 activated by Toll-like receptors regulates expression of IL-23 p19. *J Biol Chem*

49. Hegazi, R.A., K.N. Rao, A. Mayle, A.R. Sepulveda, L.E. Otterbein, and S.E. Plevy. 2005. Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. *J Exp Med* 202:1703-1713.

50. Kobayashi, T., S. Okamoto, T. Hisamatsu, N. Kamada, H. Chinen, R. Saito, M.T. Kitazume, A. Nakazawa, A. Sugita, K. Koganei, K. Isobe, and T. Hibi. 2008. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut* 57:1682-1689.

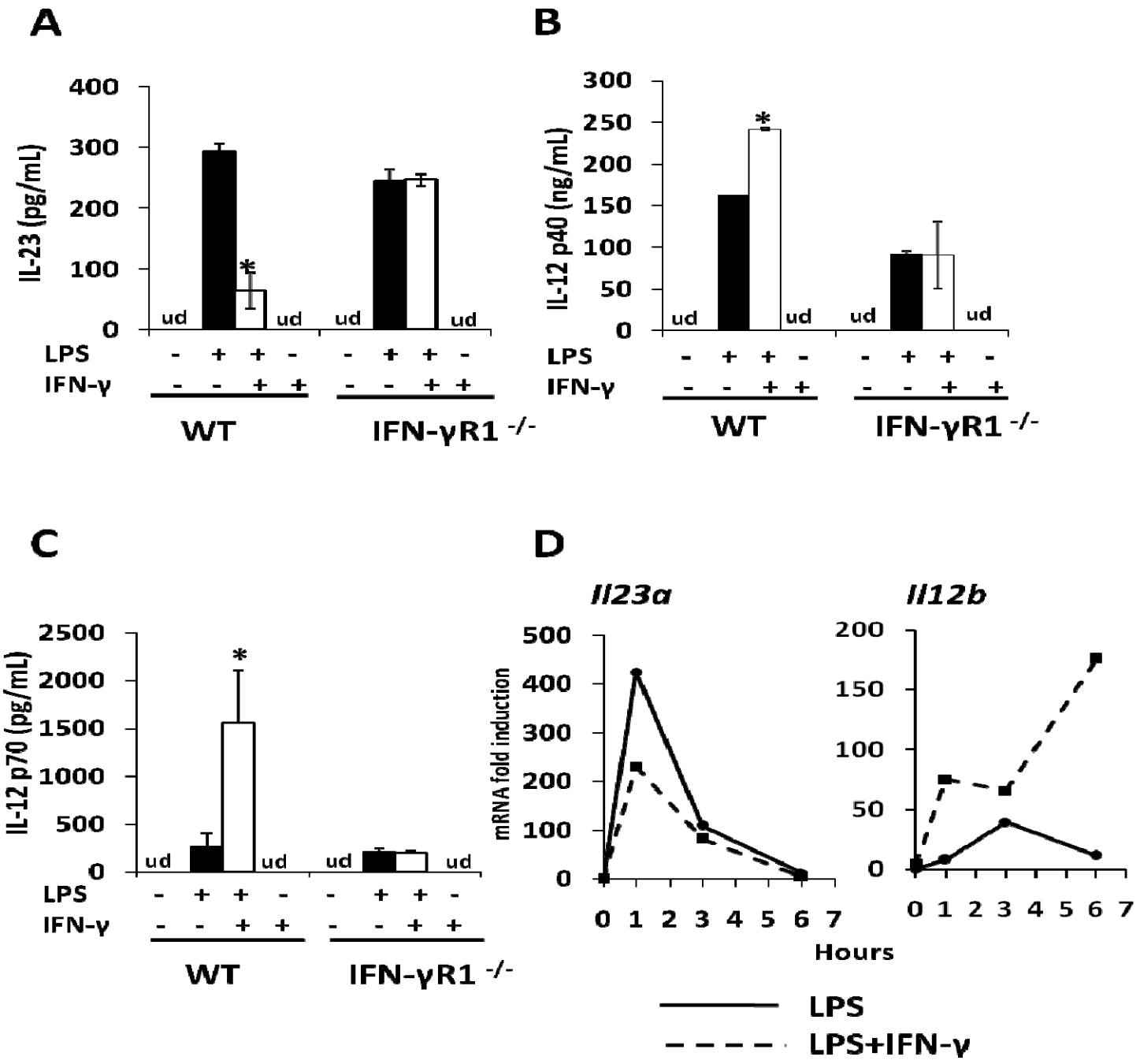


Figure 2.1. IFN-γ negatively regulates LPS-mediated IL-23 expression in macrophages. BMMs from WT and IFN-γR1 deficient mice were cultured in the presence of LPS (100 ng/ml) with or without IFN-γ (10 ng/ml). Supernatants from BMMs were analyzed for **(A)** IL-23, **(B)** IL-12 p40 and **(C)** IL-12 p70 protein expression by ELISA. Results are expressed as mean±SEM of triplicate cultures and are representative of 3 independent experiments. **(D)** WT BMMs were stimulated with LPS (100 ng/ml) and IFN-γ (10 ng/ml) for different times prior to total RNA isolation. *Il23a*, *Il12b* and β-actin mRNA expression was detected by real-time RT-PCR (Applied Biosystems). Results are expressed as fold induction normalized to β-actin and are representative of 3 independent experiments.

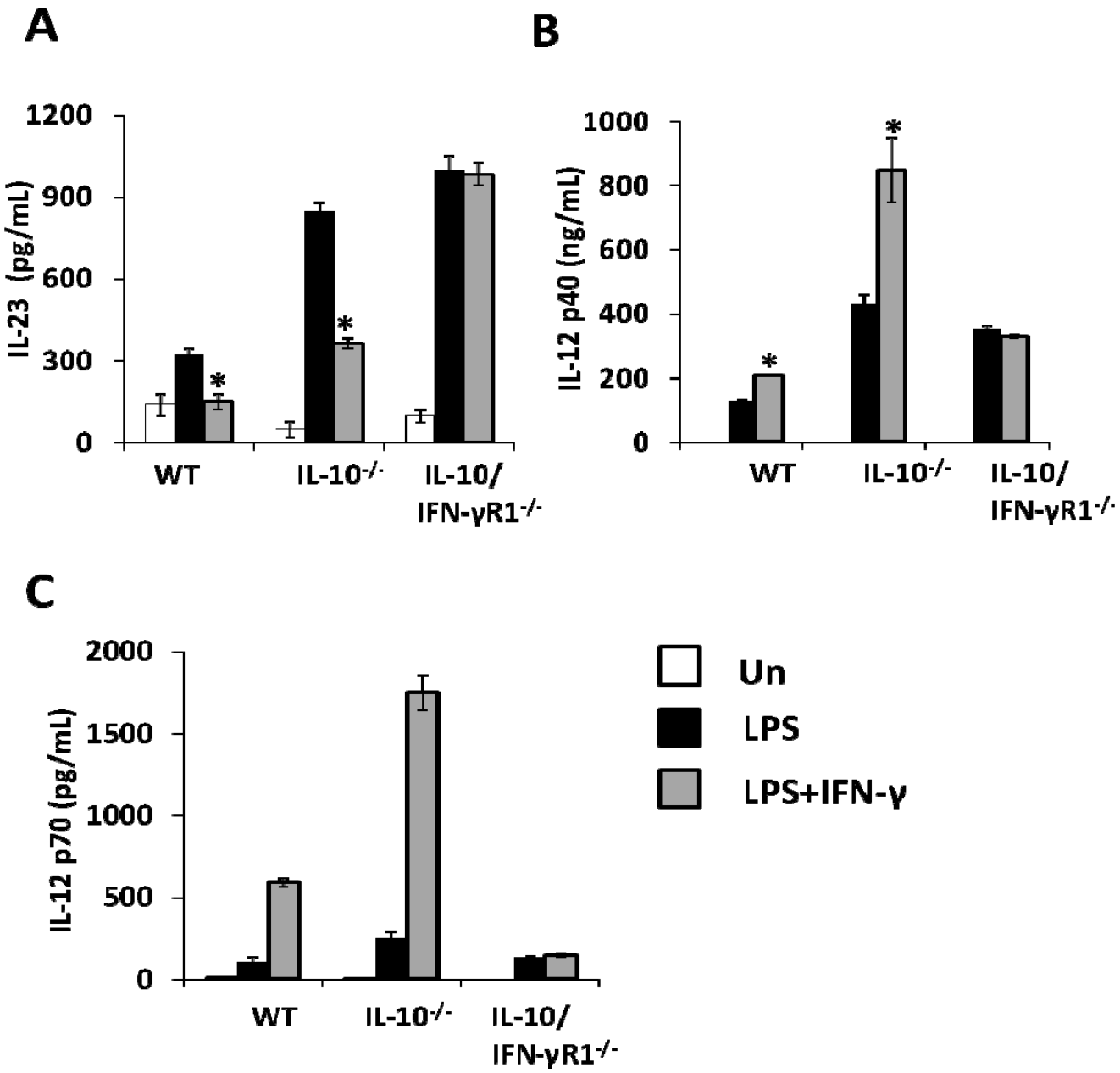


Figure 2.2. IFN-γ negatively regulates LPS-mediated IL-23 expression in IL-10$^{-/-}$ macrophages. BMMs from WT, IL-10 and IL-10/IFN-γR1 deficient mice were cultured in the presence of LPS (black bars, 100 ng/ml) and IFN-γ (grey bars, 10 ng/ml). Supernatants from cultured BMMs were analyzed for **(A)** IL-23, **(B)** IL-12 p40 and **(C)** IL-12 p70 protein expression by ELISA. Results are expressed as mean±SEM of triplicate cultures and are representative of 3 independent experiments.

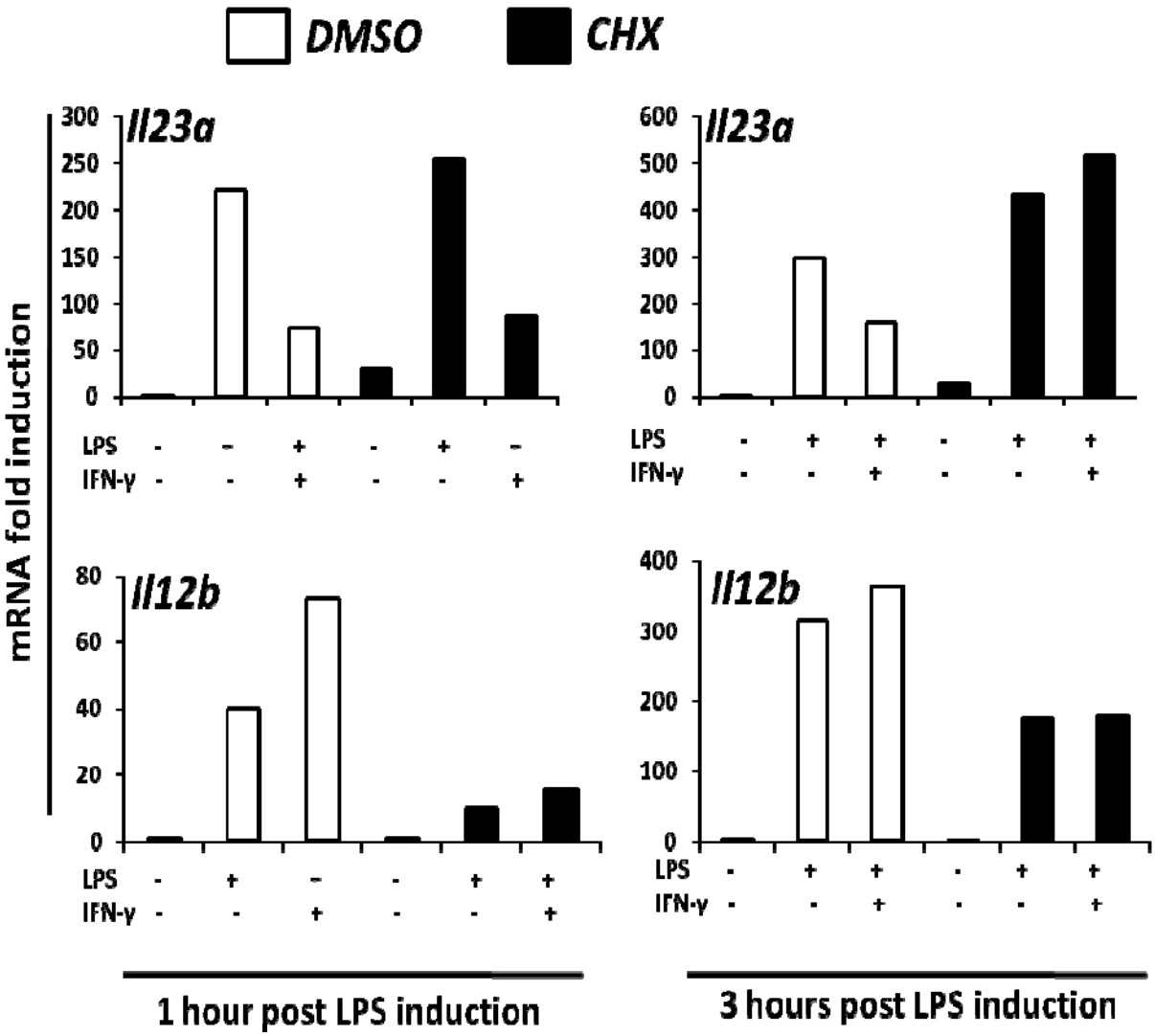


Figure 2.3. ***Il23a*** **is a primary response gene.** BMMs were incubated for 30 minutes with DMSO or cyclohexamide (5 µg/mL) and then stimulated with LPS (100 ng/mL)+IFN-γ (10 ng/mL). *Il23a* and *Il12b* mRNA was analyzed by real time RT-PCR at 1 hour (left panel) and 3 hours (right panel) post stimulation. Results are representative of 3 independent experiments with similar results.

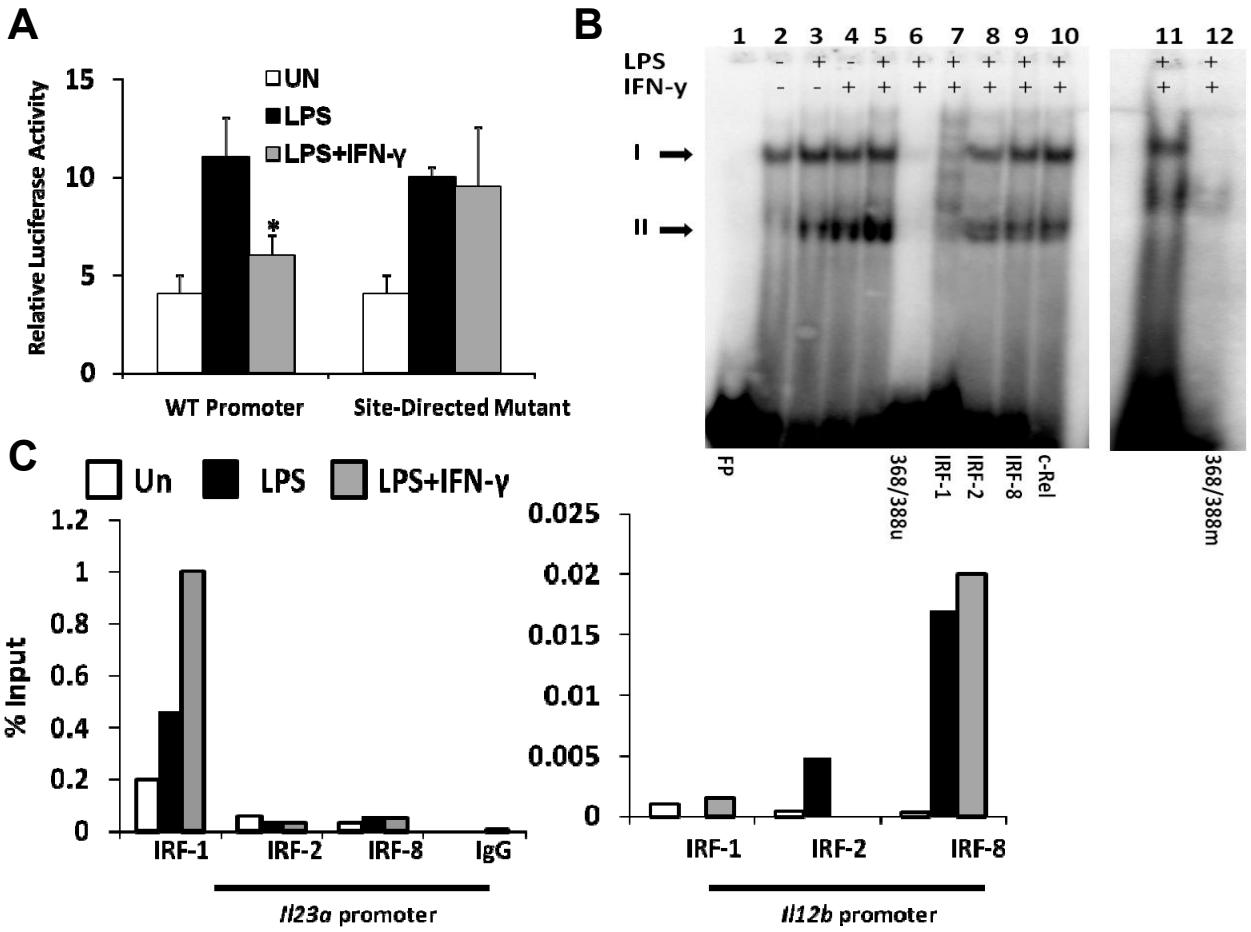


Figure 2.4. Interferon regulatory factors (IRFs) interact with an ISRE in the *Il23a* promoter. (A) BMMs were transfected with an *Il23a* promoter luciferase reporter plasmid or a promoter containing a mutant ISRE, and cultured with LPS (100 ng/ml)±IFN-γ (10 ng/ml) for 18 h. Reporter activity is presented as luciferase units normalized to HSP-promoter β-galactosidase activity. Data represent mean ± SEM of 3 independent experiments. *p<0.05 vs. LPS-stimulated *Il23a* promoter. **(B)** EMSA probe 368/388 spans the *Il23a* promoter sequence –368 to –388. The EMSA probe 368/388m contains a mutant ISRE from –378 to –384. ^{32}P-labeled probes 368/388 (lanes 2–4) and 368/388m (lane 12) were incubated with nuclear extracts from untreated (lane 2), LPS-activated (lane 3), IFN-γ-activated (lane 4), IFN-γ- and LPS-activated BMMs (lanes 5-12). Lane 1 represents free probe (FP). Two protein-DNA complexes (*I* and *II*) were detected with probe 368/388. 100-fold molar excess of unlabelled probe (368/388u, lane 6) was added to compete for binding to the labeled probe. Probe 368/388 was incubated with nuclear extracts from IFN-γ/LPS-treated BMMs cells at room temperature for 30 min, then polyclonal antibodies against c-Rel (lane 10), IRF-1, IRF-2, and IRF-8 (lanes 7–9). **(C)** Formaldehyde-cross-linked chromatin samples from BMMs treated with LPS (100 ng/ml) and IFN-γ (10 ng/ml) were immunoprecipitated with IRF-1, IRF-2, IRF-8 and control IgG antibodies. Quantitative real-time PCR analysis was performed on precipitated and input DNA. Results are reported as enrichment (% input) of IRF DNA binding. The figure represents results from one of 3 independently conducted experiments with identical results.

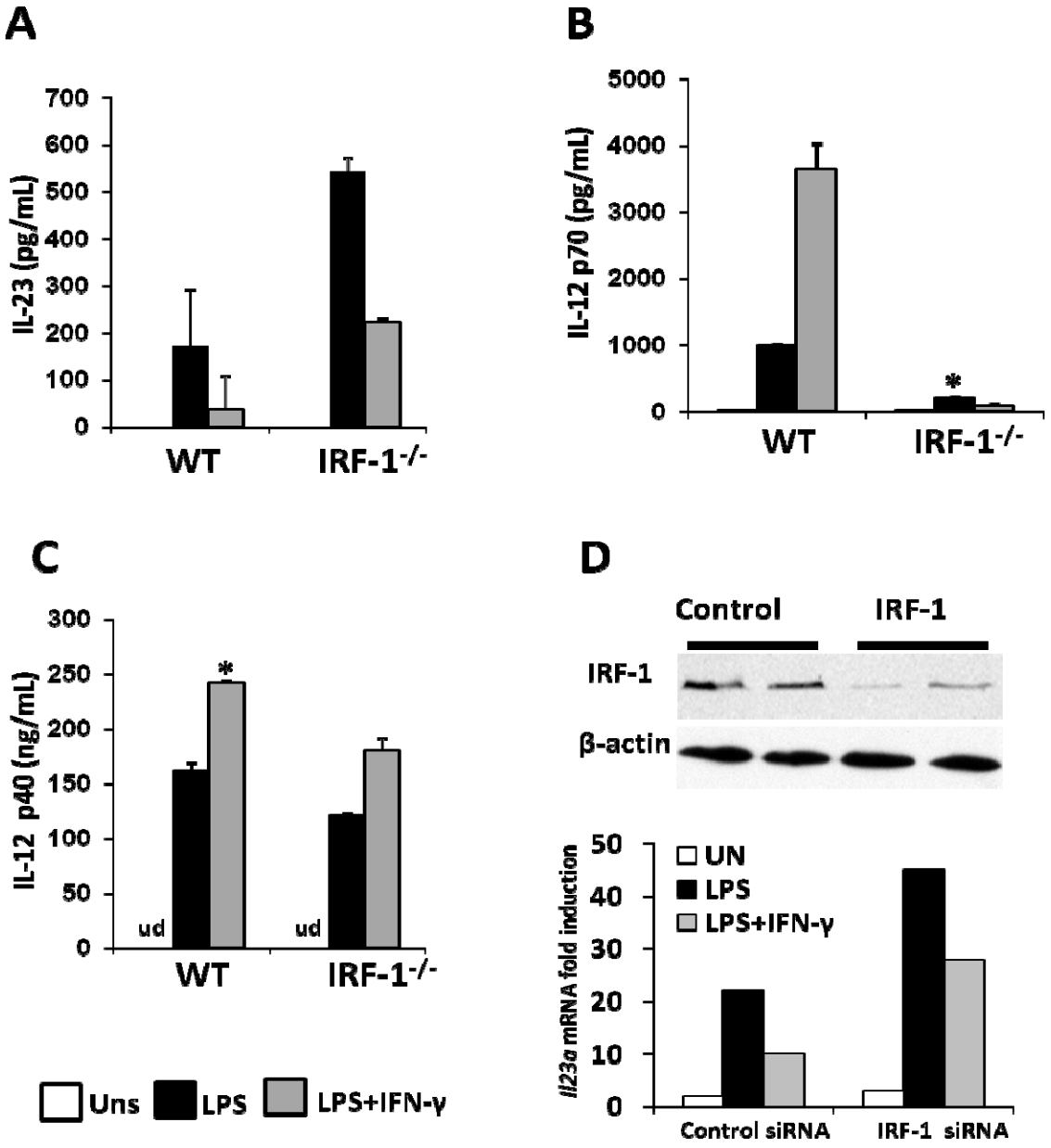


Figure 2.5. IRF-1 is a negative regulator of *Il23a* in macrophages. BMMs from WT and IRF-1$^{-/-}$ mice were cultured in the presence of LPS (100 ng/ml, black bars) and IFN-γ (10 ng/ml, grey bars). **(A)** IL-23, **(B)** IL-12 p70 and **(C)** IL-12 p40 protein was analyzed by ELISA. Data represent mean±SEM, 3 independent experiments,*p<0.05 *vs.* WT LPS **(D)** WT BMMs were transfected for 18 h with 100 nM of control scrambled siRNA or 100 nM of IRF-1 siRNA and stimulated with LPS (100 ng/ml) and IFN-γ (10 ng/ml). IRF-1 silencing was analyzed by western blot (upper panel) and *Il23a* mRNA (lower panel) was determined by RT-PCR. Results are representative of 3 independent experiments.

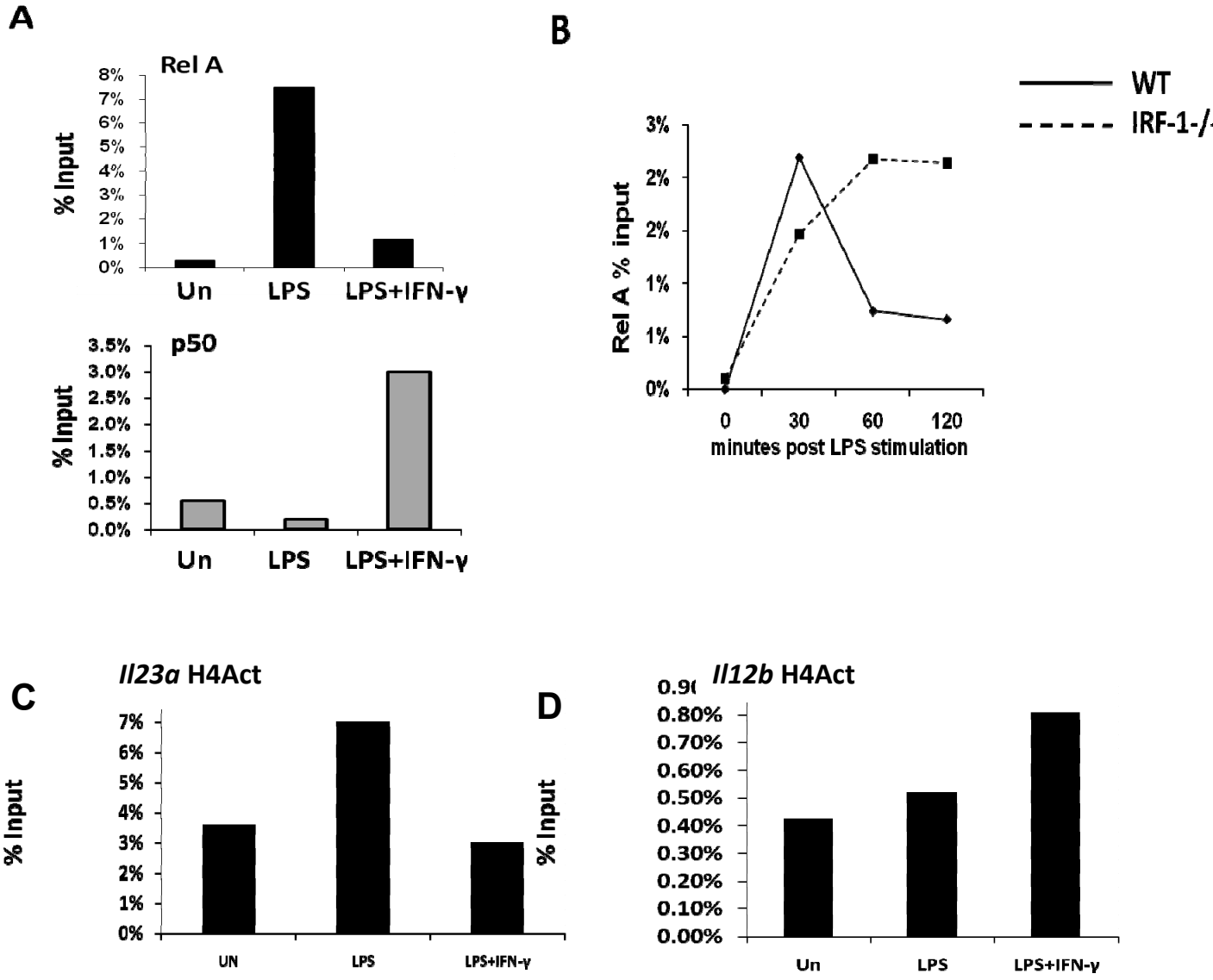


Figure 2.6. IFN-γ inhibits RelA binding to the *Il23a* promoter. (A) Binding of RelA and p50 to the distal NF-κB site on the endogenous WT *Il23a* promoter was assessed by ChIP 1 hour after incubation with LPS+IFN-y. Formaldehyde-cross-linked chromatin samples from BMMs were immunoprecipitated with anti-RelA and anti-p50 (Santa Cruz Biotechnology, CA) antibodies. Real-time PCR was performed on precipitated DNA samples. Results are presented as enrichment (% input) of RelA DNA binding are representative of 3 independent experiments with similar results. **(B)** Binding of RelA to the distal NF-κB site on the endogenous WT and IRF-1$^{-/-}$ *Il23a* promoter was assessed by ChIP at 0.5, 1 and 2 hours post LPS stimulation. Results are presented as enrichment (% input) of RelA DNA binding on the *Il23a* promoter. **(C)** Acetylation at histone 4 (H4Act) to the distal NF-κB site on the endogenous WT *Il23a* promoter and **(D)** nucleosome 1 (Nuc1) on the *Il12b* promoter was assessed by ChIP 1 hour after incubation with LPS±IFN-y. Results are presented as enrichment (% input) of H4Act binding are representative of 3 independent experiments with similar results.

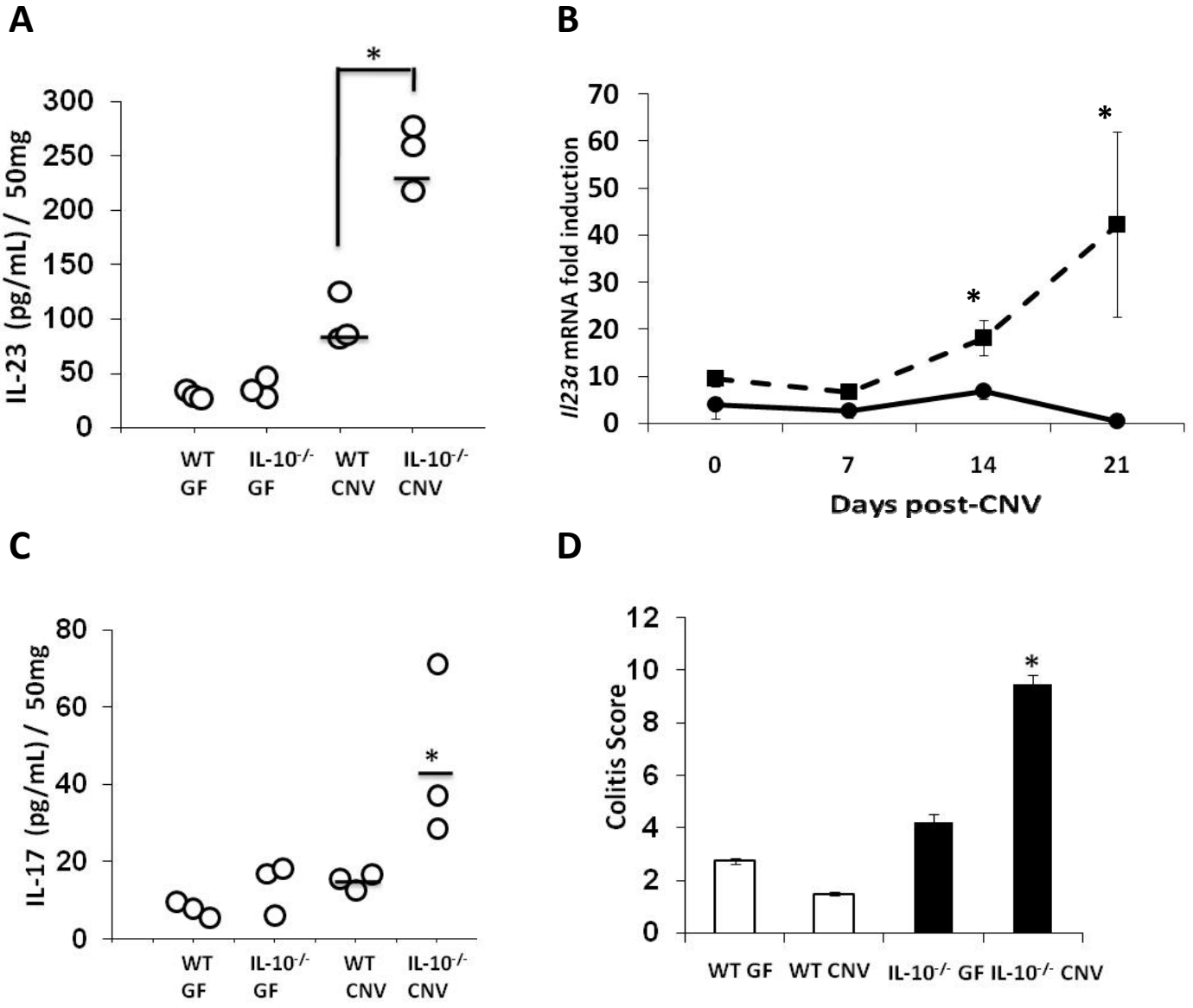


Figure 2.7. The enteric-microbiota regulate IL-23 expression in IL-10$^{-/-}$ mice. WT and IL-10$^{-/-}$ mice raised in germ free (GF) conditions were colonized with enteric-microbiota from conventionalized (CNV) mice at 8-10 weeks of age (3 mice per group). **(A)** IL-23 secretion post-colonization with enteric-microbiota in colonic explant cultures was determined by ELISA **(B)** Real time RT-PCR of colonic *Il23a* mRNA expression in WT (solid line) and IL-10$^{-/-}$ (dashed line) normalized to β-actin at 7, 14 and 21 days post-CNV with enteric-microbiota and **(C)** IL-17 secretion post-colonization with enteric-microbiota in colonic explant cultures was determined by ELISA. **(D)** Colitis scores of GF and CNV WT and IL-10$^{-/-}$ mice. Results are presented as the sum of four averaged scores from five regions of the large intestine graded by a pathologist (T.R.) blinded to the groups (49). Results are expressed as mean + SEM from 3 mice per group and are representative of 2 independent transitioned experimental cohorts, *p<0.05 vs. WT CNV.

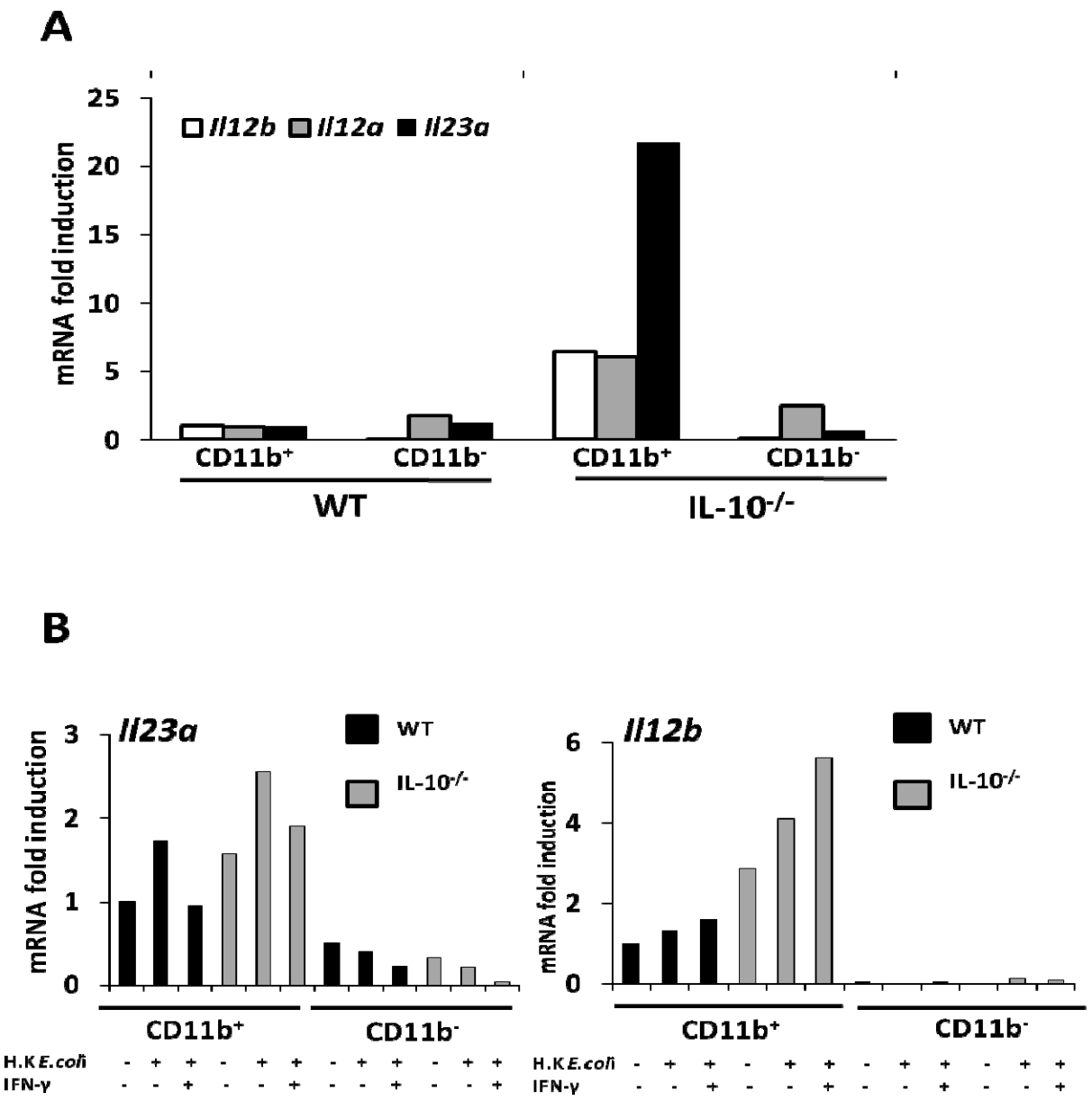


Figure 2.8. Colonic CD11b$^+$ lamina propria mononuclear cell (LPMC) in IL-10$^{-/-}$ mice are the primary source of *Il12a, Il12b* and *Il23a*. Lamina propria cells (LPMCs) were isolated from WT and IL-10$^{-/-}$ mouse colons and further separated into CD11b$^+$ cells using anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA). **(A)** Basal expression of *Il12a, Il12b* and *Il23a* mRNA was detected by real-time RT-PCR. **(B)** CD11b$^+$ and CD11b$^-$ LPMCs were activated with heat killed *E.coli* (multiplicity of infection 10:1) + IFN-γ (10 ng/mL) for 2 hours prior to total RNA isolation. *Il23a*, *Il12b* and β-actin mRNA expression was detected by real-time RT-PCR (Applied Biosystems). Results are expressed as fold induction normalized to β-actin and represent LPMCs pooled from 3 individual mouse colons, and replicated 3 times.

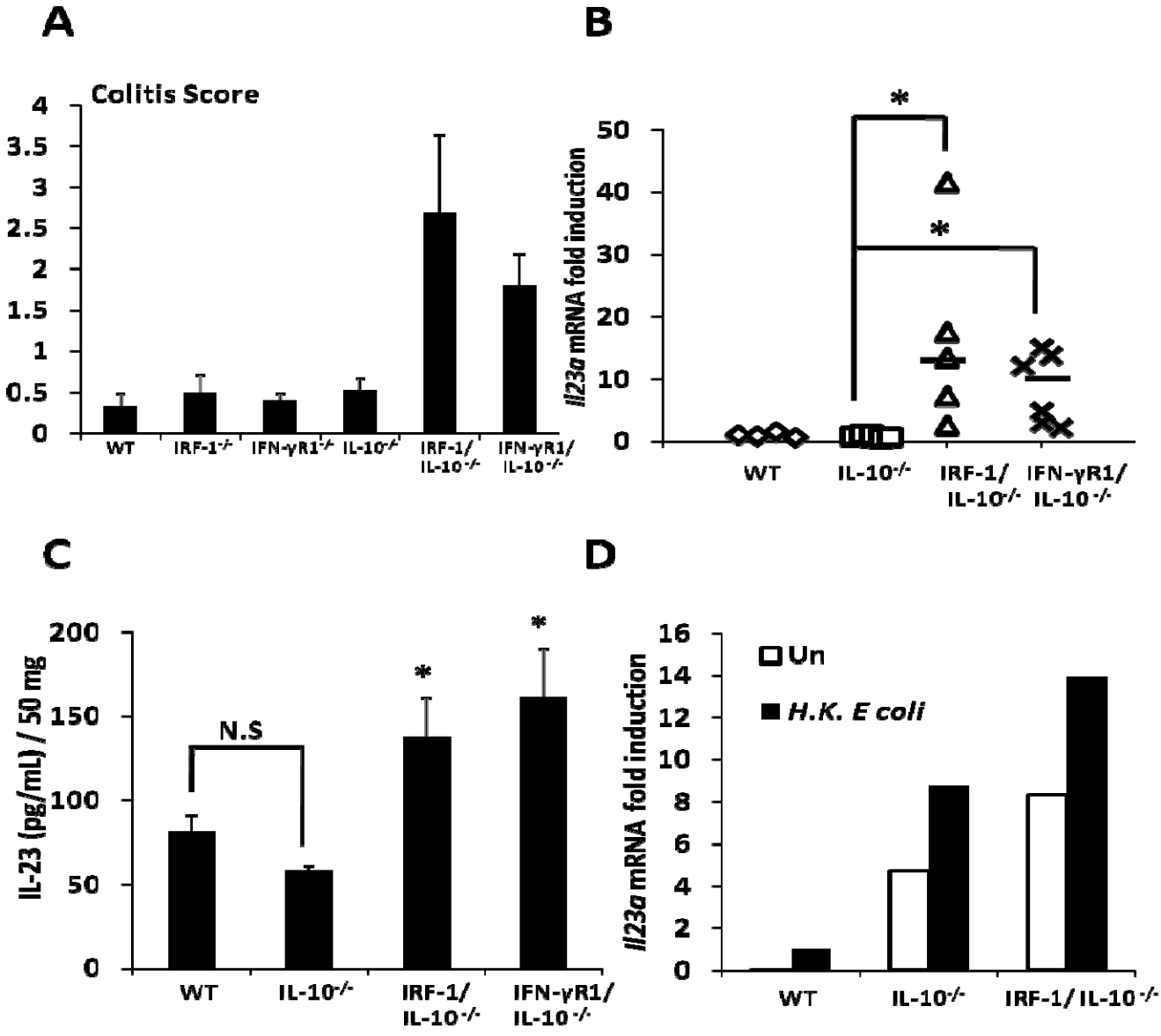
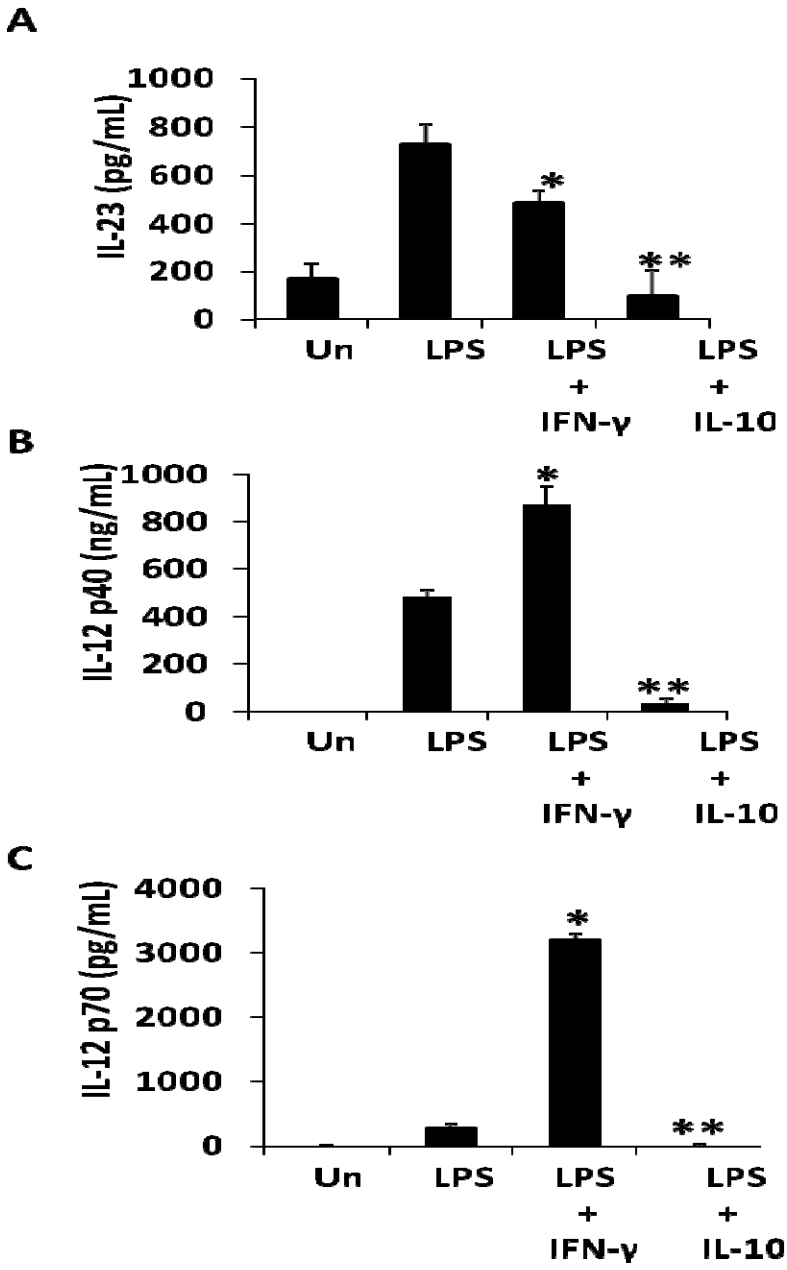
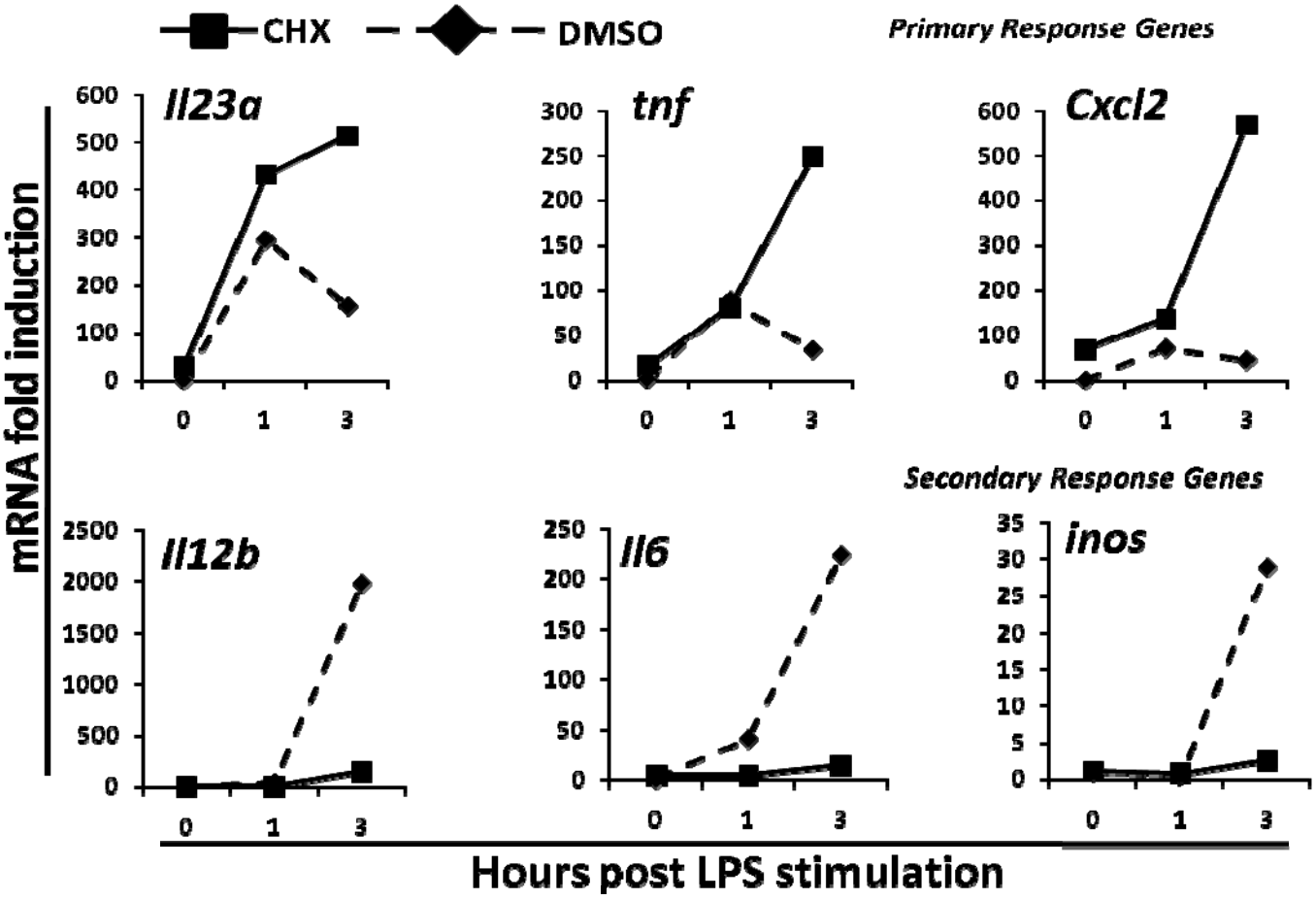


Figure 2.9. Increased mucosal expression of IL-23 in IFN-γR1/IL-10 $^{-/-}$ and IRF-1/IL-10$^{-/-}$ mice. (A) Colitis scores of WT, IRF-1$^{-/-}$, IFN-γR1$^{-/-}$, IL-10$^{-/-}$, IFN-γR1/IL-10 $^{-/-}$ and IRF-1/IL-10$^{-/-}$ mice raised in conventionalized housing at 8 weeks of age. Results are presented as the sum total of four averaged scores from five regions of the large intestine graded by a pathologist (T.R.) blinded to the groups using standard scoring system. **(B)** Colonic *Il23a* mRNA was examined using real-time RT-PCR and **(C)** IL-23 protein in supernatants from colon explants cultures were analyzed using cytokine specific ELISA and from WT, IL-10$^{-/-}$, IFN-γR1/IL-10 $^{-/-}$ and IRF-1/IL-10$^{-/-}$ mice. Results are expressed as mean $\pm$ SEM from 4-5 mice per group, *p<0.05 vs. IL-10$^{-/-}$ mice. **(D)** CD11b^{+} and CD11b^{-} LPMCs Lamina isolated from *WT, IL-10$^{-/-}$,* and *IL-10/IRF-1$^{-/-}$* mouse were unstimulated (Un) or activated with heat killed *E.coli (H.K. E.coli),* multiplicity of infection (10:1). Total RNA was isolated (Qiagen RNeasy Mini Kit) and *Il23a* and β-actin mRNA expression was detected by real-time RT-PCR. Results are expressed as fold induction relative to WT CD11b^{+} stimulated with *H.K.*

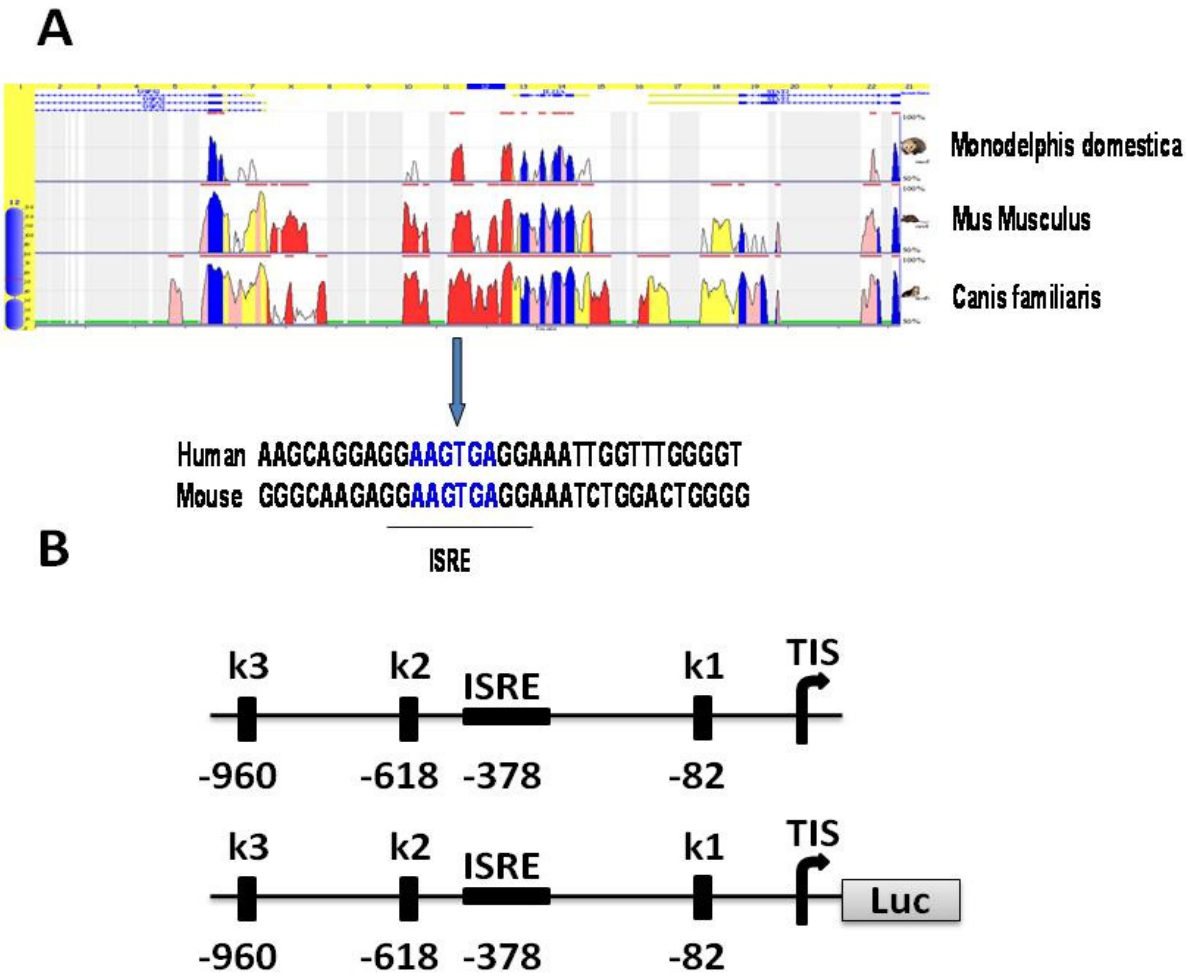
E.coli and normalized to β-actin and represent LPMCs pooled from 3 individual mouse colons.



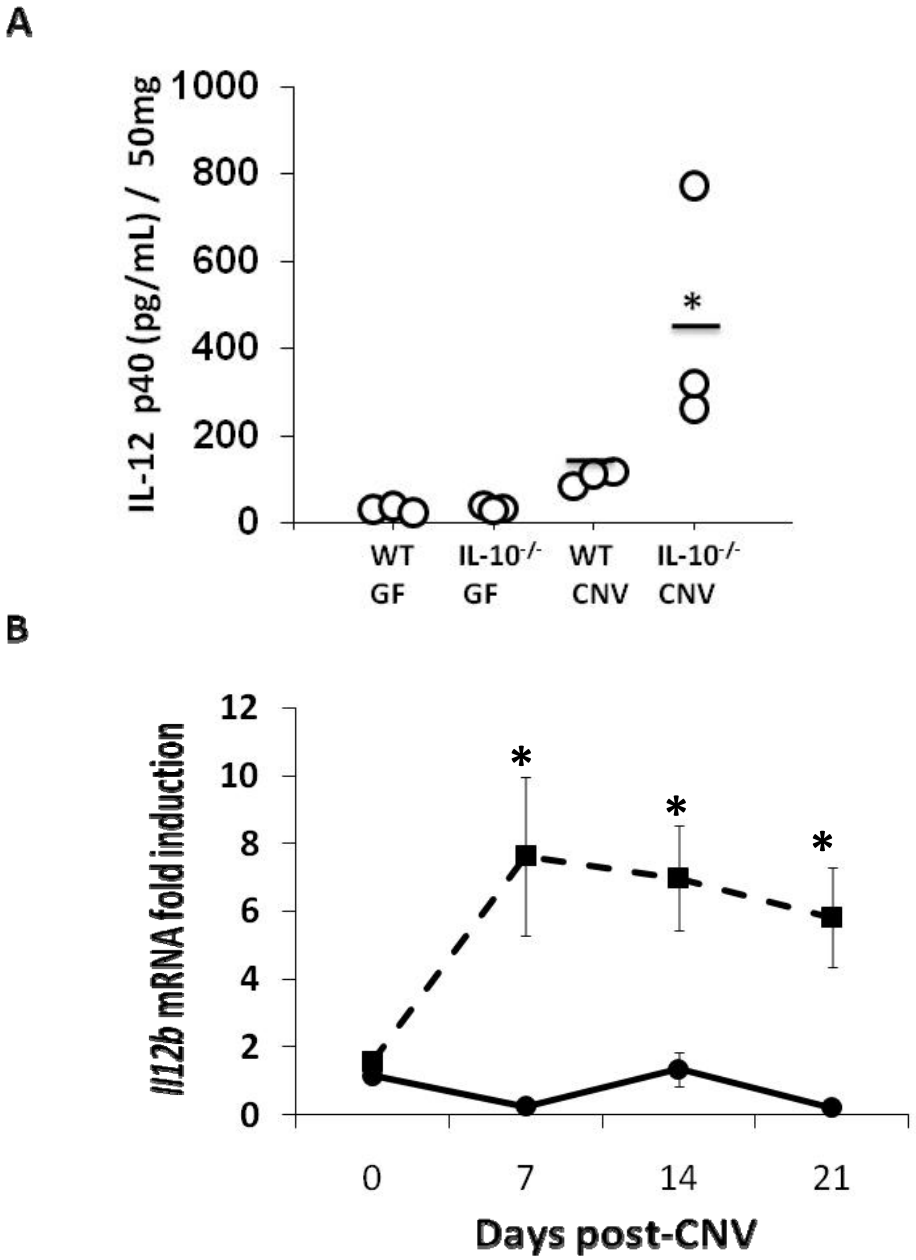
Supplemental Figure 2.1. IL-10 is a negative regulator of IL-23 in macrophages. BMMs from WT mice were cultured in the presence of LPS (100 ng/ml) and recombinant IL-10 (10 ng/ml). Supernatants from cultured BMMs were analyzed for **(A)** IL-23, **(B)** IL-12 p40 and **(C)** IL-12 p70 protein expression by ELISA. Results are expressed as mean±SEM of triplicate cultures and are representative of 3 independent experiments ($p<0.05$ vs. LPS stimulated BMMs).



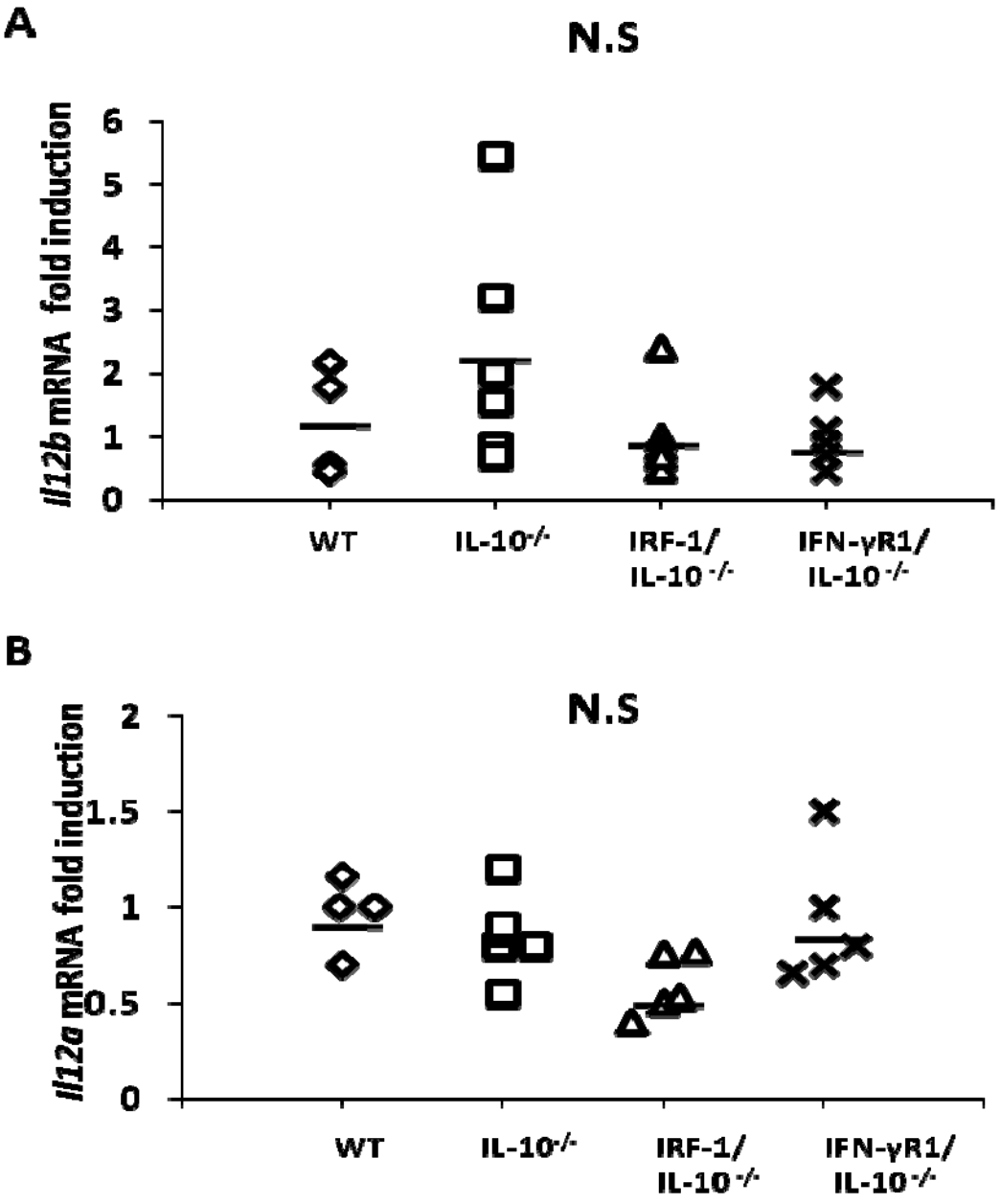
Supplemental Figure 2.2. *Il23a* is a primary response gene. BMMs were incubated for 30 minutes with DMSO (dashed line) or cyclohexamide (5 µg/mL, solid line) and then stimulated with LPS (100 ng/mL). Primary response (*Il23a, tnf* and *Cxcl2*) and secondary response gene (*Il12b, Il6* and *inos*) mRNA induction was analyzed using RT-PCR. Results are representative of 3 independent experiments with similar results



Supplemental Figure 2.3. Characterization of an ISRE within *a* conserved nucleotide sequence (CNS) in the *Il23a* promoter. (A) Using ECR Genome Browser (http://ecrbrowser.dcode.org/), CNS's (red) were identified across multiple species in the *Il23a* murine promoter relative to the human genome. A putative interferon stimulated response element (ISRE) was identified from -378 to -384 with respect to the transcription start site. **(B)** Schematic representation of the murine *Il23a* promoter region (*top panel*) and the luciferase reporter construct generated (*bottom panel*). The NF-κB sites designated κ1, κ2, and κ3 are -82, -618, and -960 bp upstream of the transcription start site (TIS), respectively (48). The luciferase reporter construct contains all three putative NF-κB sites upstream of a *luciferase* open reading frame. The ISRE (-378 to -384) is located between the NF-κB binding sites κ1 and κ2.



Supplemental Figure 2.4. The enteric-microbiota regulate IL-12 expression in IL-10$^{-/-}$ mice. WT and IL-10$^{-/-}$ mice raised in germ free (GF) conditions were colonized with enteric microbiota from conventionalized (CNV) mice at 8-10 weeks of age (3 mice per group). **(A)** IL-12 p40 secretion post-colonization with enteric-microbiota was determined by ELISA in colonic explant cultures. **(B)** Real time RT-PCR of colonic *Il12b* mRNA expression in WT (solid line) and IL-10$^{-/-}$ (dashed line) normalized to β-actin at 7, 14 and 21 days post-CNV with enteric- microbiota Results are expressed as mean + SEM from 3 mice per group and are representative of 2 independent transitioned experimental cohorts, *p<0.05 vs. WT CNV.



Supplemental Figure 2.5. Expression of IL-12 family members in IFN-γR1/IL-10 $^{-/-}$ and IRF-1 /IL-10$^{-/-}$ mice. Colonic *Il12b* and *Il12a* mRNA and was examined using real-time RT-PCR from WT, IL-10$^{-/-}$, IFN-γR1/IL-10 $^{-/-}$ and IRF-1/IL-10$^{-/-}$ mice. Results are expressed as mean ± SEM from 4-5 mice per group and were not statistically different vs. IL-10$^{-/-}$ mice.

CHAPTER 3

HEME OXYGENASE 1 (HO-1) EXPRESSION AND FUNCTION IS PROTECTIVE AGAINST MUCOSAL IMMUNE RESPONSES TO THE ENTERIC- MICROBIOTA

SHEHZAD Z. SHEIKH, KATSUYOSHI MATSUOKA, HOUDA ELLOUMI, ERIN STEINBACH, FENGLING LI, TAKU KOBAYASHI, TARA RUBINAS, SCOTT E. PLEVY

3.1. ABSTRACT

Heme oxygenase-1 (HO-1) is a cytoprotective enzyme that plays a critical role in host defense against oxidant-induced injury. HO-1 catalyzes the first and rate-limiting step in the oxidative degradation of heme to carbon monoxide (CO), biliverdin and ferrous iron. We have previously demonstrated that carbon monoxide (CO) suppresses chronic intestinal inflammation in IL-10 deficient (IL-$10^{-/-}$) mice through a HO-1 dependent pathway. Here, we elucidate mechanisms through which HO-1 expression and function impact innate immune responses to the enteric-microbiota. Wild type (WT) and colitis-prone IL-$10^{-/-}$ mice (n=5) raised in a germ-free environment (GF) were transitioned to conventionalized specific pathogen free housing (SPF). HO-1 mRNA (*Hmox1*) and protein were induced in the colons from WT SPF-transitioned but not in colons from IL-$10^{-/-}$ mice. In addition, in SPF-transitioned WT and IL-$10^{-/-}$ mice, colonic HO-1 expression inversely correlated with colonic inflammation and expression of IL-12, IL-23, and TNF. Pharmacological induction of HO-1 prevents the initiation of enteric-microbiota induced colitis in IL-$10^{-/-}$ mice. Based on these results, we further explored the role of IL-10 in the regulation of HO-1 in murine macrophages. IL-$10^{-/-}$ bone marrow-derived macrophages (BMMs) showed significantly reduced LPS-stimulated *Hmox1* and protein expression compared to WT BMMs. Furthermore, the addition of IL-10 restored HO-1 expression in IL-$10^{-/-}$ BMMs and enhanced expression in WT BMMs. Defects in intracellular bactericidal activity in macrophages have been implicated in the pathogenesis of IBD. To assess the role of CO and HO-1 in intracellular eradication of enteric bacteria, BMMs were cultured with *E. coli* (K-12) and a gentamicin protection assay was used to assess bactericidal responses. CO-exposed BMMs demonstrated increased HO-1 expression and enhanced bacterial killing compared to air-exposed BMMs. CO-induced enhanced bactericidal activity correlated with reduced secretion of IL-12 p40. To specifically study the role of HO-1, retrovirus transduced RAW

264.7 macrophages that over-express HO-1 were cultured with *E. coli.* Enhanced bacterial killing was demonstrated in RAW 264.7 macrophages over-expressing HO-1. In conclusion, the enteric-microbiota regulates HO-1 expression in the murine colon through IL-10 dependent mechanisms. Macrophage HO-1 expression enhances enteric bactericidal activity. These finding elucidate important homeostatic pathways mediated by HO-1 I mucosal immunity, and suggest that HO-1 induction is am attractive therapeutic target in chronic inflammatory bowel diseases.

3.2. Introduction

The pathogenesis of the human inflammatory bowel diseases (IBD) involves interactions between complex genetic, immunologic and environmental factors (1). The role of the environment in the pathogenesis of IBD is perhaps best evident by the epidemiological observation that cigarette smoking is protective against the development of human ulcerative colitis (UC) (2). However, factors that contribute to these protective effects remain unclear. Carbon monoxide (CO), a major component of cigarette smoke, has been shown to have anti-inflammatory effects in murine models of sepsis, postoperative ileus and organ xenotransplantation (3) (4) (5) (6). Mammalian cells generate CO endogenously as a product of heme degradation by the heme oxygenase (HO) enzymes. Heme oxygenase-1 (HO-1) is a cytoprotective enzyme that plays a critical role in defending the body against oxidant-induced injury (7). HO-1 catalyzes the first and rate-limiting step in the oxidative degradation of heme to carbon monoxide (CO), biliverdin and ferrous iron. Biliverdin is converted to bilirubin, a potent endogenous anti-oxidant with recently described anti-inflammatory properties (7). CO has numerous biological functions including anti-inflammatory properties (8). In acute models of inflammation, such as endotoxin exposure, HO-1 deficient ($Hmox1^{-/-}$) mice are susceptible to oxidant-induced tissue injury and death (9). In contrast, administration of CO or biliverdin to animals exposed to endoxin decreases inflammation and attenuates end-organ injury (10) (11) (12) (13). Although these results demonstrate beneficial effects of HO-1 and its products during purely acute inflammatory processes, there is less certainty to the role of HO-1 in chronic models of inflammation such as experimental colitis, collagen induced arthritis (CIA) and experimental autoimmune encephalitis (EAE). Our group was the first to demonstrate that CO ameliorates active

inflammation in a model of chronic IBD, IL-10 deficient (IL-$10^{-/-}$) mice, through induction of HO-1 (14).

The intestinal tract contains the largest number of macrophages in the body and these cells are strategically located directly underneath the epithelial layer (15). Intestinal macrophages have a different phenotype from other tissue macrophages in that they ingest and may kill microbes but they do not mediate strong pro-inflammatory responses upon microbial recognition (16). These properties make the intestinal macrophage an important component of host innate immune responses to enteric-microbiota. HO-1 and CO have been demonstrated to play an important role in antimicrobial processes. Endogenous HO-1 enhances bacterial clearance, in part, by increasing phagocytic activity against *E. faecalis* in a murine model of polymicrobial sepsis. These effects have been attributed to CO, as a CO-releasing molecule (CORM) enhanced phagocytosis in mice and rescued Hmox$1^{-/-}$ mice from lethality in polymicrobial sepsis (17).

In this study, we demonstrate that LPS induces HO-1 in wild type (WT) but not IL-$10^{-/-}$ bone marrow derived macrophages (BMMs). Expression of HO-1 is dependent on IL-10 and MyD88. Additionally, we demonstrate that the enteric-microbiota induce colonic expression of HO-1 in WT but not IL-$10^{-/-}$ mice. HO-1 expression inversely correlates with colonic inflammation and IL-12, IL-23 and TNF expression in IL-$10^{-/-}$. Moreover, HO-1 derived CO is important in enhancing macrophage bactericidal activity, which in turn correlates with protection against colitis in IL-$10^{-/-}$ mice. Overall, these studies further advance the understanding of the balance between pro- and anti-inflammatory mediators in enteric-microbiota induced intestinal inflammation, and highlight HO-1 as an important therapeutic target in IBD.

3.3. Results

3.3.1 Enteric-microbiota regulate heme oxygenase-1 (HO-1) expression in the colon.

To determined the effects of enteric-microbiota in regulating colonic HO-1 gene (*Hmox1)* expression in the colon, WT and colitis-prone IL-10$^{-/-}$ mice born and raised in GF environment were colonized with a specific pathogen free enteric-microbiota (conventionalized, CNV). WT but not IL-10$^{-/-}$ mice demonstrated increased *Hmox1* expression in the colon (Figure 3.1A). Attenuated colonic *Hmox1* expression correlated with the development of colonic inflammation in IL-10$^{-/-}$ mice 14 days post-CNV (Figure 3.1B).Pro-inflammatory cytokines gene expression, *Il12b* (Figure 3.1C and *tnf* (Figure 3.1D) were increased in IL-10$^{-/-}$ but not WT colons.

Next, we performed a kinetic analysis of HO-1 expression in the colons of WT and IL-10$^{-/-}$ GF mice transitioned into a CNV microbiota at 3, 7 and 14 days post-colonization. HO-1 expression was induced in WT colons but not IL-10$^{-/-}$ colons at day 3, 17 and 14 post-CNV (Figure 3.2A). The increase in colonic HO-1 was also demonstrated by immunohistochemistry in WT (Figure 3.2B, upper panel) compared with IL-10$^{-/-}$ mice (Figure 2B, lower panel). Finally, we determined the expression of HO-1 in colonic CD11b+ lamina propria mononuclear cells (LPMCs), representing predominantly an intestinal macrophage population. Colonic CD11b+ LPMCs from IL-10$^{-/-}$ mice demonstrate markedly reduced expression of *Hmox1* expression compared to WT CD 11b+ LPMC (Figure 3.2C). These results suggest that enteric-microbiota induced HO-1 expression in the colon is protective against initiation of intestinal inflammation.

3.3.2 LPS and IL-10 regulate HO-1 expression in macrophages. Next, we determined mechanisms through which enteric-bacteria regulate HO-1 in murine macrophages. BMMs from WT and IL-10$^{-/-}$ mice were stimulated with LPS ± IL-10 and expression of *Hmox1* mRNA and HO-1 protein was determined. IL-10$^{-/-}$ BMMs demonstrated decreased expression of *Hmox1* compared to WT BMMs. Addition of recombinant IL-10 restored *Hmox1* mRNA and protein expression in IL-10$^{-/-}$ BMMs (Figure 3.3A and B). Moreover, incubation of WT BMMs with an IL-10 antibody inhibited LPS induced expression of HO-1. Furthermore, addition of recombinant IL-10 augmented LPS induced HO-1 expression in WT BMMs and restored expression in IL-10$^{-/-}$BMMs (Figure 3.3B). Consequently, IL-12 p40 expression was increased in IL-10$^{-/-}$ BMMs compared to WT BMMs, and suppressed with the addition of IL-10 (Supplemental Figure 3.1A). These results demonstrate that LPS and IL-10 are regulators of HO-1 in macrophages.

3.3.3 TLR induced HO-1 in macrophages is MyD88 dependent. Toll-like receptors (TLRs) recognize specific molecular patterns present in a broad range of microbial pathogens. TLR activation utilizes a common signal transduction pathway initiated by the adaptor protein MyD88. MyD88-independent signaling is unique to TLR3 and TLR4 activation (18). To further elucidate TLR mediated induction of HO-1, WT and MyD88$^{-/-}$ BMMs were stimulated with MyD88 dependent (sBLP, LPS, flagellin and CpG DNA) and MyD88/TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent ligands (poly iC). Interestingly, sBLP, poly iC, LPS, flagellin and CpG DNA induced HO-1 expression in WT but not MyD88$^{-/-}$ BMMs (Figure 3.3C). Expression of inducible nitric oxide (iNOS), a TRIF dependent gene (19), is equivalent in MyD88$^{-/-}$ and WT BMMs, while HO-1 expression was absent (Figure 3.3C). Addition of recombinant IL-10 restored HO-1 expression in MyD88$^{-/-}$ BMMs,

suggesting that IL-10 induced expression of HO-1 in macrophages is independent of TLR mediated signaling pathways. As previously reported, TLR mediated IL-10 expression, another MyD88 dependent gene, was abrogated in MyD88$^{-/-}$ BMMs (Supplemental Figure 3.1B). These results demonstrate that HO-1 expression in macrophages is dependent on both MyD88 and IL-10.

3.3.4 Other signal transduction pathways and HO-1 expression in macrophages.

Inducers of HO-1 activate protein phosphorylation-dependent signaling cascades that ultimately converge on the transcription factors that regulate the *Hmox1* gene. MAP kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate varied cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis (10). MAPK kinases have been implicated in the induction of HO-1. We examined the role of two primary signaling cascades of the MAPK superfamily. First, treatment of WT BMMs with pharmacological inhibitor of p38 MAPK SB203580 resulted in reduction in LPS induced HO-1 protein expression (Figure 3.4A). Second, treatment with ERK1/2 inhibitor PD98059 had no effect on LPS induced HO-1 protein expression. Interestingly, p38 MAPK inhibition did not affect IL-10 induced HO-1 expression in WT BMMs (Figure 3.4A).

A limited number of studies have examined the role of the PI3K cell survival pathway in *Hmox1* gene regulation. PI3K, a ubiquitous lipid-modifying enzyme consisting of a p85 regulatory subunit and a p110 catalytic subunit, responds to activation by diverse stimuli including growth factors, cytokines, and cytotoxic agents (10). WT BMMs treated with PI3K inhibitor LY294002 resulted in significant reduction in LPS activated HO-1 protein induction (Figure 3.4B). Likewise, relatively few studies have implied roles for protein kinases (PK)

A/G/C in *Hmox1* regulation. WT BMMs treated with PKC inhibitor Ro31-8220 had no effect on LPS induced HO-1 protein expression in macrophages (Figure 3.4B). Overall, our studies indicate that the regulation of *Hmox1* gene expression in macrophages appears to involve protein phosphorylation cascades that converge on transcriptional activators or repressors leading to HO-1 induction.

3.3.5 Induction of HO-1 is protective against the initiation of colitis induced by the enteric-microbiota. We investigated the protective effects of colonic HO-1 expression in the prevention of enteric-microbiota induced colitis in IL-10$^{-/-}$ mice. GF IL-10$^{-/-}$ mice were administered 5 mg/kg cobalt protoporphyrin (CoPP), an inducer of HO-1, or vehicle (DMSO) intraperitoneally (IP), for two weeks prior to conventionalization. CoPP treated GF IL-10$^{-/-}$ mice demonstrated a robust increase in colonic HO-1 protein expression compared with vehicle treated mice (Figure 3.5A). Post-conventionalization, WT (vehicle treated) and IL-10$^{-/-}$ mice treated with CoPP demonstrated increased colonic HO-1 expression compared to vehicle treated CNV IL-10$^{-/-}$ mice (Figure 3.5B). IL-10$^{-/-}$ mice treated with CoPP demonstrated less severe colitis compared to vehicle treated group (Figure 3.5C). IL-12 p40 secretion in supernatants from colonic explants cultures was also significantly reduced in CoPP treated IL-10 mice compared to vehicle treated controls (Figure 3.5D). Our results demonstrate that induction of colonic HO-1 expression is protective against enteric -microbiota induced colitis in IL-10$^{-/-}$ mice.

3.3.6 HO-1 derived CO is enhances macrophage bactericidal activity. Resident macrophages adapt to the antigen rich intestinal environment by acquiring a phenotype that

is refractory to the induction of proinflammatory cytokine production by PAMPs (20). However, intestinal macrophages are not impaired in their phagocytic activity of enteric bacteria like *E. coli* which is exceptionally potent (21). Patients with Crohn's disease also demonstrate defective bacterial clearance by macrophages (22). Recently, HO-1 derived CO has been shown to enhance bacterial clearance by increasing the endogenous antimicrobial response (17). Compounds known as CO-releasing molecules (CORM) such as CORM-186 have the ability to release CO in vivo (23). CO is able to diffuse across cell boundaries and exhibit biological functions on neighboring cells. Therefore, studies were performed to determine whether HO-1 or CO affect the ability of macrophages to eradicate intracellular enteric bacteria. WT BMMs were transfected with *Hmox1* siRNA using AMAXA nucleofector technology. Significant knockdown of HO-1 was observed in *Hmox1* siRNA transfected BMMs compared with scrambled siRNA transfected controls (Figure 3.6A). Using gentamicin protection assays, *Hmox1* siRNA transfected BMMs showed impaired killing of intracellular commensal K12 *E. coli* compared with scrambled siRNA transfected controls (Figure 3.6B). Similarly, in RAW 264 macrophages stably transfected to over-express HO-1 (provided by Leo Otterbein, Beth Israel-Deaconess Medical Center,, Boston, MA) also demonstrated enhanced *E. coli* clearance compared with control transfected RAW 264 cells (Figure 3.6C). Finally, to demonstrate that bactericidal activity mediated by HO-1 is mediated by its metabolitic product CO, WT BMMs were incubated with CORM-186 (100 ug/ml) or inactive (i) CORM-186. Incubation with CORM-186 enhanced macrophage bactericidal activity in control and *Hmox1* siRNA transfected cells compared to iCORM-186 treated macrophages (Figure 3.6D). IL-$10^{-/-}$ BMMS, defective in TLR mediated HO-1 expression, also demonstrated impaired killing of intracellular *E. coli* compared with WT BMMs (Figure 3.6D). Incubation with CORM-186 (100 ug/ml) resulted in enhanced clearance of intracellular *E. coli* in WT and IL-$10^{-/-}$ BMMs. Our results demonstrate that HO-1

derived CO enhances macrophage bactericidal activity against a commensal enteric bacterium.

3.3.7 HO-1 derived carbon monoxide (CO) is protective against progression of murine colitis. Short term administration of the cyclooxygease inhibitor piroxicam has been shown to accelerate the development and severity of chronic colitis in IL-$10^{-/-}$ mice. Homozygous IL-$10^{-/-}$ mice on C57BL/6 background (5–6 weeks of age) were given piroxicam for 14 days at a dose of 200 ppm in the diet. As an *in vivo* correlate, we next determined whether CO would have an impact on colitis severity in piroxicam treated IL-$10^{-/-}$ mice. Following piroxicam treatment for 14 days, 30 mg/kg of CORM-186 or iCORM-186 was injected into the peritoneum twice daily for 2 weeks. Endogenously generated CO and exogenous CO gas, inhaled at doses whereby the oxygen-carrying capacity of hemoglobin is not severely compromised (carboxy-hemoglobin, HbCO<20%), has been shown to elicit protection and beneficial outcomes, covering a vast array of responses against multiple organ injury, inflammation, apoptosis, cell proliferation, vasoconstriction and both systemic and pulmonary hypertension. HbCO levels in CORM-186 treated mice ranged from 9-13% compared to <1-3% in iCORM-186 treated group (data not shown). CORM-186 treated mice had a significant decrease in colitis scores compared with iCORM-treated mice (Figure 3.7A). CORM-186 treated IL-$10^{-/-}$ mice also demonstrated less colonic secretion of IL-12 p40 in colon explant cultures (Figure 3.B).

3.3.8 Pharmacological induction of HO-1 and CORM-186 enhance phagolysosome formation in macrophages. HO-1 and CO have been implicated in enhancing macrophage

phagocytosis. HO-1 and CO also both enhance the ability to eradicate intracellular *E. coli*. Recently, alterations in phagosomal function have emerged as a central focus in the macrophage's ability to eradicate intracellular bacteria. However, mechanisms responsible these defects remain elusive. We next sought to determine if induction of HO-1 (CoPP) or treatment with CORM-186 affect phagolysosome formation in macrophages. WT BMMS were incubated with medium containing LysoTracker®, a weak base that permeated cell membranes and fluoresces upon protonation in low-pH environments. LysoTracker® detects phagolysosomal acidification (Figure 3.8A). eGFP labeled E.coli were used as a positive control to demonstrate phagolysosomal activation. CoPP and CORM-186 treated WT BMMs had significantly increased percentage of LysoTracker®-positive cells (Figure 3.8B) demonstrating that HO-1 and CO may enhance phagolysosomal activation.

3.4. Discussion

These experiments demonstrate the importance of colonic HO-1 expression in protecting against mucosal innate immune responses to enteric-microbiota. The enteric -microbiota induce colonic HO-1 in WT but not colitis-prone IL-$10^{-/-}$ mice in a GF environment when transitioned to CNV housing. Pharmacological induction of HO-1 in IL-$10^{-/-}$ GF mice prior to colonization with enteric-microbiota is protective against the initiation of colitis. Mechanistically, LPS and IL-10 regulate HO-1 expression in BMMs and colonic CD11b+ LPMCs. TLR mediated regulation of HO-1 in macrophages is MyD88 dependent. Our experiments also reveal that protective effects of HO-1 in colitis are through CO, one of the by-products of heme metabolism, and suggest that, in part, CO and HO-1 may exert mucosal protection through enhancement of bactericidal pathways..

In a murine model of acute mucosal injury (dextran sodium sulfate–induced colitis), the HO-1 inducer CoPP modestly ameliorated inflammatory changes, but CO had no effect (24). In addition, pharmacologic inhibition of HO-1 exacerbated TNBS-induced acute colitis in rats (25). Our group was the first to suggest an anti-inflammatory role for CO in chronic intestinal inflammation and the first to modulate the HO-1 pathway in established chronic intestinal inflammation (14). Accumulating evidence suggests that the dynamic balance between the enteric-microbiota and host innate responses in the intestine has a pivotal role in the initiation and pathogenesis of chronic IBD (26). The importance of the microbiota is directly supported by studies in a variety of murine strains in which 'spontaneous' chronic colitis is dependent on the presence of the enteric-microbiota (27). Utilizing GF WT and colitis-prone IL-$10^{-/-}$ mice, this study is the first to demonstrate that the enteric-microbiota regulate expression of HO-1 in the murine colon. Enteric-microbiota induced colonic HO-1 inversely correlates with colonic inflammation and pro-inflammatory cytokine secreation in

IL-10$^{-/-}$ mice. Decreased *Hmox1* expression was also evident in CD 11b+ LPMCs from IL-10$^{-/-}$ mice compared to WT mice.

A variety of physiological and non-physiological stressors, including endotoxin, inflammatory cytokines, ultraviolet A radiation (UVA), hyperthermia, and heavy metals, stimulate HO-1 expression and activity, primarily via activation of the *Hmox*1 gene. LPS stimulates HO-1 in WT but not IL-10$^{-/-}$ BMMs. However, IL-10 still induced HO-1 in MyD88$^{-/-}$ BMMs, suggesting an alternative signaling pathway from TLR ligands (Figure 3.3C). Lee and colleagues demonstrated that IL-10 induces expression of HO-1 via a p38 mitogen-activated protein kinase (MAPK)-dependent pathway (28). Interestingly, inhibition of p38 also repressed LPS induced HO-1 in BMMs but inhibition of ERK had no apparent effects (Figure 3.4). This is in agreement with a substantial amount of data that supports a role for the MAPK cascades in signal-mediated *Hmox1* activation (29) (30). Interestingly, decreased HO-1 induction by MAPK p38 inhibition may be specific to macrophages. Studies utilizing the p38 inhibitor SB 203580 in other cell types such as rat hepatocytes is not inhibited. This may point to effects of specific isoforms of p38, as the α- and β-isozymes of p38 that are direct targets of p38 MAPK inhibitor SB 203580(30). We also show the importance of PI3K in LPS mediated induction of HO-1 in macrophages. This is supported by studies demonstrating that specific inhibitors of PI3K blocked Nrf2 activation, the major transcription factor involved in *Hmox1* gene expression, upon oxidative stress in H4IIE hepatoma cells(31). Similarly, inhibitors of PI3K blocked the activation of *Hmox1* by 15d-PGJ_2 in human lymphocytes (32). In similar studies using murine macrophages, the PI3K inhibitor LY294002 also blocked *ho-1* activation by LPS (33).

LPS and inflammatory cytokines (IL-1β and TNF) are widely recognized as potent inducers of HO-1 expression, and several studies have linked NF-κB and AP-1 factors in this

response (34) (35). However, given the absence of a clearly identified, functional NF-κB binding site, how NF-κB promotes *Hmox1* gene transcription becomes a matter of speculation (36). Prior studies have provided support for NF-κB in *Hmox1* regulation with the use of chemical and gene-based inhibitors (37) (38). Our study is the first to demonstrate that TLR mediated regulation of HO-1 is dependent on MyD88 in BMMs.

Patients with Crohn's disease demonstrate defective macrophage bacterial clearance (22). HO-1 derived CO has been shown to enhance bacterial clearance by increasing the endogenous antimicrobial response without inhibiting the inflammatory response (17). Similarly, Otterbein and colleagues have shown *in vitro* that CO increased macrophage phagocytosis of E.coli in the RAW 264.7 cell line (11). CO has also been reported to inhibit TLR pathways that results in a decrease production of pro-inflammatory cytokines, such as TNF in macrophages (39). We were interested in studying whether suppression of an inflammatory response by HO-1 and CO could involve the ability of macrophages to eradicate commensal bacteria like *E.coli*. *Hmox1* gene knockdown using siRNA markedly reduced the ability of macrophages to eradicate intracellular E.coli. Importantly, IL-$10^{-/-}$ BMMs, defective in LPS mediated induction of HO-1, also demonstrated decreased bactericidal activity. CO enhanced macrophage bactericidal activity against E.coli in both WT and IL-$10^{-/-}$ BMMs. Administration of CORM-186 to colitis-prone IL-$10^{-/-}$ mice decreased intestinal inflammation and colonic IL-12 p40 secretion. Chung and colleagues recently demonstrated that CORMs increase phagocytosis, decrease circulating bacterial counts and rescue HO-$1^{-/-}$ mice from the exaggerated mortality of polymicrobial sepsis.

Our study also reveals a novel pathway through which HO-1 and CO affects macrophage bactericidal activity. Pharmacological induction of HO-1 with CoPP or incubation with CORM-186 results in significantly increased phagolysosomal activation in

macrophages associated with enhanced killing of intracellular *E. coli*. Macrophages can kill or limit the replication of microorganisms through many possible mechanisms including the limitation of available nutrients, production of antimicrobial peptides, reactive oxygen and nitrogen species (ROS/RNS) and lysosomal enzymes (1). Phagolysosome formation is an early but critical aspect through which macrophages eradicate intracellular pathogens. Indeed, in humans, chronic granulomatous disease (CGD) is a consequence of a genetic alteration that results in defective phagolysosomal activation. CGD patients are not only susceptible to bacterial infections but also develop IBD. Furthermore, the importance of microbicidal pathways in the pathogenesis of IBD was highlighted by the discovery that a synonymous SNP in the auto-phagocytic gene ATG16L1 and a SNP in the phagosomal gene NCF4 are associated with enhanced risk for IBD (40, 41).

In conclusion the enteric-microbiota induce HO-1 in the colons of WT but not colitis prone IL-10$^{-/-}$ mice. Pharmacological induction of HO-1 in GF IL-10$^{-/-}$ mice prior to colonization with enteric-microbiota reduces colonic inflammation. HO-1 and its metabolic product CO exert their anti-inflammatory effects through enhancement of macrophage bactericidal activity. Defective HO-1 induction in macrophages may lead to defective intracellular responses to commensal enteric bacteria, therefore contributing to the pathogenesis of IBD.

3.5. Materials and Methods

Mice. Wild-type and genetically deficient mice (WT, IL-10$^{-/-}$, MyD88$^{-/-}$) in specific pathogen free conventionalized housing (CNV) were on the C57BL/6 background (Jackson Laboratories) and matched for age in all experiments. All genetically deficient mice assessed for spontaneous colitis were littermates. 129S6/SvEv GF mice (WT and IL-10 $^{-/-}$) were Caesarian derived and were maintained according to standard techniques (42) in Trexler flexible film isolators at the Gnotobiotic Animal Facility of the Center for Gastrointestinal Biology and Disease at the University of North Carolina, Chapel Hill. CNV mice were maintained in a dedicated room at the University of North Carolina Laboratory Animal Resources Facility. GF status was monitored every 2 weeks by aerobic and anaerobic culture and gram stain of stool samples and/or bedding material. Mice were colonized with enteric-microbiota at 10-12 weeks of age with a murine microbiota that was isolated from WT mice raised in SPF conditions (43). GF IL-10$^{-/-}$ mice were injected intra-peritoneal (IP) with 5 mg/kg of cobalt protoporphyrin (CoPP). Similarly IL-10$^{-/-}$ mice raised in CNV environment were injected IP with 30 mg/kg of CORM-186 or inactive CORM-186, twice daily for two weeks. Additionally, CNV mice were determined to be negative for *Helicobacter* species (*Helicobacter bilis, Helicobacter hepaticus, Helicobacter rodentium, Helicobacter trogontum and Helicobacter sp.*) using *Helicobacter* PCR profile performed on freshly harvested fecal pellets (RADIL Laboratories, Columbia MO). All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. At the end of the study period, animals were euthanized using excess CO2 inhalation.

Murine bone marrow-derived macrophages. Bone-marrow-derived macrophages (BMMs) were harvested as previously described (44). Briefly, BMMs were grown for 7 days in RPMI 1640 containing 10% FCS, 10mM HEPES, 1% penicillin/streptomycin and supplemented with 40 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ). BMMs were stimulated with 100 ng/ml of high purity LPS and/or 10 ng/ml of IFN-γ (Invivogen, San Diego, CA).

Cytokine ELISAs. Murine IL-12 p40 immunoassay kits (R&D Systems) was used according to the manufacturers' instructions.

Western immunoblot. Western blot analyses were performed on whole cell extracts as described previously (44). Anti–HO-1 antibodies were from Stressgen, MI and β-actin antibodies were purchased from Abcam, MA.

Soluble inhibitors. Soluble inhibitors for ERK1/2 (PD 98059), p38 MAPK (SB 203680), PKC (Ro31-8220) and PI3K (LY 294003) were obtained from Calbiochem, CA..

RNA extraction and quantitative real-time RT PCR (qRT-PCR) analysis. Total RNA was extracted with RNeasy kit (Qiagen) and reverse-transcribed with ramdom hexamers using Superscript reverse transcriptase II (Invitrogen). Complementary DNA was analyzed by quantitative real-time PCR using SYBR Green Master Mix (Applied Biosystems) on a HT-7900 (Applied Biosystems). Single-product amplification was confirmed by melting-curve analysis. Primer sequences are as follow: *Hmox1* Forward 5'-ccagagtttccgcatacaacc-3', Reverse 5'-tctctggacacctgacccttcg-3', *Il12b* Forward 5'-cgcaagaaagaaaagatgaaggag-3', Reverse 5'-ttgcattggacttcggtagatg-3'; tnf Forward 5' *β-Actin* Forward 5'-agccatgtacgtagccatccag-3', Reverse 5'-tggcgtgagggagagcatag-3'. Expression was normalized to β-Actin and represented as fold induction over unstimulated cells.

Transient siRNA transfections. Bone marrow-derived murine macrophages were transiently transfected with ON-TARGET*plus Hmox1* siRNA, Chicago, IL using *AMAXA Nucleofector Technology* (AMAXA) by the described protocol for murine macrophages. After incubation for 12 hours at 37^{o}C, the cells were either unactivated or activated with 100 ng/ml LPS, 10 ng/ml IFN-γ, or both. Control scrambled siRNA was purchased from Dharmacon, IL.

Colonic tissue explant cultures. Sections of the transverse colon were processed as previously described (14). Tissue fragments (0.5 g dry weight) were incubated in 1.0 ml RPMI 1640 supplemented with 50 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone (GIBCO BRL), and 5% heat-inactivated fetal calf serum. Tissue fragment supernatants were collected after 24 hours for cytokine ELISAs.

Preparation of heat-killed bacteria. *E. coli* in log-phase growth was harvested and washed twice with ice-cold PBS. Bacterial suspensions were, then, heated at 80°C for 30 minutes, washed, resuspended in PBS, and stored at –80°C. Complete killing was confirmed by 72-hour incubation at 37°C on plate medium. Heat-killed bacteria were added at multiplicity of infection (M.O.I.) = 10.

Isolation of colonic macrophages. Lamina propria cells (LPCs) were isolated from mouse colon by a modified enzymatic method (45). Briefly, colons were dissected into small pieces, and washed three times in Hank's buffered saline solution (HBSS) containing 2.5% FBS. The last wash was done with 1mM DTT to remove mucus. The pieces were, then, incubated in HBSS containing 1mM EDTA three times for 20 min each at 37°C. The remaining tissue was digested in HBSS containing 1 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO) for 1.5 hours at 37°C. The supernatant was collected, filtered and centrifuged to obtain a cell pellet. LPCs were isolated by density gradient centrifugation using 40 % and 75 % Percoll solution (GE Healthcare, Piscataway, NJ). The intermediate layer containing LPCs

was collected. LPCs were further separated into CD11b+ cells using anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA). Purity was more than 90% by flow cytometric analysis (data not shown).

Histology. Colonic tissue sections were fixed in 10% buffered formalin and embedded in paraffin. 4-μm-thick sections were stained with hematoxylin and eosin. Spontaneous colitis scoring was adapted from the criteria reported by Berg et al, as previously described (14). All histological scores were determined by a staff pathologist (T. Rubinas) who was blinded to the experimental protocols.

Immunohistochemistry. Colonic tissue sections were fixed in 10% formalin and cut into 4 μm-thick paraffin sections and placed on microscope slides. Tissue sections were stained with rabbit anti-HO-1 (Stressgen, MI), at a 1:500 dilution for overnight at 4^{o}C. Samples were then incubated for 1 hour at room temperature with secondary antibody (1:500 dilution of rabbit anti-mouse (SantaCruz, CA). Rabbit nonimmune serum or secondary antibody alone was used as controls and nonspecific immunostaining was not observed (data not shown). Slides were viewed on an Olympus Flouview 1000 confocal microscope (Olympus America, Melville, NY).

Gentamicin protection assay. 1x10^{6} WT and IL-10$^{-/-}$ BMMs were cultured in 12-well plates in triplicate and incubate with *E.coli* K12 at 10:1 ratio in antibiotic-free medium for 1 hour. Cells were then washed with PBS plus gentamicin (200 μg/ml) and incubated in medium supplemented with gentamicin (200 μg/ml) at 37°C for an additional hour to eliminate extracellular bacteria. New medium supplemented with gentamicin (100 mg/ml is added to the cells. At 12 hours, a batch of cells are lysed with 1% Triton X-100, diluted, plated on BHI agar plates, and incubated at 37°C overnight. Colony-forming units (CFUs) calculated at 1 hour were determine total uptake of bacteria (phagocytosis). The CFUs recovered at 12

hours were represented as the percent survival of total bacteria phagocytosed at the 1 hour time point. CORM-186 and inactive CORM-186 were added to the cells after the initial elimination of extracellular bacteria and incubated for 12 hours thereafter.

Statistical Analysis. Statistical significance for data subsets from experiments performed in cells was assessed by the two-tailed Student's *t*-test. Statistical significance for *in vivo* data subsets was assessed by the Mann-Whitney U test (SPSS, Chicago, IL, USA)) with Bonferroni correction.

3.6. References

1. Xavier, R.J., and D.K. Podolsky. 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448:427-434.

2. Jick, H., and A.M. Walker. 1983. Cigarette smoking and ulcerative colitis. *N Engl J Med* 308:261-263.

3. Ryter, S.W., and A.M. Choi. 2007. Cytoprotective and anti-inflammatory actions of carbon monoxide in organ injury and sepsis models. *Novartis Found Symp* 280:165-175; discussion 175-181.

4. Sato, K., J. Balla, L. Otterbein, R.N. Smith, S. Brouard, Y. Lin, E. Csizmadia, J. Sevigny, S.C. Robson, G. Vercellotti, A.M. Choi, F.H. Bach, and M.P. Soares. 2001. Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. *J Immunol* 166:4185-4194.

5. Hoetzel, A., T. Dolinay, R. Schmidt, A.M. Choi, and S.W. Ryter. 2007. Carbon monoxide in sepsis. *Antioxid Redox Signal* 9:2013-2026.

6. Moore, B.A., L.E. Otterbein, A. Turler, A.M. Choi, and A.J. Bauer. 2003. Inhaled carbon monoxide suppresses the development of postoperative ileus in the murine small intestine. *Gastroenterology* 124:377-391.

7. Otterbein, L.E., M.P. Soares, K. Yamashita, and F.H. Bach. 2003. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 24:449-455.

8. Choi, A.M., and T. Dolinay. 2005. "Therapeutic" carbon monoxide may be a reality soon. *Am J Respir Crit Care Med* 171:1318-1319.

9. Wiesel, P., A.P. Patel, N. DiFonzo, P.B. Marria, C.U. Sim, A. Pellacani, K. Maemura, B.W. LeBlanc, K. Marino, C.M. Doerschuk, S.F. Yet, M.E. Lee, and M.A. Perrella. 2000. Endotoxin-induced mortality is related to increased oxidative stress and end-organ dysfunction, not refractory hypotension, in heme oxygenase-1-deficient mice. *Circulation* 102:3015-3022.

10. Ryter, S.W., J. Alam, and A.M. Choi. 2006. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 86:583-650.

11. Otterbein, L.E., F.H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R.J. Davis, R.A. Flavell, and A.M. Choi. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 6:422-428.

12. Morse, D., S.E. Pischke, Z. Zhou, R.J. Davis, R.A. Flavell, T. Loop, S.L. Otterbein, L.E. Otterbein, and A.M. Choi. 2003. Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. *J Biol Chem* 278:36993-36998.

13. Mazzola, S., M. Forni, M. Albertini, M.L. Bacci, A. Zannoni, F. Gentilini, M. Lavitrano, F.H. Bach, L.E. Otterbein, and M.G. Clement. 2005. Carbon monoxide pretreatment prevents respiratory derangement and ameliorates hyperacute endotoxic shock in pigs. *FASEB J* 19:2045-2047.

14. Hegazi, R.A., K.N. Rao, A. Mayle, A.R. Sepulveda, L.E. Otterbein, and S.E. Plevy. 2005. Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. *J Exp Med* 202:1703-1713.

15. Chieppa, M., M. Rescigno, A.Y. Huang, and R.N. Germain. 2006. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med* 203:2841-2852.

16. Coombes, J.L., and K.J. Maloy. 2007. Control of intestinal homeostasis by regulatory T cells and dendritic cells. *Semin Immunol* 19:116-126.

17. Chung, S.W., X. Liu, A.A. Macias, R.M. Baron, and M.A. Perrella. 2008. Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *J Clin Invest* 118:239-247.

18. Rakoff-Nahoum, S., and R. Medzhitov. 2008. Innate immune recognition of the indigenous microbial flora. *Mucosal Immunol* 1 Suppl 1:S10-14.

19. Lee, J.Y., C.A. Lowell, D.G. Lemay, H.S. Youn, S.H. Rhee, K.H. Sohn, B. Jang, J. Ye, J.H. Chung, and D.H. Hwang. 2005. The regulation of the expression of inducible nitric oxide synthase by Src-family tyrosine kinases mediated through MyD88-independent signaling pathways of Toll-like receptor 4. *Biochem Pharmacol* 70:1231-1240.

20. Smith, P.D., C. Ochsenbauer-Jambor, and L.E. Smythies. 2005. Intestinal macrophages: unique effector cells of the innate immune system. *Immunol Rev* 206:149-159.

21. Smythies, L.E., M. Sellers, R.H. Clements, M. Mosteller-Barnum, G. Meng, W.H. Benjamin, J.M. Orenstein, and P.D. Smith. 2005. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* 115:66-75.

22. Smith, A.M., F.Z. Rahman, B. Hayee, S.J. Graham, D.J. Marks, G.W. Sewell, C.D. Palmer, J. Wilde, B.M. Foxwell, I.S. Gloger, T. Sweeting, M. Marsh, A.P. Walker, S.L. Bloom, and A.W. Segal. 2009. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med* 206:1883-1897.

23. Foresti, R., C. Shurey, T. Ansari, P. Sibbons, B.E. Mann, T.R. Johnson, C.J. Green, and R. Motterlini. 2005. Reviewing the use of carbon monoxide-releasing molecules (CO-RMs) in biology: implications in endotoxin-mediated vascular dysfunction. *Cell Mol Biol (Noisy-le-grand)* 51:409-423.

24. Berberat, P.O., A.R. YI, K. Yamashita, M.M. Warny, E. Csizmadia, S.C. Robson, and F.H. Bach. 2005. Heme oxygenase-1-generated biliverdin ameliorates experimental murine colitis. *Inflamm Bowel Dis* 11:350-359.

25. Wang, W.P., X. Guo, M.W. Koo, B.C. Wong, S.K. Lam, Y.N. Ye, and C.H. Cho. 2001. Protective role of heme oxygenase-1 on trinitrobenzene sulfonic acid-induced colitis in rats. *Am J Physiol Gastrointest Liver Physiol* 281:G586-594.

26. Sartor, R.B. 2006. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 3:390-407.

27. Sartor, R.B. 2008. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 134:577-594.

28. Lee, T.S., and L.Y. Chau. 2002. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med* 8:240-246.

29. Elbirt, K.K., A.J. Whitmarsh, R.J. Davis, and H.L. Bonkovsky. 1998. Mechanism of sodium arsenite-mediated induction of heme oxygenase-1 in hepatoma cells. Role of mitogen-activated protein kinases. *J Biol Chem* 273:8922-8931.

30. Kietzmann, T., A. Samoylenko, and S. Immenschuh. 2003. Transcriptional regulation of heme oxygenase-1 gene expression by MAP kinases of the JNK and p38 pathways in primary cultures of rat hepatocytes. *J Biol Chem* 278:17927-17936.

31. Kang, K.W., S.J. Lee, J.W. Park, and S.G. Kim. 2002. Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-related factor 2 through actin rearrangement in response to oxidative stress. *Mol Pharmacol* 62:1001-1010.

32. Alvarez-Maqueda, M., R. El Bekay, G. Alba, J. Monteseirin, P. Chacon, A. Vega, J. Martin-Nieto, F.J. Bedoya, E. Pintado, and F. Sobrino. 2004. 15-deoxy-delta 12,14-prostaglandin J2 induces heme oxygenase-1 gene expression in a reactive oxygen species-dependent manner in human lymphocytes. *J Biol Chem* 279:21929-21937.

33. Chung, S.W., Y.H. Chen, and M.A. Perrella. 2005. Role of Ets-2 in the regulation of heme oxygenase-1 by endotoxin. *J Biol Chem* 280:4578-4584.

34. Camhi, S.L., J. Alam, L. Otterbein, S.L. Sylvester, and A.M. Choi. 1995. Induction of heme oxygenase-1 gene expression by lipopolysaccharide is mediated by AP-1 activation. *Am J Respir Cell Mol Biol* 13:387-398.

35. Camhi, S.L., J. Alam, G.W. Wiegand, B.Y. Chin, and A.M. Choi. 1998. Transcriptional activation of the HO-1 gene by lipopolysaccharide is mediated by 5' distal enhancers: role of reactive oxygen intermediates and AP-1. *Am J Respir Cell Mol Biol* 18:226-234.

36. Xiao, W. 2004. Advances in NF-kappaB signaling transduction and transcription. *Cell Mol Immunol* 1:425-435.

37. Wijayanti, N., S. Huber, A. Samoylenko, T. Kietzmann, and S. Immenschuh. 2004. Role of NF-kappaB and p38 MAP kinase signaling pathways in the lipopolysaccharide-dependent activation of heme oxygenase-1 gene expression. *Antioxid Redox Signal* 6:802-810.

38. Hill-Kapturczak, N., V. Thamilselvan, F. Liu, H.S. Nick, and A. Agarwal. 2001. Mechanism of heme oxygenase-1 gene induction by curcumin in human renal proximal tubule cells. *Am J Physiol Renal Physiol* 281:F851-859.

39. Nakahira, K., H.P. Kim, X.H. Geng, A. Nakao, X. Wang, N. Murase, P.F. Drain, M. Sasidhar, E.G. Nabel, T. Takahashi, N.W. Lukacs, S.W. Ryter, K. Morita, and A.M. Choi. 2006. Carbon monoxide differentially inhibits TLR signaling pathways by regulating ROS-induced trafficking of TLRs to lipid rafts. *J Exp Med* 203:2377-2389.

40. Reinhard, C., and J.D. Rioux. 2006. Role of the IBD5 susceptibility locus in the inflammatory bowel diseases. *Inflamm Bowel Dis* 12:227-238.

41. Cho, J.H. 2008. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 8:458-466.

42. MacDonald, T.T., and P.B. Carter. 1978. Contact sensitivity in the germ-free mouse. *J Reticuloendothel Soc* 24:287-293.

43. Kim, S.C., S.L. Tonkonogy, C.A. Albright, J. Tsang, E.J. Balish, J. Braun, M.M. Huycke, and R.B. Sartor. 2005. Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. *Gastroenterology* 128:891-906.

44. Xiong, H., C. Zhu, F. Li, R. Hegazi, K. He, M. Babyatsky, A.J. Bauer, and S.E. Plevy. 2004. Inhibition of Interleukin-12 p40 Transcription and NF-{kappa}B Activation by Nitric Oxide in Murine Macrophages and Dendritic Cells. *J Biol Chem* 279:10776-10783.

45. Kobayashi, T., S. Okamoto, T. Hisamatsu, N. Kamada, H. Chinen, R. Saito, M.T. Kitazume, A. Nakazawa, A. Sugita, K. Koganei, K. Isobe, and T. Hibi. 2008. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut* 57:1682-1689.

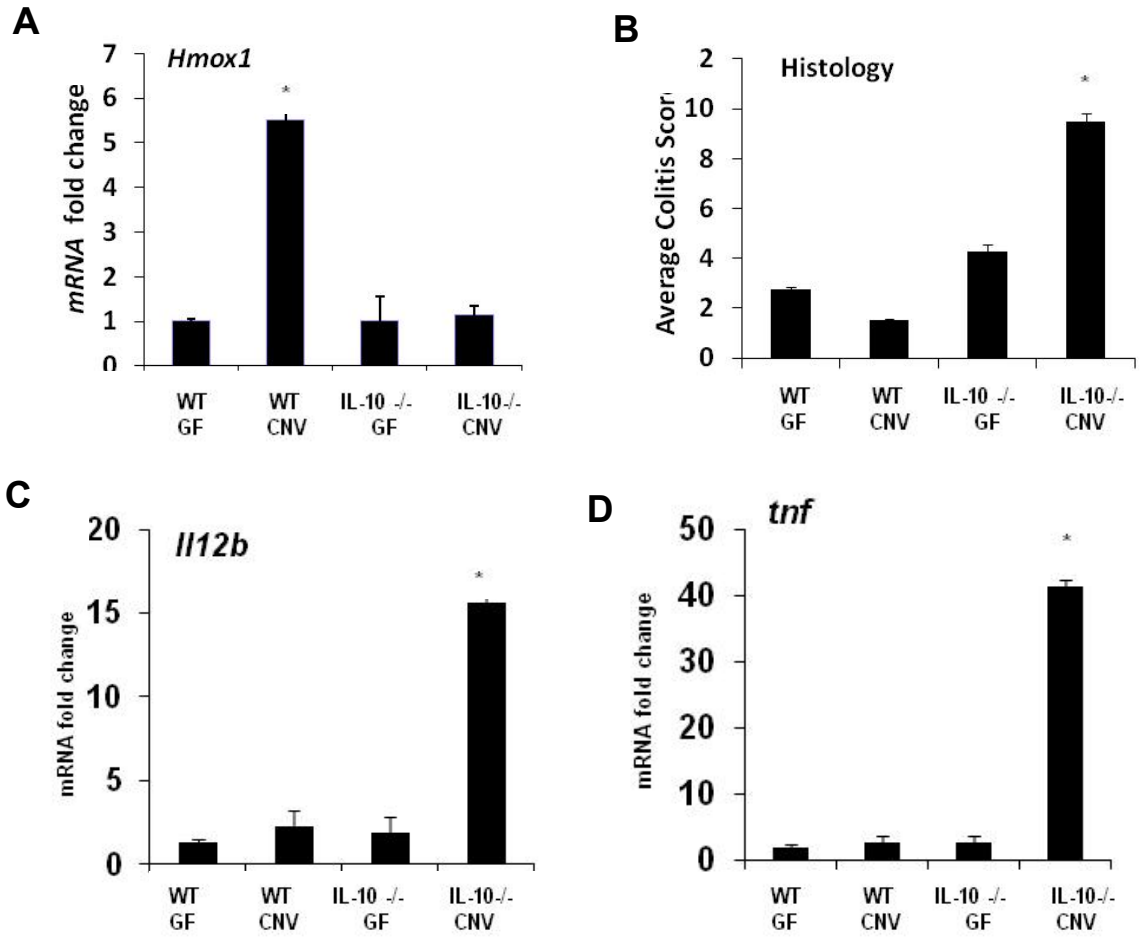


Figure 3.1. The enteric-microbiota regulate colonic *Hmox1* expression in WT but not IL-10$^{-/-}$ mice. WT and IL-10$^{-/-}$ mice raised in germ free (GF) conditions were colonized with enteric-microbiota from conventionalized (CNV) mice at 8-10 weeks of age (3 mice per group). Colons were harvested at 14 days post-CNV. **(A)** Total RNA was isolated (Qiagen RNeasy Mini Kit) and and β-actin mRNA expression was detected by real-time RT-PCR (Applied Biosystems). Results are expressed as mean ± SEM from 3 mice per group and are representative of 3 independent transitioned experimental cohorts, *p<0.05 vs. WT CNV. **(B)** Colitis scores of 8-wk-old GF WT and IL-10$^{-/-}$ mice. Results are presented as the sum total of four averaged scores from five regions of the large intestine graded by a pathologist (T.R.) blinded to the groups using a standard scoring system (14). Results are expressed as mean + SD from 3 mice per group and are representative of 3 independent transitioned experimental cohorts, *p<0.05 vs. WT CNV. **(C)** Total colonic RNA was isolated and *Il12b* and **(D)** *tnf* expression detected by real-time RT-PCR (Applied Biosystem). Results are expressed as mean ± SEM from 3 mice per group and are representative of 3 independent transitioned experimental cohorts, *p<0.05 vs. WT CNV.

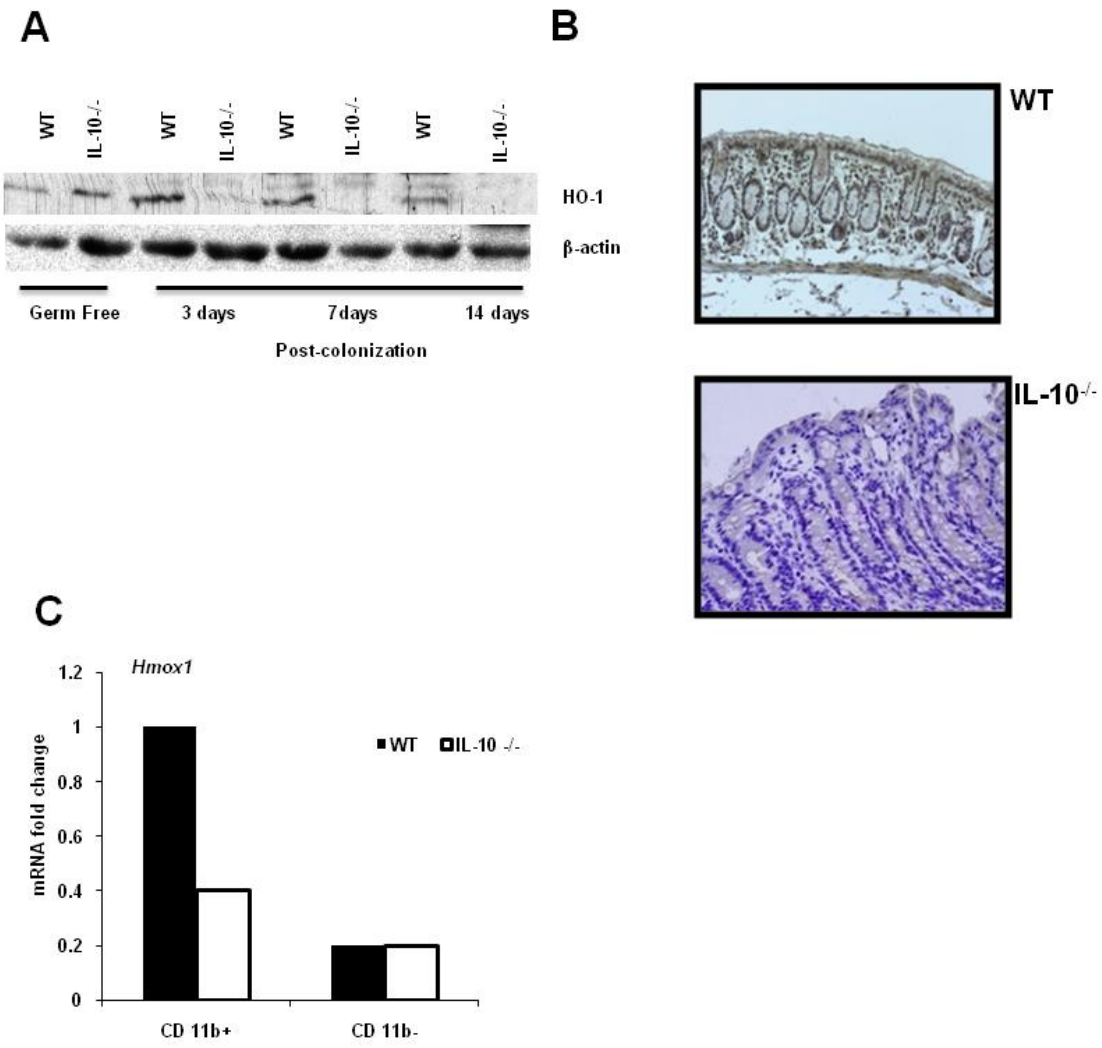


Figure 3.2. Kinetic analysis of HO-1 protein expression in WT but not IL-10$^{-/-}$ mice. WT and IL-10$^{-/-}$ mice raised in germ free (GF) conditions were colonized with enteric- microbiota from conventionalized (CNV) mice at 8-10 weeks of age (3 mice per group). Colons were harvested at day 3, 7, 14 and 21 days post-CNV. **(A)** Total colonic protein was isolated and analyzed for HO-1 expression using Western blot. Data is representative of 3 similar blots from 3 different mice per time point. **(B)** Immunohistochemical analysis of intestinal HO-1 protein expression from WT and IL-10$^{-/-}$ CNV mice. Depicted intestinal sections were matched for similar histologic colitis activity. **(C)** CD11b+ and CD11b- LPMCs isolated from WT and IL-10$^{-/-}$ mice were analyzed for baseline *Hmox1* expression. Total RNA was isolated (Qiagen RNeasy Mini Kit) and *Hmox1* and β-actin mRNA expression was detected by real-time RT-PCR (Applied Biosystems). Results are expressed as fold induction relative to WT represent LPMCs pooled from 3 individual mouse colons.

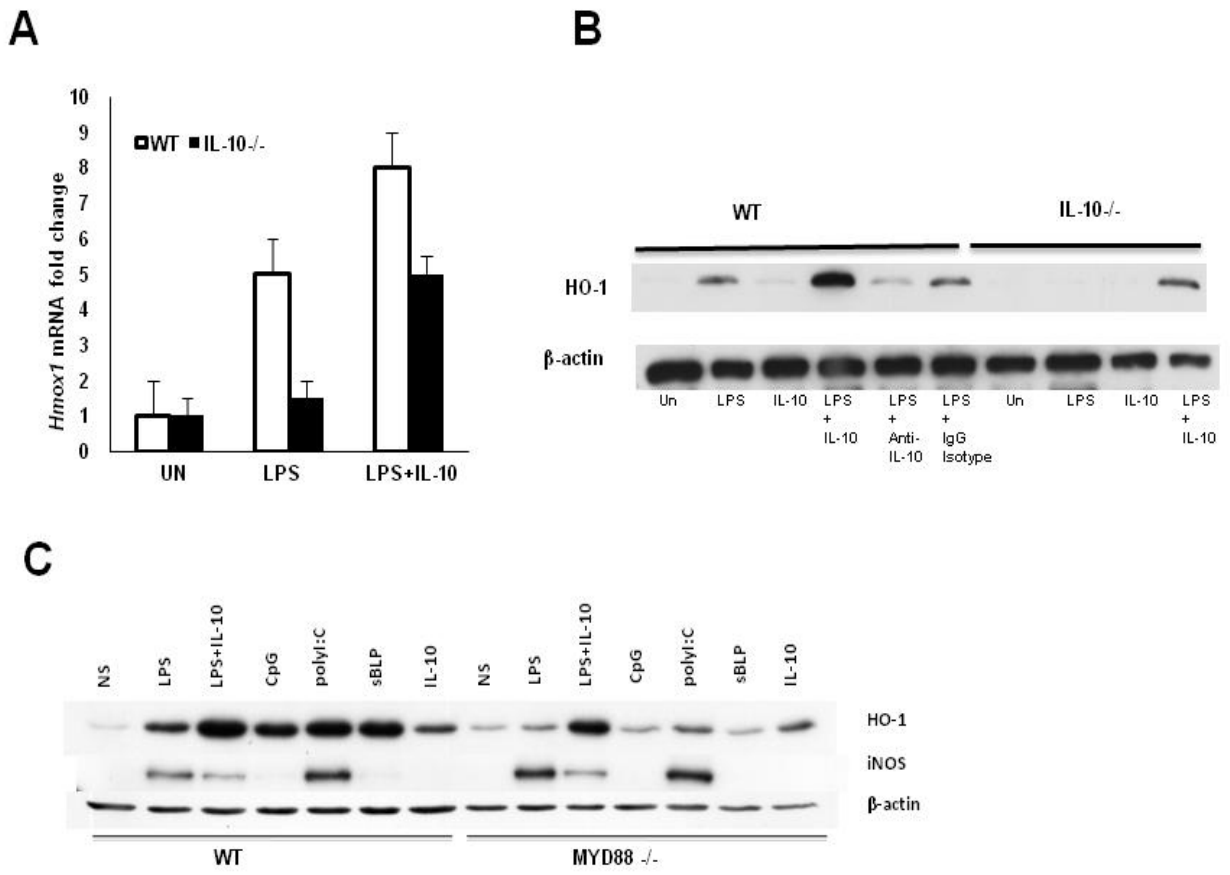


FIGURE 3.3. LPS and IL-10 induce HO-1 in murine macrophages. WT and IL-10^{-}bone marrow derived macrophages (BMMs) were stimulated with **(A)** LPS (100 ng/mL) ± IL-10 (10 ng/mL). Total RNA was isolated (Qiagen RNeasy Mini Kit) and analyzed for *Hmox1* and β-actin mRNA expression was detected by real-time RT-PCR (Applied Biosystems). Results are expressed as mean ± SEM from 3 independent experiments. **(B)** WT and IL-10$^{-/-}$ BMMs were stimulated with LPS (100 ng/mL) in the presence of IL-10 (10 ng/mL), and anti-1IL-10 antibody (10 ug/mL). HO-1 protein was analyzed by western blotting. Data is representative of 5 independent experiments with similar results. **(C)** WT and MyD88$^{-/-}$ BMMs were stimulated with LPS (100 ng/mL), CpG (1 μM), SbLP (100 ng/mL), polyI:C (50 ng/mL) and IL-10 (10 ng/mL), ug/mL). HO-1 protein was analyzed by western blotting. Data is representative of 3 independent experiments.

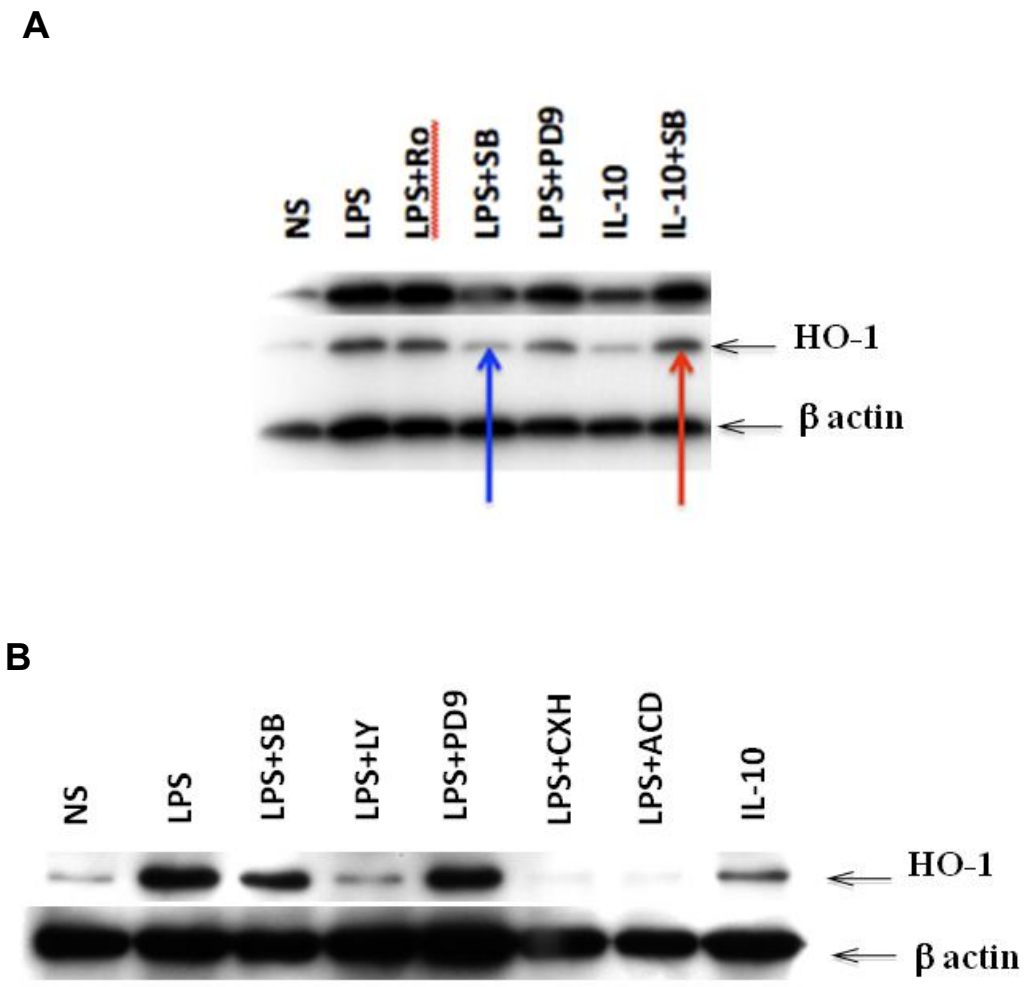


Figure 3.4. Other signal transduction pathways in HO-1 induction in macrophages. WT BMMs were stimulated with LPS in the presence of MAPK p38 inhibitor **(A)** SB 203580 (10μM), ERK1/2 inhibitor PD 98059 (50 μM), protein kinase C (PKC) inhibitor Ro31-8220 (5μM) and IL-10 (10 ng/mL), **(B)** PI3K inhibitor LY 294002 (10μM), cyclohexamide (10 μg/mL) and actinomycin D (1 μg/mL). HO-1 protein was analyzed by Western blotting. Results are representative of 3 independent experiments.

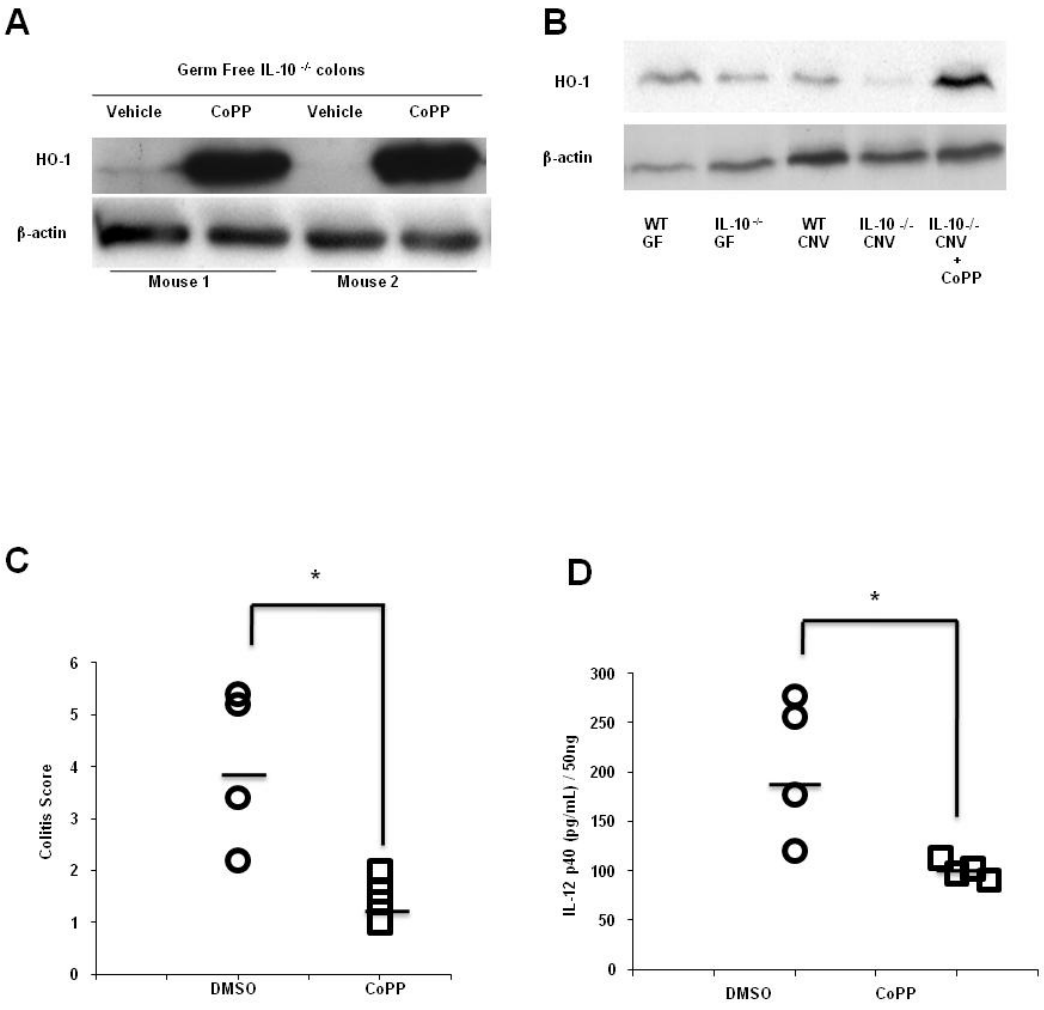


Figure 3.5. HO-1 induction prevents enteric-microbiota induced colitis in IL-10$^{-/-}$ mice. Germ free (GF) IL-10 $^{-/-}$ mice were injected intraperitoneally (IP) with 5ug/kg of cobalt protoporphyrin (CoPP). **(A)** Colons harvested 7 days after CoPP administration revealed increased colonic expression of HO-1 compared to vehicle treated mice. **(B)** WT CNV and CoPP treated IL-10$^{-/-}$ mice demonstrated colonic HO-1 protein expression compared with vehicle treated IL-10 CNV mice. **(C)** CoPP treated IL-10$^{-/-}$ mice revealed less intestinal inflammation compared to vehicle group. **(D)** Spontaneous IL-12 p40 secretion was determined in cell free supernatants from colonic explants by ELISA. ($p<0.05$ vs. vehicle treated IL-10$^{-/-}$ mice, n=4 mice per group)

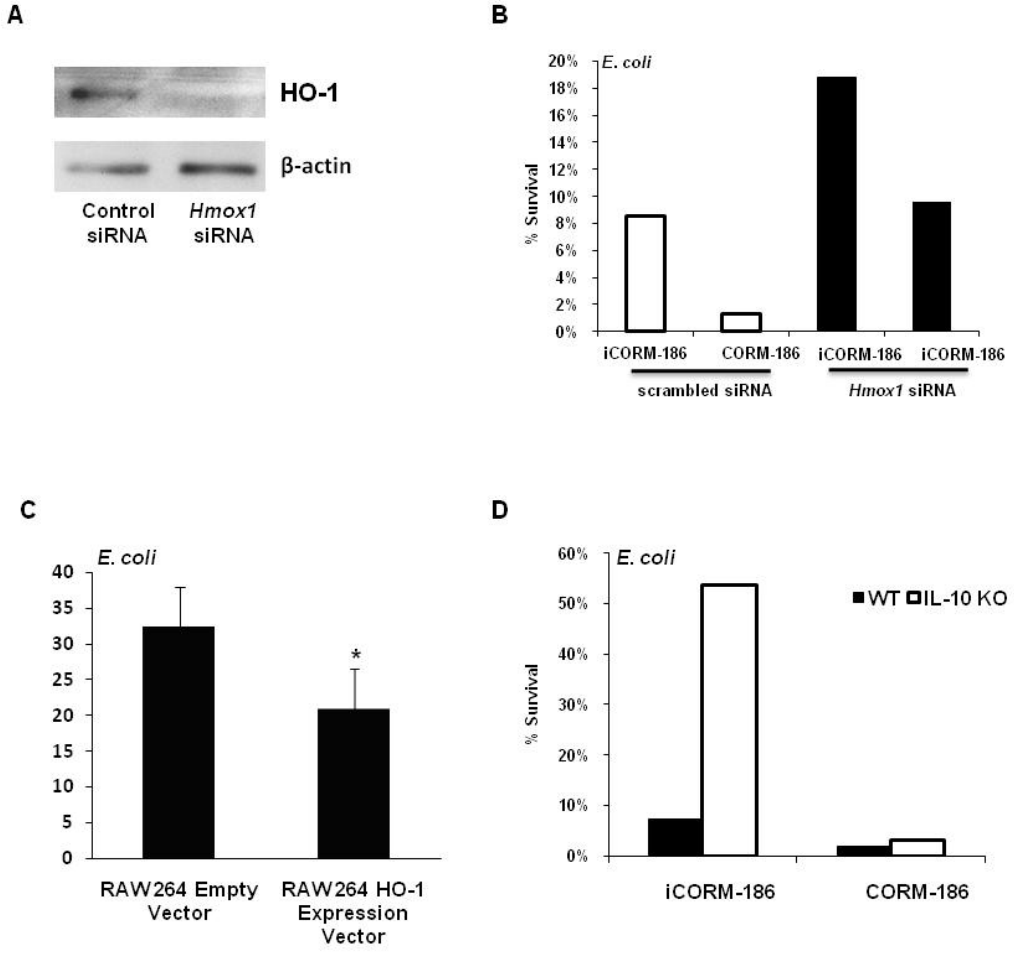


Figure 3.6. *Hmox1* derived carbon monoxide (CO) is important for macrophage bactericidal activity. (A) siRNA was used to knock down *Hmox1* in WT BMMs. Total protein was isolated and analyzed for HO-1 expression to assess effective gene silencing. **(B)** Cells were analyzed for macrophage bactericidal activity against *E.coli* K12 using gentamicin protection assay. siRNA transfected cells were cultured in the presence of CORM-186 to study the effect of CO on bactericidal activity. Results are representative of 3 independently performed experiments. **(C)** RAW 264 macrophages engineered to over-express *Hmox1* were used to perform a gentamicin protection assay against *E.coli* K12. Results are representative of 3 independently performed experiments. **(D)** WT and IL-10$^{-/-}$ BMMs were analyzed for macrophage bactericidal activity against *E.coli* K12 using gentamicin protection assay. BMMs were also cultured in the presence of CORM-186 or inactive CORM-186 (iCORM-186) to study the effects of CO on bactericidal activity. Results are representative of 3 independently performed experiments.

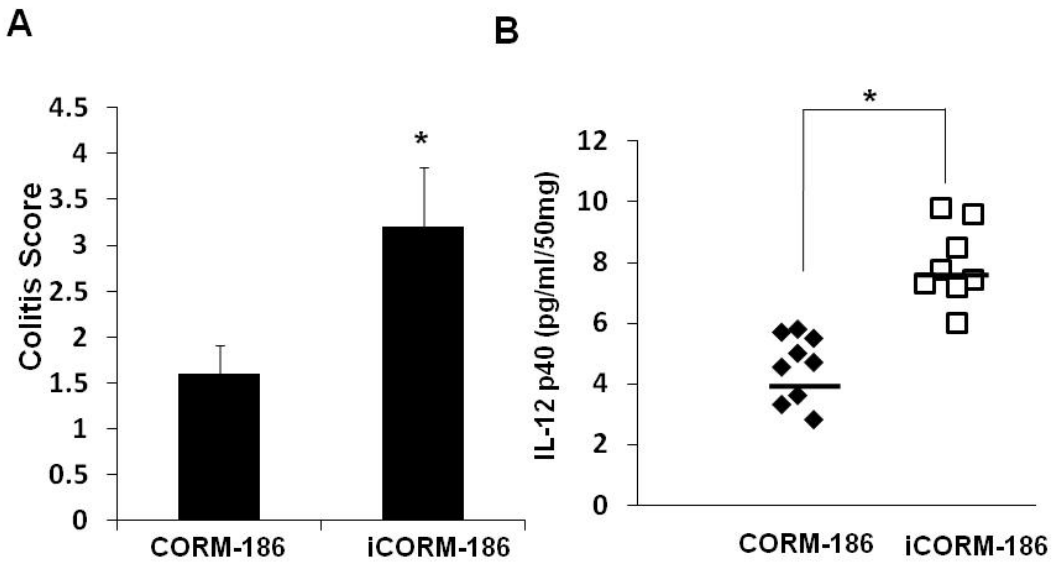
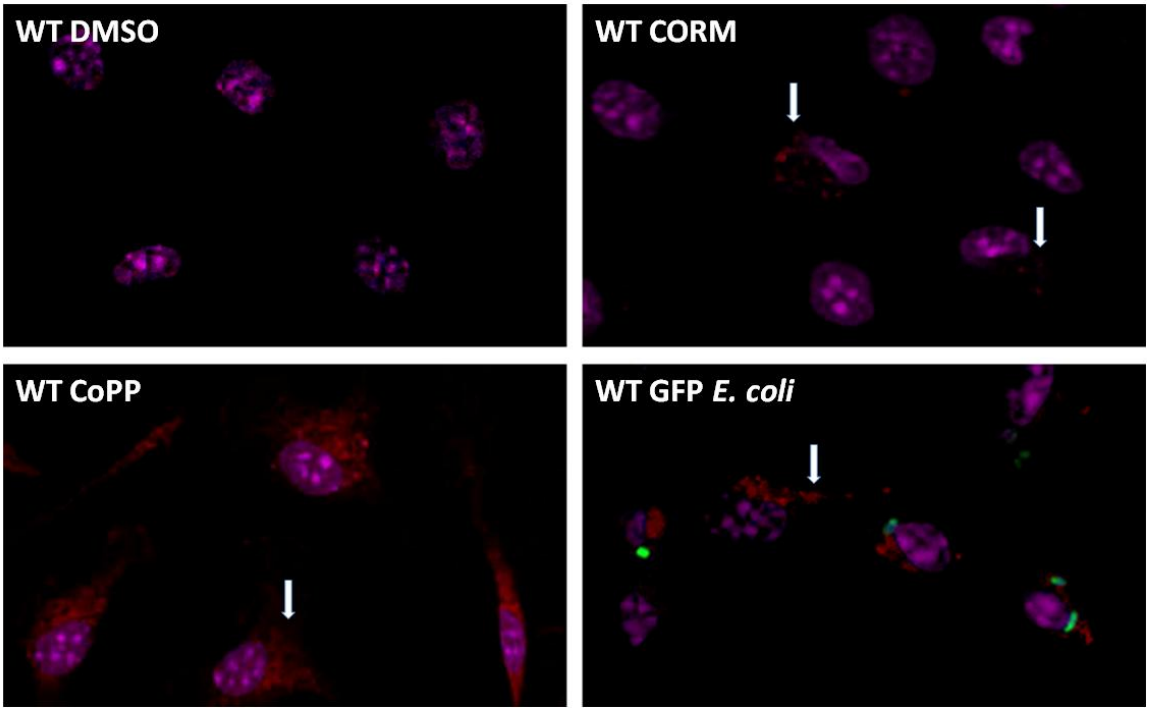


Figure 3.7. CO-Releasing Molecule (CORM-186) ameliorates piroxicam induced colitis in IL-10$^{-/-}$ mice. (A) Piroxicam treated IL-10$^{-/-}$ mice were administered , CO-Releasing Molecule, CORM-186 (30mg/kg) IP for 2 weeks. CORM-186 treated mice showed less inflammation as compared to inactive CORM-186 (iCORM186) treated controls. **(B)** Spontaneous IL-12 p40 secretion was determined in cell free supernatant from colonic explants by ELISA. (*$p<0.05$ vs. iCORM-186 treated IL-10 $^{-/-}$ mice, n=8-9 mice per group)

A



B

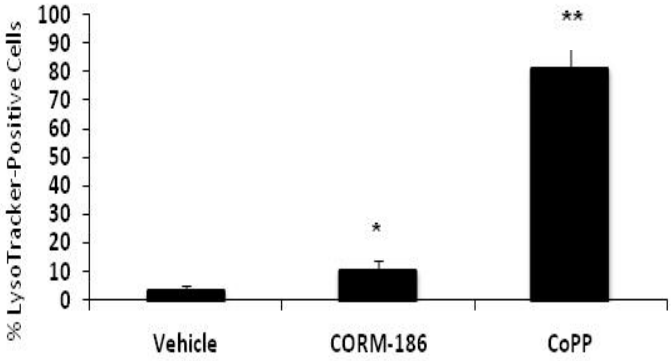
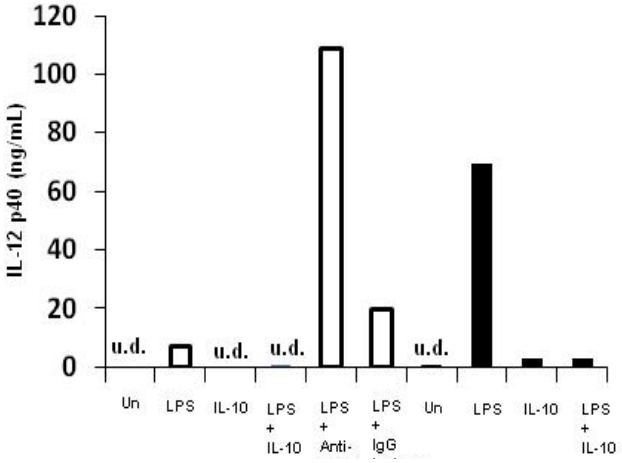
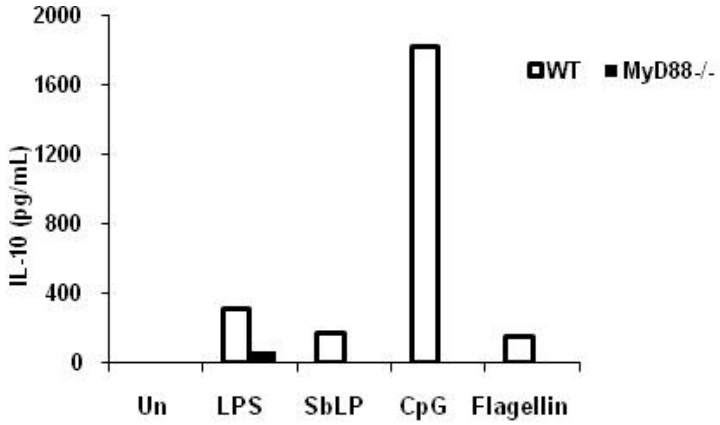


Figure 3.8. Cobalt protoporphyrin (CoPP) and CORM-186 enhance phagolysosomal activation. BMMs from WT were incubated with CoPP (10 μM) and CORM-186 (100 μM) following which cells were treated with LysoTracker® (100 nM), fixed in 4% paraformaldehyde and stained with nuclear stain Topro-3, 5nM (Invitrogen). **(A)** Representative LysoTracker® –positive cells (red) and nuclei (magenta) from vehicle treated (left upper panel), CORM-186 treated (upper right panel), CoPP treated (lower left panel) and eGFP- E.coli incubated, positive control (lower right panel) were visualized by confocal carl zeiss LSM 710 laser scanning microscopemicroscopy. **(B)** LysoTracker-positive cells were calculated as a percentage of total cells. At least 10 high powered fields and at least 100 cells were counted. Error bars represented mean ± SEM. * $p<0.05$ **$p<0.01$ vs. vehicle treated control BMMs.

A



B



Supplemental Figure 3.1. LPS and IL-10 regulate IL-12 and HO-1 in murine macrophages. WT and IL-10$^{-/-}$ bone marrow derived macrophages (BMMs) were stimulated with **(A)** LPS (100 ng/mL) ± IL-10 (10 ng/mL) and anti-IL-10 antibody (10 ug/mL). IL-12 p40 secretion was measured in cell free supernantants 24 hours after stimulation using cytokine specific ELISA. Results are a representative from 3 independent experiments with similar results. **(B)** WT and MYD88-/- BMMs were stimulated with LPS (100 ng/mL), CpG (5 ug/mL), SbLP (100 ng/mL), and IL-10 (10 ng/mL), ug/mL). IL-10 secretion in cell free supernatants was analyzed using cytokine specific ELISA. Data is representative of 3 independent experiments.

CHAPTER 4

AN ANTI-INFLAMMATORY ROLE FOR THE HEME OXYGENASE-1 (HO-1) PATHWAY IN CHRONIC T HELPER-2 MEDIATED MURINE COLITIS

SHEHZAD Z. SHEIKH, REFAAT HEGAZI, KATSUYOSHI MATSUOKA, TAKU KOBAYASHI, ANTONIA R. SEPULVEDA, FENGLING LI, LEO E. OTTERBEIN, SCOTT E. PLEVY

4.1. Abstract

Rationale: The metabolic product of the heme oxygenase-1 (HO-1) pathway, carbon monoxide (CO) exerts immunomodulatory effects in numerous disease models. We have previously demonstrated that CO suppresses chronic inflammation in Th1/17-mediated experimental colitis in IL-10$^{-/-}$ mice through an HO-1 dependent pathway. In this study, homeostatic effects of the HO-1 pathway were determined in Th2-mediated chronic colonic inflammation in T cell receptor- alpha (TCRα) deficient ($^{-/-}$) mice. Methods. TCRα $^{-/-}$ mice were exposed to CO or treated with the pharmacologic HO-1 inducer cobalt protoporphyrin (CoPP) to assess anti-inflammatory effects on colitis and cytokine expression. Results: TCRα $^{-/-}$ mice exposed to CO or treated with CoPP demonstrated significant amelioration of active colitis compared to the respective control groups. CO and CoPP suppressed colonic IL-1β, TNF and IL-4 production, while intestinal IL-10 protein secretion was induced. In macrophages, CO induced IL-10 expression through an HO-1 dependent pathway. Conclusions: CO and induction of HO-1 suppresses Th2 mediated colitis in TCRα$^{-/-}$ mice. The anti-inflammatory effect of CO and HO-1 is likely in part through induction of IL-10. This study provides further evidence that HO-1 is an important homeostatic pathway in mucosal inflammation with pleiotropic anti-inflammatory effects, and that targeting HO-1 is a promising therapeutic strategy in chronic inflammatory bowel diseases.

4.2. Introduction

Cigarette smoking is perhaps the most significant environmental risk factor identified in the human inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC). Meta-analyses have shown that the risk of developing UC in current smokers is approximately 40% that of nonsmokers (1). Furthermore, former smokers are at approximately a 1.7 times increased risk of developing UC (2). Some studies even suggest a dose-response, with heavier smokers having greater protection (1). Even passive smoking in childhood may confer protection against UC (3). Based on these compelling epidemiological observations, one of the important unanswered questions in IBD is how does cigarette smoking mediate this protective effect? Remarkably, the influence of cigarette smoking on CD is opposite: CD patients who smoke have an aggressive disease course compared to non-smokers, with more rapid progression to surgery and faster postoperative recurrences (4).

One possible mediator that may contribute to the beneficial association between smoking and ulcerative colitis is the gaseous molecule, carbon monoxide (CO). Carbon monoxide is a prominent component of cigarette smoke. Blood carboxyhemoglobin levels, a measure of systemic exposure to CO, have been reported to range from 1% to 18% in active smokers (5). The endogenous enzyme, heme oxygenase-1 (HO-1) mediates the degradation of heme into equimolar quantities of carbon monoxide (CO), iron, and biliverdin (BV). HO-1 and its metabolic products regulate immune responses, tissue injury and repair (6). We have previously shown that CO ameliorates active inflammation in an experimental model of chronic IBD, IL-10 deficient ($^{-/-}$) mice, through induction of HO-1(7).

Cytokines elaborated by CD4+ helper T cells play a key role in the regulation of immune responses in the intestine. CD4+ T cells have been divided into functionally

important subsets based on the cytokines they produce (8). Although these subdivisions represent a reduction of complex biology, most applicable to the mouse, they provide a framework to understand mucosal T cell responses in human IBD. T-helper-1 cells (Th1) produce the inflammatory cytokines IFN-γ and IL-2. These cells and cytokines are the hallmarks of cell-mediated immunity, necessary for the eradication of intracellular pathogens and the development of long-term immunity against infectious agents (8). Numerous mouse models of IBD are characterized by an overabundance of intestinal Th1 cytokines. CD was initially described as a prototype Th1-mediated chronic inflammatory disorder, characterized by mucosal granulomas (the histologic hallmark of a Th1 response), increased expression of IFN-γ, as well as increased IL-12 and IL-18, two cytokines necessary for the Th1 development (9, 10). However, the discovery of the IL-12 cytokine-family member IL-23 which shares a common p40 subunit with IL-12 has lead to a paradigm shift in our understanding of inflammatory responses in IBD (11) (12). IL-23, unlike IL-12, promotes a distinct CD4+ T cell activation state characterized by the production of the cytokine IL-17. These Th17 cells develop distinct from the Th1 lineage. IL-23 enhances Th17 function and survival by acting on differentiated Th17 cells which express the IL-23 receptor (13). A pivotal role for IL-23 and Th17 cells has been demonstrated in experimental IBD models such as the IL-$10^{-/-}$ mouse, and recent genetic and immunologic findings highlight the importance of this pathway in human IBD (14) (15)

T-helper-2 cells produce the cytokines IL-4, IL-5, and IL-13 (16). These cytokines provide help for B cell antibody production, and in the mucosal immune system are involved in host defense against extracellular helminthic parasites (17). Inflammation in UC has been characterized as mediated by Th2 cytokines (18). Lamina propria T cells from UC patients produce IL-13 and IL-5, and little IFN-γ (19). Although multiple experimental models of chronic Th1/17-driven intestinal inflammation have been elucidated, few have been

described where disease occurs in a Th2 cytokine milieu. Mice with targeted disruption of the T cell receptor alpha gene (TCRα$^{-/-}$) perhaps most closely resemble the colonic Th2 signature that characterizes human UC. IL-4 and IL-1β play an important role in the development of colitis in TCR alpha$^{-/-}$ mice (20). T cells, B cells and autoantibodies are essential for the development of colitis in TCRα$^{-/-}$ mice. However, the role of macrophages in the pathogenesis of chronic intestinal inflammation in TCRα$^{-/-}$ mice is unknown.

Our previous work has shown that CO and HO-1 induction ameliorates colonic inflammation in a Th1/Th17 mediated model of intestinal inflammation, IL-10$^{-/-}$ mice (7). To model the protective effects of cigarette smoking in human UC, we now study CO and the HO-1 pathway in a murine model with immunologic similarities to ulcerative colitis. We demonstrate anti-inflammatory effects of CO in spontaneous Th2-mediated colitis in TCRα$^{-/-}$ mice. Exposure of TCRα$^{-/-}$ mice to CO is associated with histologic improvement, and suppression of the inflammatory cytokines IL-1β, IL-4 and TNF in the colon. Pharmacological induction of HO-1 recapitulates the immunomodulatory effects of CO, attenuates colonic inflammation and inhibits colonic inflammatory cytokine secretion. Histological improvement with CO exposure and HO-1 induction in TCRα-/- mice also correlates with induction of the anti-inflammatory cytokine IL-10 in colonic macrophages.

4.3. Results

4.3.1. CO exposure ameliorates Th2 mediated colitis in TCRα $^{-/-}$ mice. TCRα $^{-/-}$mice were exposed to 250 ppm of CO from 10 to 15 weeks of age (n=10) and compared to a control group (n=10) exposed to ambient air. Mice in both treatment groups were matched for age, sex and initial body weight. CO-exposed mice showed an increase in body weight compared to mice housed in ambient air (Figure 4.1A). Assessment of histological improvement was performed by a pathologist blinded to treatment groups. Mice exposed to CO demonstrated significantly reduced histologic inflammation (Figure 4.1B).

Whether CO exposure affects colonic cytokine expression in TCRα $^{-/-}$ mice was next determined. Colonic explant cultures from TCRα $^{-/-}$ mice exposed to CO in vivo for 4 weeks produced less IL-1β, IL-4, TNF and IL-17 (Figure 4.2A and B), correlating with histological improvement. CO also induced colonic IL-10 secretion compared to explant cultures from air exposed TCRα $^{-/-}$ mice (Figure 4.2A).

As IL-10 is an important regulatory cytokine, correlations between CO exposure and colonic IL-10 induction were further explored. Ten week old TCRα$^{-/-}$ mice were divided into three groups: Group 1 was exposed to CO (250 ppm) for four weeks, group 2 was exposed to air for four weeks, and group 3 was exposed to CO for two weeks and then transferred to ambient air for two weeks. Mice exposed to CO demonstrated increased secretion of IL-10 in colonic explants. Mice transferred from CO exposure to air after 2 weeks showed intermediate IL-10 secretion, with more colonic IL-10 compared to air exposed mice, but less than mice continually exposed to CO (Figure 4.3). These findings suggest that CO may ameliorate inflammation through induction of IL-10. Furthermore, as IL-10 secretion is increased 2 weeks after removing mice from CO, CO may induce a durable change in a cell population that secretes IL-10 in the colon.

4.3.2. CO induces IL-10 in macrophages through induction of HO-1. To study regulation of IL-10 by CO in macrophages, bone marrow-derived macrophages (BMMs) from wild type and TCRα $^{-/-}$ mice were stimulated with LPS in CO (250 ppm) or ambient air. As previously described (21), CO augmented LPS stimulated IL-10 secretion in BMMs (Figure 4.4A). We have previously shown that CO inhibits LPS/IFN-γ mediated IL-12 p40 expression through a HO-1 dependent mechanism. BMMs from HO-1 deficient (*Hmox1*$^{-/-}$) mice were stimulated with LPS in presence or absence of CO and then cultured for 24 hours. Compared to wild type macrophages, in *hmox*$^{-/-}$ BMMs, CO failed to induce IL-10 secretion (Figure 4.4B). These results suggest that IL-10 secretion in murine macrophages in response to CO is HO-1 dependent.

4.3.3. HO-1 induction recapitulates immunomodulatory effects of CO in vivo. To understand the role of HO-1 in the anti-inflammatory effects of CO in vivo, TCRα $^{-/-}$ mice were treated with a pharmacological inducer of HO-1, cobalt protoporphyrin (CoPP, 5 mg/kg) intraperitoneally twice weekly for two weeks and compared to PBS treated controls. CoPP treatment resulted in robust induction of HO-1 mRNA (*Hmox1*) expression in colonic CD11b- and CD11b+ lamina propria mononuclear cell (LPMC) populations (Figure 4.5A). CoPP treatment resulted in decreased colonic inflammation compared to PBS treated mice as indicated by improved histological scores (Figure 4.5B). Colonic explant cultures revealed decreased IL-4, IL-1β, and TNF secretion in CoPP-treated TCRα$^{-/-}$ mice compared to PBS treated control mice (Figure 4.6A and 4.6B).

Next, we sought to determine the effects of HO-1 induction on IL-10 secretion in the colon. Intestinal CD11b+ colonic LPMCs were the primary source of IL-10 from CoPP treated TCRα$^{-/-}$ mice compared with PBS treated controls (Figure 4.7A). As a control, no differences in IL-12 p40 were detected from CD11b+ LPMCs between the two groups (Figure 4.7B).

Regulatory FoxP3+ T and CD11d+ B cells have been previously demonstrated to be a source of IL-10 production in the intestine and have important anti-inflammatory roles in murine IBD (22). However, no differences in numbers of IL-10 expressing CD1d+ B cells from mesenteric lymph nodes (MLN) lymphocytes were detected between air-exposed and CO-exposed TCRα$^{-/-}$ mice (Supplemental Figure 4.1). Interestingly, a marked decrease in numbers of Cd4+ FoxP3+ T cells are found in TCRα $^{-/-}$ mice compared to wild type mice (Supplemental Figure 4.2). These results strongly implicate CD11b+ LPMCs as the primary source of IL-10 in the TCRα$^{-/-}$ mice.

4.4. Discussion

In summary, CO exposure ameliorates Th2-mediated chronic colitis in TCRα $^{-/-}$ mice. The effects of CO were recapitulated by pharmacologic HO-1 induction. CO and heme oxygenase-1 induction resulted in increased intestinal IL-10 expression in the CD11b+ population of cells, and inhibition of inflammatory cytokines.

We have previously demonstrated that CO ameliorates colitis in IL-10$^{-/-}$ mice. IL-10$^{-/-}$ mice exhibit a predominant Th1/17-mediated immune pathology. The immune protective effects of CO in IL-10$^{-/-}$ mice was attributed in part to inhibition of the synergistic activation of IL-12 p40 by LPS/IFN-γ (7). Our current study elucidates the anti-inflammatory effects of CO in TCRα $^{-/-}$ mice characterized by increased colonic Th2 cytokine expression.

Several regulatory B cell populations have been characterized in TCRα $^{-/-}$ mice. A subset of regulatory B cells has been identified as an important source of IL-10 and was responsible for inhibiting IL-1β and amelioration of colitis in TCRα $^{-/-}$ mice (23, 24). However, we could not discern any difference in numbers of CD1d+ MLNs from CO-exposed TCRα $^{-/-}$ mice compared to air exposed mice. An IL-12-producing regulatory B-cell subset that develops in the presence of IL-10 has also been shown to be involved in the regulation of colonic inflammation in this model (22). During experimental protocols of CO exposure and pharmacologic HO-1 induction, we demonstrate induction of colonic IL-10 in the CD11b+ LPMC fraction, containing predominantly macrophages but dendritic cells as well. We also detected expression of the IL-12 p40 subunit exclusively in the lamina propria CD11b+ cell population, and were unable to detect IL-12 p70 in colonic explants (data not shown). These findings may suggest that CO and HO-1 induction, possibly through IL-10, has anti-inflammatory effects that extend beyond induction of previously described regulatory B cell populations in this model.

IL-10 is expressed in may cell types, including macrophages, dendritic cells, B cells and regulatory T cells (Treg) (25). Our analysis revealed a significant numerical deficiency of FoxP3+ T cells in the spleen and MLNs of TCRα $^{-/-}$ mice compared to wild type mice. This finding allows for speculation that Treg deficiency in TCRα $^{-/-}$ mice may contribute to chronic intestinal inflammation. CO has been shown to induce Tregs in other model systems (26). Hence, we conclude that Tregs are not an important source of IL-10 in TCRα $^{-/-}$ mice (26).

Recently, the IL-10 family member IL-22 expressed by Th17 cells was demonstrated to ameliorate colitis in TCRα $^{-/-}$ mice (27). We unexpectedly detected a Th17 cytokine signature in colonic explants from TCRα $^{-/-}$ mice with abundant levels of IL-17. The IL-17 producing cell population(s) remains to be determined. Given recent reports, it is interesting to speculate that γδ T cells may be a source of IL-17 (28). Interestingly, IL-17 levels decreasing following CO exposure or HO-1 induction, correlating with histologic improvement. It will be of interest in future studies to determine whether IL-22 is involved in the protective effects of CO/HO-1 in this model, as IL-22 is a potent inducer of IL-10. The description of a Th17 signature in TCRα $^{-/-}$ mice is also likely validates this as a model for human UC, where the same susceptibility genes that lie within the IL-23/Th17 pathway confer susceptibility to CD and UC (15). Likewise, current biological interventions that inhibit TNF and are widely employed for the treatment of moderate to severe UC and CD (29, 30). CO and pharmacological induction of HO-1 with CoPP resulted in a significant decrease in TNF secretion from TCRα $^{-/-}$ colons which may underlie the therapeutic effect.

Activated macrophages and DCs are an abundant source of IL-10 (25). CO augments LPS induced IL-10 secretion from TCRα $^{-/-}$ BMMs. We have previously demonstrated that CO ameliorates colitis in the IL-10$^{-/-}$ mouse, a Th1/Th17-mediated model of chronic intestinal inflammation through induction of HO-1 (7). Administration of CoPP also

ameliorates colitis in TCRα $^{-/-}$ mice. Specifically, TCRα$^{-/-}$ mice treated with CoPP resulted in a specific increase in IL-10 secretion from only CD11b+ LPMCs. These findings further support the recent identification of IL-10 producing CD11b+ LPMCs that act in part on Treg cells to maintain their expression of Foxp3 that is lost in inflammatory conditions in T cell-transfer murine colitis (31).

These results are unique since they are the first to characterize the anti-inflammatory properties of CO in a Th2-mediated model of inflammation. The anti-inflammatory effects of CO are attributed to the induction of HO-1 and IL-10, and highlight the importance of CO and HO-1 now in several experimental models of intestinal inflammation. These studies suggest that HO-1 is a central regulator of intestinal homeostasis through pleiotropic mechanisms and that understanding its role is of mechanistic and therapeutic relevance in human IBD.

4.5. Materials and Methods

Mice. Wild type and TCR$\alpha^{-/-}$ mice were obtained from The Jackson Laboratory. All wild-type and genetically deficient mice used in this study were on the C57BL/6 background and matched for age and sex in all experiments. All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and the University of North Carolina Schools of Medicine. At the end of the study period, animals were euthanized using excess CO_2 inhalation. Immediately afterward, blood by cardiac puncture (carboxyhemoglobin determination), spleens, intestinal tissue samples, and femurs were collected. BM-derived macrophages and splenocytes were cultured as described previously (7).

CO exposure. Mice or macrophages were exposed to compressed air or CO at a concentration of 250 ppm as previously described (7). Briefly, CO at a concentration of 1% (10,000 ppm) was mixed with compressed air before delivery into the exposure chamber. Flow into animal chamber was maintained at rate of 12 liters/min and into the cell culture chamber at a rate of 2 liters/min. The cell culture chamber was humidified and maintained at 37^{o}C. A CO analyzer (Interscan) was used to measure CO levels continuously in the chambers. Cardiac blood samples (0.2 ml) were taken immediately after the mice were sacrificed to measure carboxyhemoglobin using a hemoximeter (OSM3; Radiometer Copenhagen).

Cytokine ELISAs. Linco Cytokine-16 plex Mouse ELISA was performed for IL-4, IL-1β, IL-10, TNF and IL-17 ((Millipore, Billerica, MA) as per manufacturer's instructions. Murine IL-12 p40 and IL-10 were measure with cytokine specific immunoassay kits (R&D Systems, Minneapolis, MN).

Intestinal tissue explant cultures. Colonic tissue fragments (0.5 g dry weight) were isolated and incubated in 1.0 ml RPMI 1640 supplemented with 50 µg/ ml gentamicin, 100 U/ml penicillin, 100 _g/ml streptomycin, 0.25 µg/ml fungizone (GIBCO BRL), and 5% heat-inactivated fetal calf serum as previously described (7). Tissue fragment supernatants were collected after 24 h for cytokine ELISAs.

Isolation of colonic macrophages. Lamina propria cells were isolated from the mouse colon by an enzymatic method as previously described (32). Briefly, colons were dissected into small pieces, and washed three times in Hank's buffered saline solution (HBSS) containing 2.5% FBS. The last wash was done with 1mM DTT to remove mucus. The pieces were, then, incubated in HBSS containing 1mM EDTA three times for 20 min each at 37°C. The remaining tissue was digested in HBSS containing 1 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO) for 1.5 hours at 37°C. The supernatant was collected, filtered and centrifuged to obtain a cell pellet. LPCs were isolated by density gradient centrifugation using 40 % and 75 % Percoll solution (GE Healthcare, Piscataway, NJ). The intermediate layer containing LPCs was collected. LPCs were further separated into CD11b positive cells using anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA). Purity was more than 90% by flow cytometric analysis.

Flow cytometry. Splenocytes from CO and air exposed mice were collected and were stained for cell surface markers as previously described (33). Cells were first stained extracellularly with fluorescein isothiocyanate–conjugated anti-$CD4^+$ (RM4-5) were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen) and then were stained intracellularly with allophycocyanin-conjugated anti-FoxP3 (FJK-16s). Mesenteric lymph node cells were isolated as previously described(33) and stained with the B cell marker fluorescein isothiocyanate (FITC) anti-mouse B220 and cell surface marker phycoerythrin

(PE) anti-mouse CD1d (1B1) (eBioscience). Samples were acquired on a FACSCalibur (Becton Dickinson and Company, NJ, USA) and data were analyzed with CellQuest Pro software (BD Biosciences, San Jose, CA).

Histology. Colons were removed after mice were euthanized and rinsed with cold PBS to remove fecal material. Tissue sections were fixed in 10% buffered formalin and embedded in paraffin. 5-μm-thick sections were stained with hematoxylin and eosin. Colitis scores (0–4) were determined by a staff pathologist who was blinded to the experimental protocol using the criteria reported by Berg et al (34). 20 separate microscopic fields (magnification of 100X) were evaluated for each mouse by a pathologist (A.R. Sepulveda) blinded to the treatment groups.

Data analysis. Statistical significance for data subsets from experiments performed in cells was assessed by the two-tailed Student's *t*-test. Statistical significance for *in vivo* data subsets was assessed by the Mann-Whitney U test (SPSS, Chicago, IL, USA)) with Bonferroni correction.

4.6. References

1. Jick, H., and A.M. Walker. 1983. Cigarette smoking and ulcerative colitis. *N Engl J Med* 308:261-263.

2. Boyko, E.J., T.D. Koepsell, D.R. Perera, and T.S. Inui. 1987. Risk of ulcerative colitis among former and current cigarette smokers. *N Engl J Med* 316:707-710.

3. Sandler, R.S., D.P. Sandler, C.W. McDonnell, and J.I. Wurzelmann. 1992. Childhood exposure to environmental tobacco smoke and the risk of ulcerative colitis. *Am J Epidemiol* 135:603-608.

4. Rutgeerts, P. 2003. Strategies in the prevention of post-operative recurrence in Crohn's disease. *Best practice & research* 17:63-73.

5. Smith, C.J., T.D. Guy, M.F. Stiles, M.J. Morton, B.B. Collie, B.J. Ingebrethsen, and J.H. Robinson. 1998. A repeatable method for determination of carboxyhemoglobin levels in smokers. *Hum Exp Toxicol* 17:29-34.

6. Ryter, S.W., J. Alam, and A.M. Choi. 2006. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 86:583-650.

7. Hegazi, R.A., K.N. Rao, A. Mayle, A.R. Sepulveda, L.E. Otterbein, and S.E. Plevy. 2005. Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. *J Exp Med* 202:1703-1713.

8. Maynard, C.L., and C.T. Weaver. 2009. Intestinal effector T cells in health and disease. *Immunity* 31:389-400.

9. Kanai, T., M. Watanabe, A. Okazawa, T. Sato, M. Yamazaki, S. Okamoto, H. Ishii, T. Totsuka, R. Iiyama, R. Okamoto, M. Ikeda, M. Kurimoto, K. Takeda, S. Akira, and T. Hibi. 2001. Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterology* 121:875-888.

10. Okazawa, A., T. Kanai, M. Watanabe, M. Yamazaki, N. Inoue, M. Ikeda, M. Kurimoto, H. Ishii, and T. Hibi. 2002. Th1-mediated intestinal inflammation in Crohn's disease may be induced by activation of lamina propria lymphocytes through synergistic stimulation of interleukin-12 and interleukin-18 without T cell receptor engagement. *The American journal of gastroenterology* 97:3108-3117.

11. Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S.A. Lira, D. Gorman, R.A. Kastelein, and J.D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.

12. Uhlig, H.H., B.S. McKenzie, S. Hue, C. Thompson, B. Joyce-Shaikh, R. Stepankova, N. Robinson, S. Buonocore, H. Tlaskalova-Hogenova, D.J. Cua, and F. Powrie. 2006. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* 25:309-318.

13. Korn, T., E. Bettelli, M. Oukka, and V.K. Kuchroo. 2009. IL-17 and Th17 Cells. *Annual review of immunology* 27:485-517.

14. Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M.A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D.J. Cua, R.A. Kastelein, and D. Rennick. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *The Journal of clinical investigation* 116:1310-1316.

15. Duerr, R.H., K.D. Taylor, S.R. Brant, J.D. Rioux, M.S. Silverberg, M.J. Daly, A.H. Steinhart, C. Abraham, M. Regueiro, A. Griffiths, T. Dassopoulos, A. Bitton, H. Yang, S. Targan, L.W. Datta, E.O. Kistner, L.P. Schumm, A.T. Lee, P.K. Gregersen, M.M. Barmada, J.I. Rotter, D.L. Nicolae, and J.H. Cho. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314:1461-1463.

16. Lucey, D.R., M. Clerici, and G.M. Shearer. 1996. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clinical microbiology reviews* 9:532-562.

17. Finkelman, F.D., T. Shea-Donohue, S.C. Morris, L. Gildea, R. Strait, K.B. Madden, L. Schopf, and J.F. Urban, Jr. 2004. Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunological reviews* 201:139-155.

18. Targan, S.R., and L.C. Karp. 2005. Defects in mucosal immunity leading to ulcerative colitis. *Immunol Rev* 206:296-305.

19. Fuss, I.J., F. Heller, M. Boirivant, F. Leon, M. Yoshida, S. Fichtner-Feigl, Z. Yang, M. Exley, A. Kitani, R.S. Blumberg, P. Mannon, and W. Strober. 2004. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest* 113:1490-1497.

20. Mizoguchi, A., E. Mizoguchi, and A.K. Bhan. 1999. The critical role of interleukin 4 but not interferon gamma in the pathogenesis of colitis in T-cell receptor alpha mutant mice. *Gastroenterology* 116:320-326.

21. Otterbein, L.E., F.H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R.J. Davis, R.A. Flavell, and A.M. Choi. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 6:422-428.

22. Sugimoto, K., A. Ogawa, Y. Shimomura, K. Nagahama, A. Mizoguchi, and A.K. Bhan. 2007. Inducible IL-12-producing B cells regulate Th2-mediated intestinal inflammation. *Gastroenterology* 133:124-136.

23. Mizoguchi, E., A. Mizoguchi, F.I. Preffer, and A.K. Bhan. 2000. Regulatory role of mature B cells in a murine model of inflammatory bowel disease. *Int Immunol* 12:597-605.

24. Mizoguchi, A., E. Mizoguchi, H. Takedatsu, R.S. Blumberg, and A.K. Bhan. 2002. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* 16:219-230.

25. O'Garra, A., and K.M. Murphy. 2009. From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce T(H)1 development. *Nat Immunol* 10:929-932.

26. Lee, S.S., W. Gao, S. Mazzola, M.N. Thomas, E. Csizmadia, L.E. Otterbein, F.H. Bach, and H. Wang. 2007. Heme oxygenase-1, carbon monoxide, and bilirubin induce tolerance in recipients toward islet allografts by modulating T regulatory cells. *FASEB J* 21:3450-3457.

27. Sugimoto, K., A. Ogawa, E. Mizoguchi, Y. Shimomura, A. Andoh, A.K. Bhan, R.S. Blumberg, R.J. Xavier, and A. Mizoguchi. 2008. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *The Journal of clinical investigation* 118:534-544.

28. Lockhart, E., A.M. Green, and J.L. Flynn. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J Immunol* 177:4662-4669.

29. Sandborn, W.J., and S.R. Targan. 2002. Biologic therapy of inflammatory bowel disease. *Gastroenterology* 122:1592-1608.

30. Targan, S.R., S.B. Hanauer, S.J. van Deventer, L. Mayer, D.H. Present, T. Braakman, K.L. DeWoody, T.F. Schaible, and P.J. Rutgeerts. 1997. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 337:1029-1035.

31. Murai, M., O. Turovskaya, G. Kim, R. Madan, C.L. Karp, H. Cheroutre, and M. Kronenberg. 2009. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol*

32. Kamada, N., T. Hisamatsu, S. Okamoto, H. Chinen, T. Kobayashi, T. Sato, A. Sakuraba, M.T. Kitazume, A. Sugita, K. Koganei, K.S. Akagawa, and T. Hibi. 2008. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest* 118:2269-2280.

33. Murali-Krishna, K., J.D. Altman, M. Suresh, D.J. Sourdive, A.J. Zajac, J.D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8:177-187.

34. Berg, D.J., N. Davidson, R. Kuhn, W. Muller, S. Menon, G. Holland, L. Thompson-Snipes, M.W. Leach, and D. Rennick. 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* 98:1010-1020.

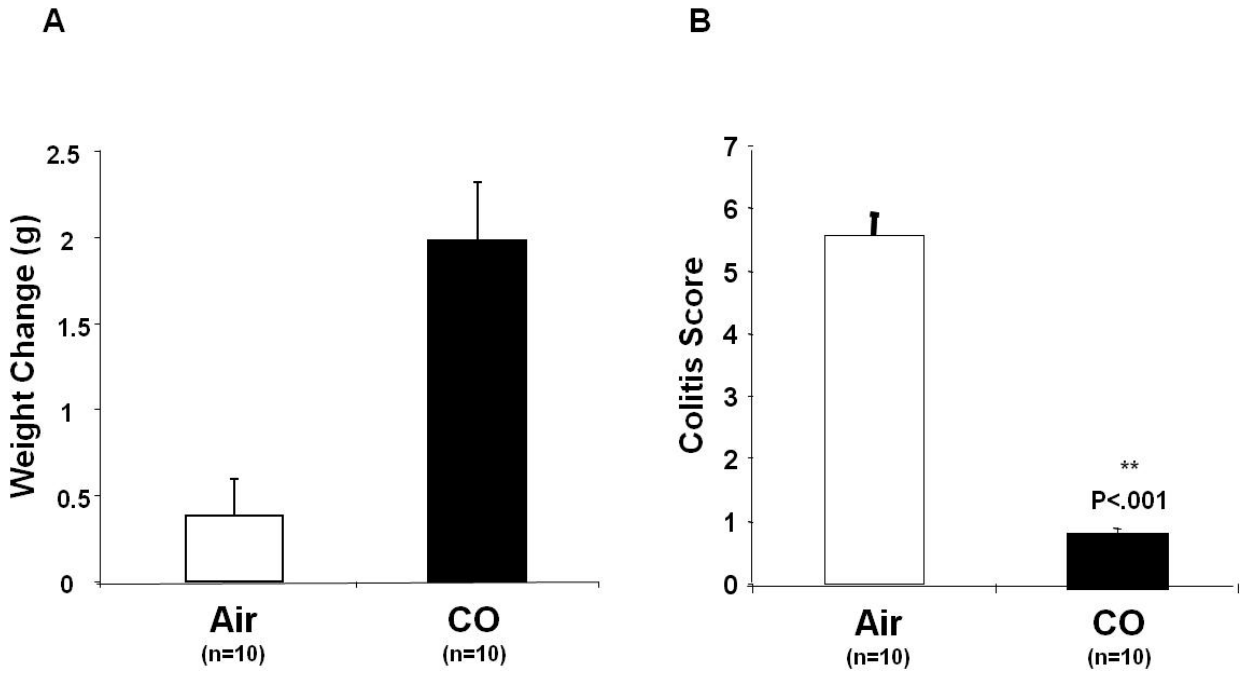


Figure 4.1. CO ameliorates Th2-mediated colitis in TCRα$^{-/-}$ mice. TCRα$^{-/-}$ mice were housed in ambient air or a chamber maintaining a constant concentration of CO at 250 ppm (*n*=10 each) from 16 through 20 weeks of life. **(A)** CO exposed mice gained more weight than air exposed mice. **(B)** Colitis scores were significantly decreased in CO exposed compared to control mice (**p<0.001).

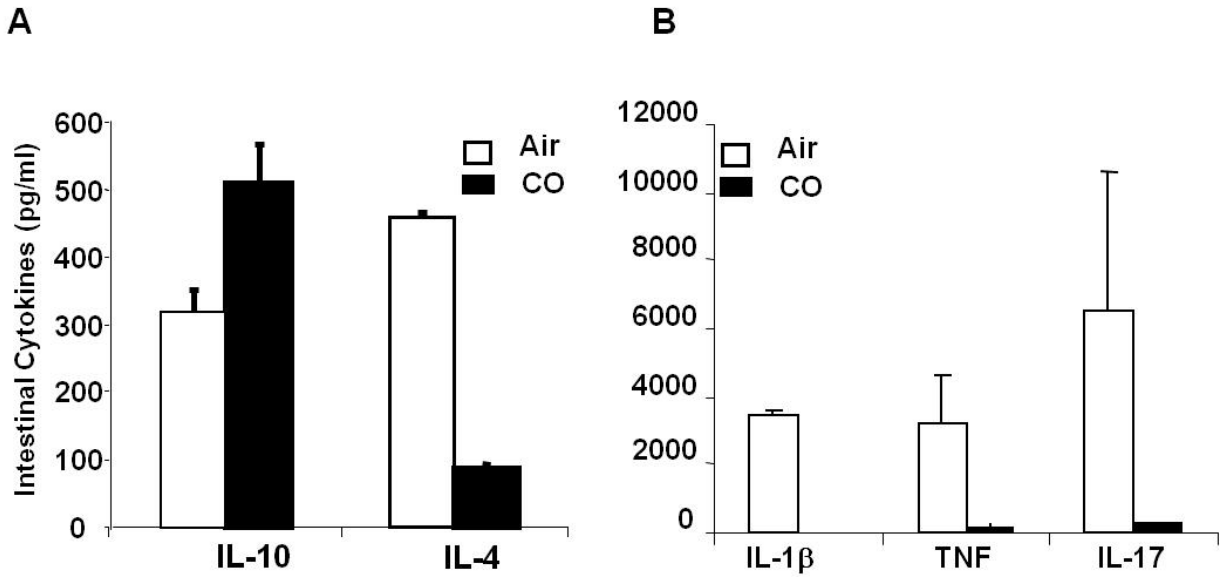


Figure 4. 2. CO exposure affects colonic cytokine secretion in *TCRα*$^{-/-}$ mice. Spontaneous protein secretion determined in 24 hour supernatants from intestinal explants from CO- and air-exposed *TCRα* $^{-/-}$ mice. **(A)** Spontaneous IL-10 and IL-4 using cytokine specific ELISA **(B)** IL-1β, TNF and IL-17 protein secretion using Linco 16-multiplex cytokine assay respectively. Each result represents the mean ± SD of triplicate assays.

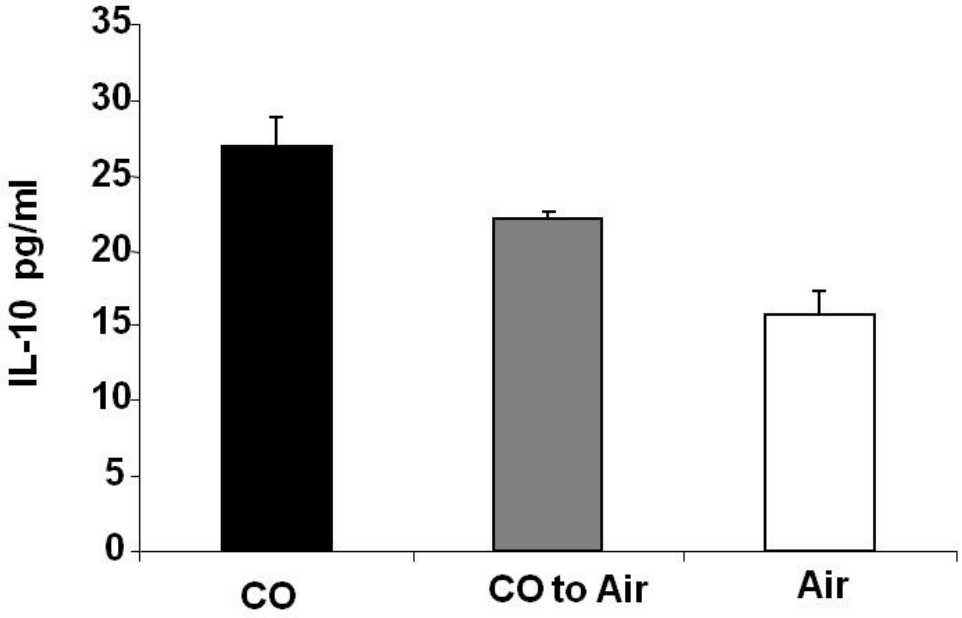


Figure 4.3. CO induces intestinal IL-10 protein secretion in *TCRα*$^{-/-}$ mice. 10 wk old *TCRα*$^{-/-}$ mice were divided into three groups: either exposed to CO (250 ppm, black bar) for four weeks, exposed to air for four weeks (white bar) or exposed to CO for two weeks and then transferred to ambient air housing condition (grey bar). Spontaneous IL-10 secretion was measured in full length colonic cell free supernatants using cytokine specific IL-10 ELISA.

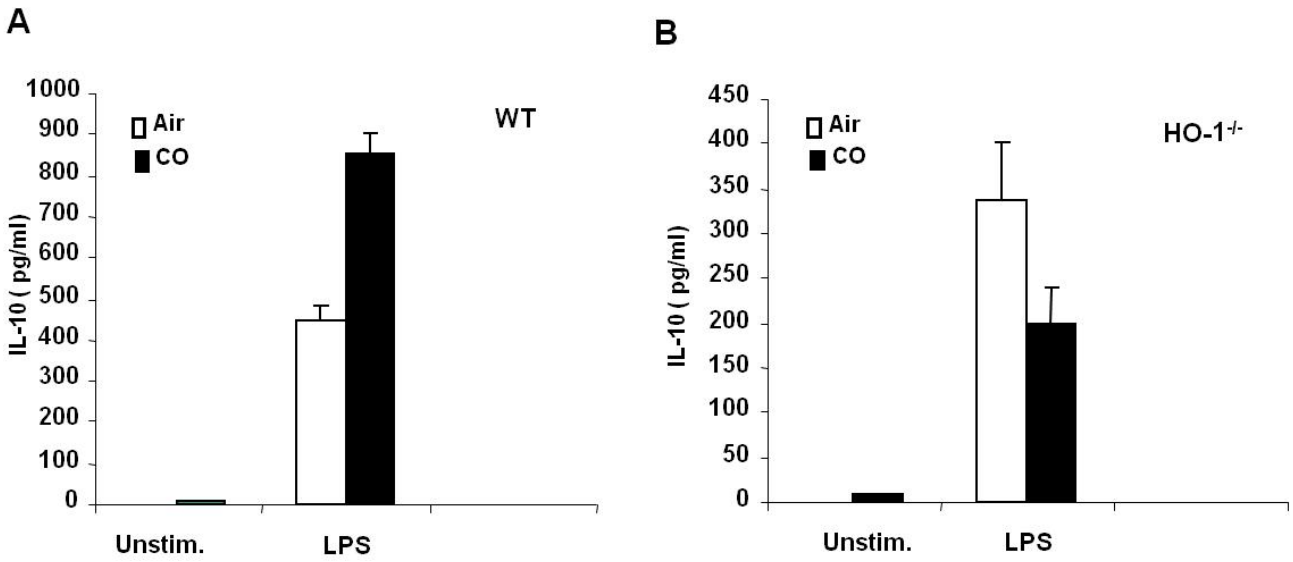


Figure 4.4. CO induced IL-10 in murine macrophages via induction of the HO-1 pathway. (A) BM-derived murine macrophages from wild type and **(B)** HO-1$^{-/-}$ mice were cultured in CO (250 ppm) or ambient air. Following activation with LPS (1 mg/ml), IL-10 protein secretion was assayed from supernatants at 24 hours by ELISA. Representative of 3 independent experiments with similar results.

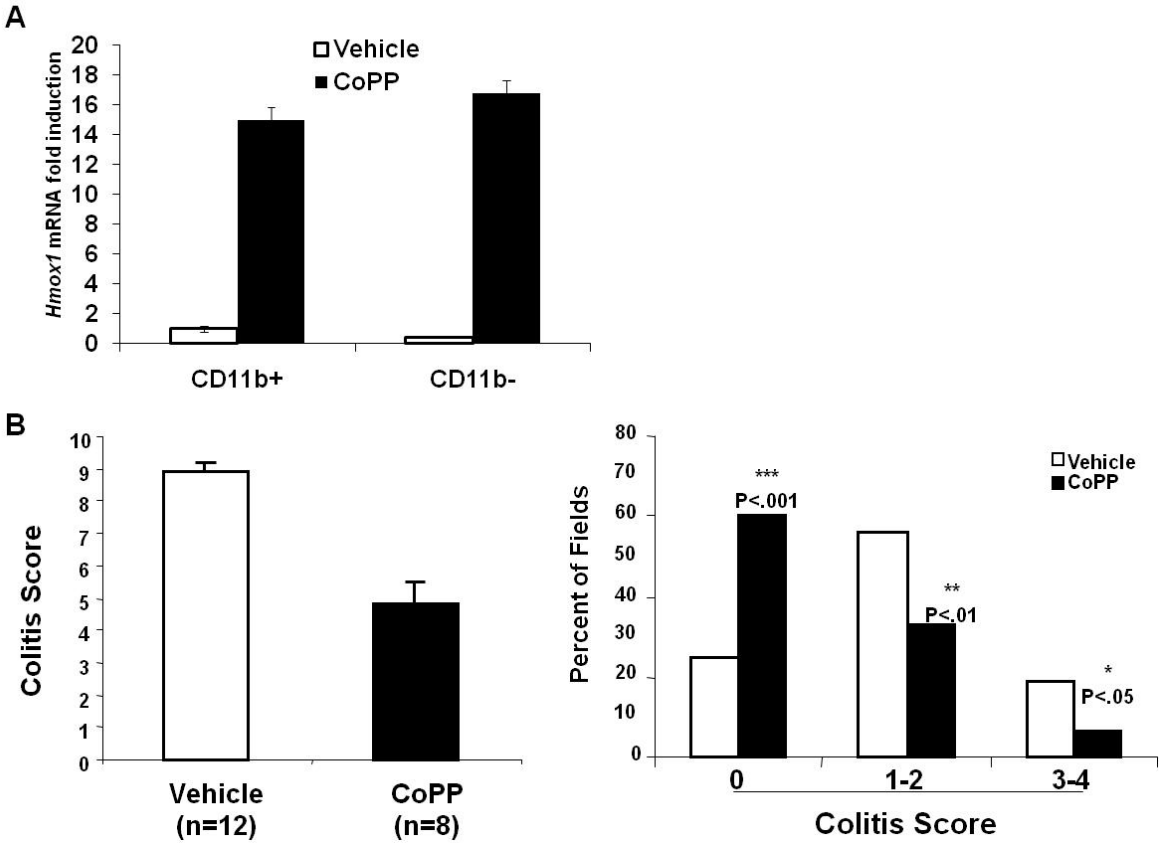


Figure 4.5. HO-1 inducer (cobalt protoporphyrin) ameliorates colitis in TCRα$^{-/-}$ mice. Twenty week old TCRα$^{-/-}$ mice were treated with i.p. injection of cobalt protoporphyrin (CoPP, 5 mg/kg twice/weekly for two weeks) (n=8) and control mice were treated with PBS vehicle i.p. (n=12). **(A)** Lamina propria cells (LPMCs) were isolated from colons of TCRα$^{-/-}$ mice treated with vehicle (white bars) and CoPP (black bars) and further separated into intestinal macrophages (CD11b+) cells using anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA) and analyzed for HO-1 mRNA (*Hmox1*) expression. **(B)** CoPP injected mice had significantly less severe colitis (left panel) and fewer fields with mild (scores of 1-2) and severe colitis (scores of 3-4) and significantly more fields with no colitis (scores of 0) (right panel).

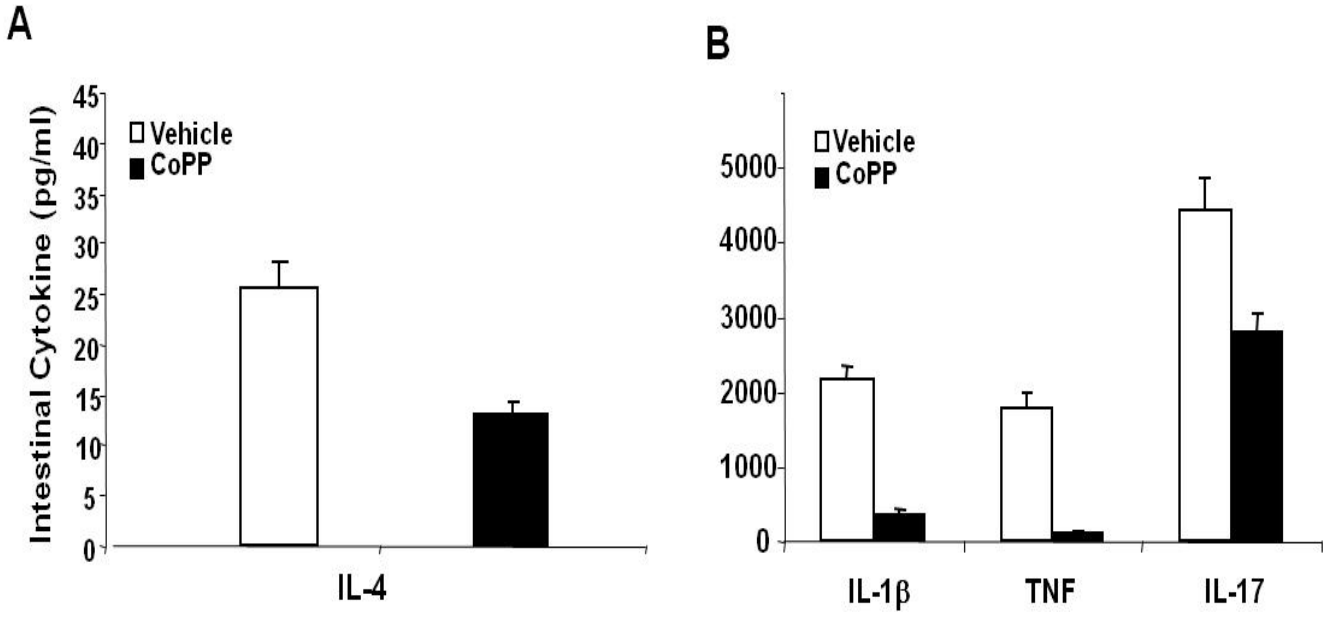


Figure 4.6. HO-1 inducer (cobalt protoporphyrin) reduces secretion of pro-inflammatory cytokines in TCRα$^{-/-}$ mice. Spontaneous protein secretion determined in 24 hour supernatants from intestinal explants from 5mg/kg of CoPP (n=12) and PBS Vehicle (n=8) i.p. treated TCRα $^{-/-}$ mice. **(A)** IL-4, **(B)** IL-1β, TNF and IL-17 secretion by Linco 16-multiplex cytokine ELISA. Results represent mean±SD of triplicate assays of pooled intestinal explants

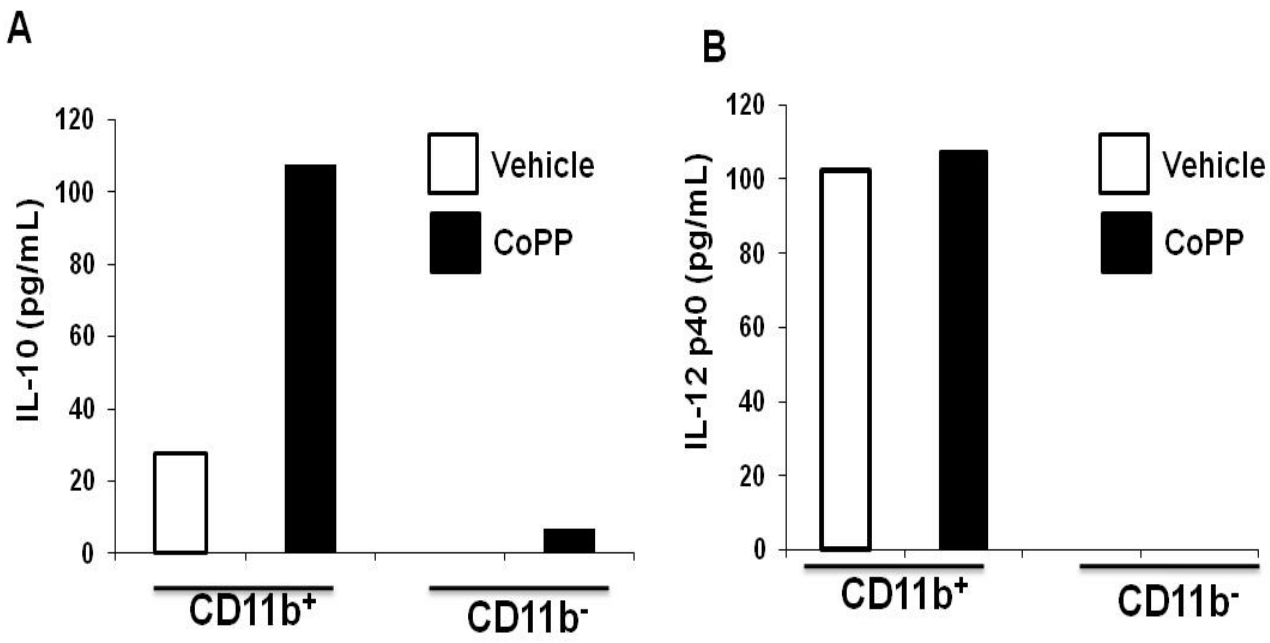
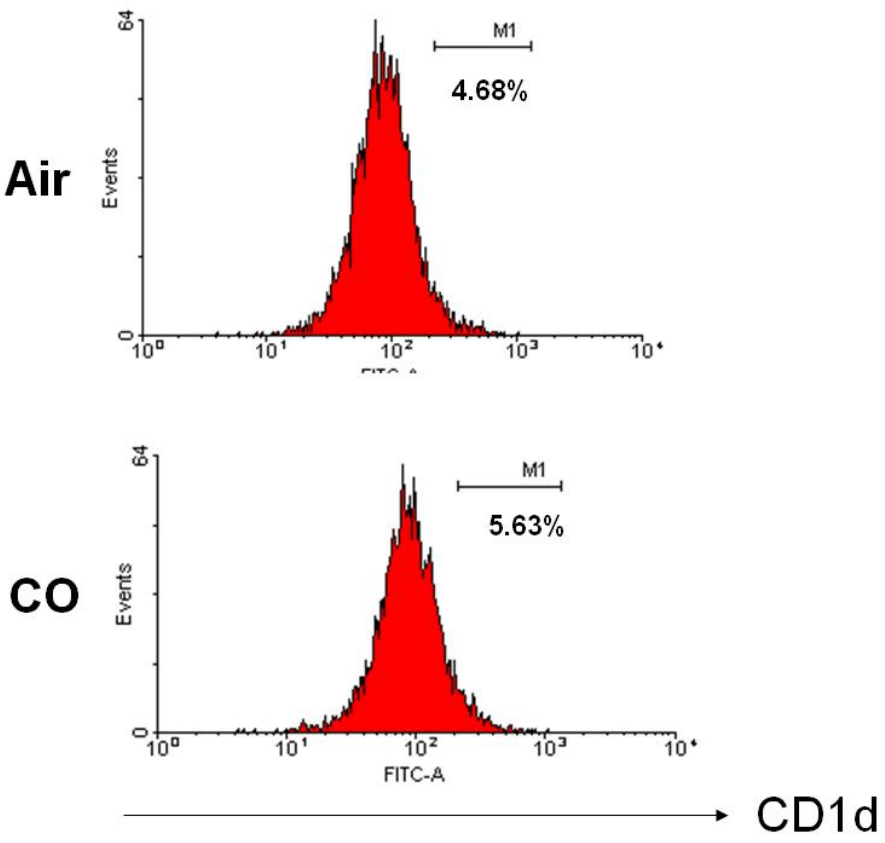
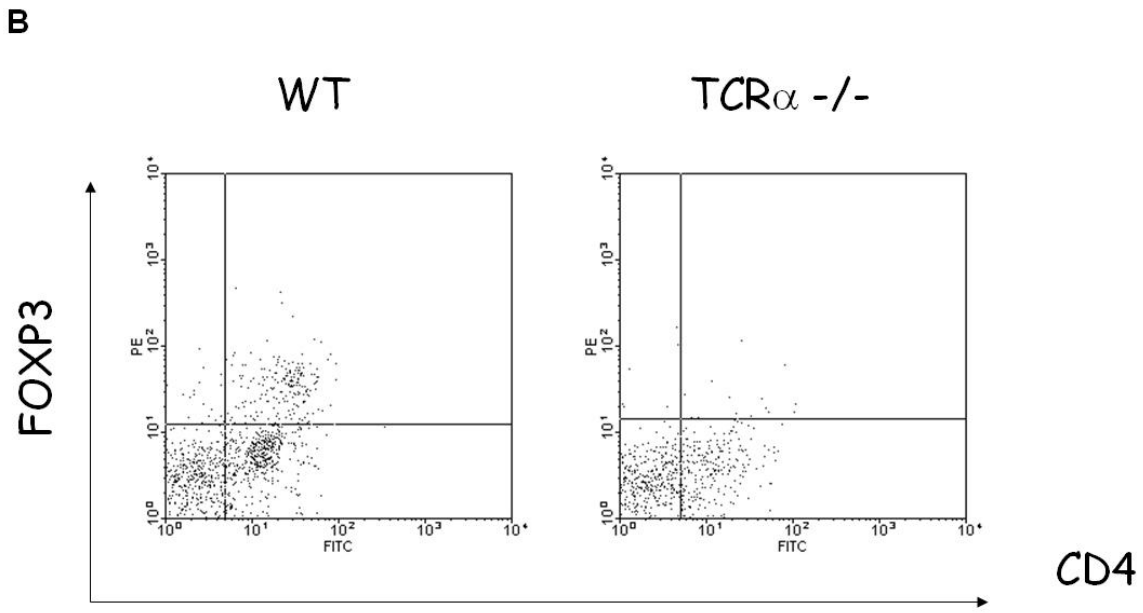


Figure 4.7. CD11b+ lamina propria mononuclear cells are the major source of IL-10 in TCRα$^{-/-}$. LPMCs were isolated from colons of TCRα$^{-/-}$ mice treated with vehicle (n=4, white bars) and CoPP (n=5, black bars) and further separated into intestinal macrophages (CD11b+) cells using anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA). Supernatants from pooled CD11b+ and CD11b- LPMCs in each group plated at a concentration of 1X10$^{\wedge 6}$ / mL in a 96 well tissue-culture plate were harvested 15 hours later and **(A)** IL-10 and **(B)** IL-12 p40 cytokine secretion determined using specific ELISA.



Supplemental Figure 4.1. CO does not induce CD1d+ B cells MLNs of CO and air exposed TCRα$^{-/-}$ mice and B220^{+} MLN cells were isolated and pooled (n=3) and stained for CD1d. Percentages of CD1d high positive cells were compared between Air- and CO-exposed mice.



Supplemental Figure 4.2. Regulatory Tcells are absent in TCRα$^{-/-}$ mice. Splenocytes from WT and TCRα$^{-/-}$ mice were isolated and pooled (n=3) and stained for CD4 and FOXP3 and compared to those of air-exposed mice.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

5.1. Summary

The gastrointestinal immune system is able to discriminate between the commensal microbiota and enteric pathogens. Macrophages are central to the maintenance of immune homeostasis in the intestines. Alterations in macrophage specific homeostatic pathways lead to chronic intestinal inflammation. Unlike resident intestinal macrophages, macrophages in the inflamed mucosa of IBD patients react vigorously to the enteric-microbiota to produce a robust inflammatory response characterized by IL-12 and IL-23 production. This in turn leads to differentiation and proliferation of pathogenic Th1/Th17 cell populations.

Our studies implicate two 'checkpoints' that are important in maintaining mucosal immune homeostasis. First, we demonstrate that IFN-γ, best known for promoting inflammation, may be protective during the initiation phase of intestinal inflammation through inhibition of IL-23 in macrophages. Second, we further characterize HO-1 as a molecular brake on innate immune activation of macrophages in Th1/Th17 and Th2 mediated models of experimental colitis.

Mechanistically, IFN-γ mediated inhibition is through two distinct pathways. First, IFN-γ alters LPS induced recruitment of NF-κB subunits to the *Il23a* murine promoter, specifically through inhibition of the RelA but potentiating p50 subunits. Similarly, inhibition of histone H4 acetylation indicates that IFN-γ may alter chromatin structure at the *Il23a* locus that in turn may directly affect the recruitment of these factors. Second, IRF-1 is also a negative regulator of LPS induced IL-23. IRF-1 deficient BMMs demonstrate more IL-23 expression compared with WT BMMs. IFN-γR1/IL-10$^{-/-}$ IRF-1/IL-10$^{-/-}$ mice are also develop more severe colitis compared to IL-10$^{-/-}$ mice. Finally, the enteric-microbiota induced a transient surge of *ifng* in GF WT mice transitioned to a CNV microbiota that inversely

correlates with *Il23a* expression. This negative regulatory checkpoint has important implications, given the inability of IFN-γ to inhibit *Il23a* in IL-10$^{-/-}$ GF mice transitioned to CNV microbiota that go on to develop colitis.

Interestingly, GF IL-10$^{-/-}$ mice also fail to induce HO-1 in the colon when compared to WT GF mice transitioned to a CNV microbiota. Our studies demonstrate that HO-1 induction in macrophages dampens innate immune responses, specifically to the enteric- microbiota. WT BMMs transfected with *Hmox1* siRNA show poor bactericidal activity against commensal E.coli. HO-1 derived CO seems to be the final mediator that enhances the ability of macrophages to eradicate intracellular bacteria. Both HO-1 and CO are protective against microbiota induced colitis in IL-10$^{-/-}$ mice. Finally, the protective effects of HO-1 and CO were also demonstrated in a Th2 mediated model of experimental colitis. CO and HO-1 induction ameliorate intestinal inflammation in the TCRα$^{-/-}$ mouse, specifically through induction of IL-10 in CD11b+ LPMCs. Our study will be the first to characterize the role of macrophages in maintaining immune homeostasis in the TCRα$^{-/-}$ mouse.

5.2. Developing models to study *in vivo* real time regulation of IL-12 family members

Despite the importance of IL-12 and IL-23 in immunity and inflammation, methods to follow the production of these cytokines or the cells that produce them *in vivo* are limited. DCs constitute heterogeneous populations that are few in number and turnover rapidly after activation. Macrophages are just as diverse, and their residence in tissues makes their recovery difficult. To further study *in vivo* regulation of IL-23 we can produce transgenic mice that are modified so that an *Il23a allele* is modified to express linked fluorescent reporter

proteins, thus efficiently marking *Il23a* expressing cells. With commercially available *Il12b* reporter mouse the generation of an *Il23a/Il12b* single and double reporter transgenic mouse will allow for the study of regulation of IL-12 family members *in vivo*. This double reporter can be utilized to study regulation of *Il23a* and *Il12b* in various models of murine IBD, including IL-10$^{-/-}$ mice.

5.3. Role of IFN-γ in shaping macrophage activation phenotypes in the intestine

We show that IFN-γ inhibits *Il23a* in CD11b+LPMC in WT and IL-10$^{-/-}$ mice. IFN-γR1/IL-10 and IRF-1/IL-10$^{-/-}$ colonic CD11b+ LPMCs produce more *Il23a* compared with WT and IL-10$^{-/-}$ CD11b+ LPMCs. CD11b+ LPMCs cells represent a heterogenous population of macrophages and DCs. However, there are subpopulations of colonic macrophages that include CD11b+CD11c- cells (low IL-12 and high IL-10) versus CD11b+CD11c+ (high IL-12 and low IL-10) (1). Further studies can be performed to correlate the effect of IFN-γ on specific macrophage activation phenotypes. It will be interesting to study phenotypic and functional characteristics of specific intestinal macrophage populations that express IL-23, whether they differ from IL-12 or IL-10 expressing populations, and whether their development is independent of the enteric- microbiota. Additionally, in the future, the contribution of IL-10 to inhibition of inflammatory innate immune responses to enteric-microbiota can be investigated through exogenous administration of IL-10 in IL-10$^{-/-}$ mice on transfer to CNV conditions and studying *Il23a* and *Il12b* expression.

We characterize *Il23a* as a primary response gene. Indeed, colonization of GF WT and colitis prone IL-10$^{-/-}$ mice with the microbiota leads to an early activation of *Il23a* in the colon. The rapid extinguishing of *Il23a* in WT GF mice colonized with enteric-microbiota is

associated with a transient surge in mucosal *ifg* expression. However, *ifng* and *Il23a* levels both continue to rise over time in the IL-10$^{-/-}$ mice, correlating with worsened colitis. This raises the intriguing hypothesis that depending on the stage of colitis, IFN-γ may diametrically regulate mucosal immune responses. Through generation of inducible macrophage/DC specific IFN-γR1$^{-/-}$ mice, the effects of IFN-γ on early versus late disease pathogenesis can be studied. Based on our study, we would predict that loss of IFN-γ signaling will worsen initiation of colitis but improve late disease course.

We also demonstrate that IFN-γ promotes the recruitment of NF-κB p50 to the *Il23a* promoter. NF-κB RelA-/- and p65$^{-/-}$ BMMs demonstrate markedly decreased TLR mediated Il23a gene induction (2). p50$^{-/-}$ and p50/p65$^{-/-}$ develop worsened *Helicobacter hepaticus* induced colitis compared with WT mice (3). It remains to be seen whether mucosal *Il23a* gene expression, specifically in intestinal macrophage populations, is altered in these mice and contributes to disease pathogenesis.

Finally, we examine potential protective effects of IFN-γ and IRF-1 in an experimental model that is dependent on both innate and adaptive immune responses. Notably, IFN-γ clearly can affect multiple immune pathways and the protective effects we observed may be multifactorial. Therefore, it will be important to determine if the role of IFN-γ in colonic IL-23 inhibition in experimental models dependent on innate immunity. Fortunately, such models have been developed and represent an important future goal of this project. To discern a macrophage specific role in colitis development, cell type specific deletions of IFN-γR1 and IRF-1; and models that are completely T cell independent (RAG$^{-/-}$ mice treated with anti-CD40 or colonized with *Helicobacter hepaticus*) will be developed (4, 5).

5.4. Epigenetic regulation of *Il23a*

Characterization of an ISRE in the *Il23a* promoter led to our conclusion that IRF-1 is an IFN-γ inducible negative regulator of *Il23a.* This ISRE is uniquely placed within an area of conserved nucleotide sequences, which also harbors two critical NF-κB binding sites, essential for induction of *Il23a* promoter activity by LPS (2, 6). Whereas cytoplasmic activation of NF-κB leads to nuclear import and binding to a multitude of gene promoters, "epigenetic" mechanisms superimposed on the primary genetic code have yet to be fully elucidated. Before the NF-κB family member RelA accesses its DNA-binding sites, a transition from a condensed to a decondensed chromatin structure needs to occur (7). We show that *Il23a* is an 'early response gene', unlike *Il12b,* which has been characterized as a 'secondary response gene' (8), implying distinct regulatory mechanisms. Further studies will look at various chromatin remodeling enzymes such as CBP/p300, BRG1/BRM1, and SWI/SNF to assess how NF-κB subunits and other IRFs may interact with co-activators/repressors in macrophages to regulate IL-12 and IL-23 gene activation *versus* inhibition.

5.5. Role of HO-1 expression and function in regulating mucosal innate immune responses

We show that GF IL-10$^{-/-}$ mice, unlike GF WT mice, failed to up-regulate colonic HO-1 on transition to a CNV microbiota. TLR ligands and IL-10 are important regulators of HO-1 in macrophages. HO-1 plays an important role in antimicrobial process while inhibiting the inflammatory response. We also demonstrate that HO-1 expression and function is important for the macrophage's ability to eradicate intracellular enteric bacteria. With

accumulating evidence demonstrating the importance of microbial communities in the pathogenesis of IBD, future studies can address the preferential induction of HO-1 by monoassociation of GF IL-$10^{-/-}$ mice with strains of colitogenic (NC 101) versus non-colitogenic (K12) *E. coli*. Likewise it is intriguing to speculate that beneficial bacterial constituents such as probiotic bacteria may exert anti-inflammatory effect through induction of intestinal HO-1.

Bacteria have been shown to generate and secrete large amounts of extracellular ATP (9). Furthermore, treatment of GF mice with ATP markedly increases the numbers of IL-17-producing $CD4^+$ cells and worsens enteric-microbiota induced coltiis (10). Regulation of HO-1 by ATP remains to be understood. Use of bacterial strains that lack the ability to generate ATP (ATPase deficient) to monoassociate GF IL-$10^{-/-}$ mice and correlate with HO-1 expression and function can highlight the importance of bacterial derived ATP in colitis pathogenesis. Similarly, retinoic acid receptor agonists (RARs) have been shown to promote regulatory $Foxp3^+CD4^+$ T cells and inhibit Th17 cells. Given that multiple regulatory cytokines, including IL-10, have been implicated in functioning through what has been popularly called '*the HO-1 therapeutic funnel' (11)*, it is also important to study whether these RARs function partly through induction of HO-1. Finally, further studies with macrophage specific *Hmox1* over expressing and knockout murine models can demonstrate the protective role of HO-1 expression in the pathogenesis of IBD, specifically when crossed with colitis-prone models like the IL-$10^{-/-}$ mouse.

5.6. Carbon monoxide (CO) as a therapeutic modality for IBD

We demonstrate that CO ameliorates colitis in both Th1/Th17 and Th2 driven models of experimental colitis. We also report for the first time the potential therapeutic benefit of a CO releasing molecule (CORM) in ameliorating chronic intestinal inflammation in IL-$10^{-/-}$ mice. CO and CORMs enhance the ability of macrophages to eradicate intracellular bacteria. Future studies will address alterations in microbial communities after treatment with CORMs using terminal restriction fragment length polymorphism (TRFLP) and pyrosequencing on stool and intestinal mucosal samples. Indeed, if the mechanisms for intracellular inhibition and killing are diminished in IBD as indicated by our work and others (12) (13), then persistent survival may stimulate inflammation through numerous innate and adaptive pathways. The role of CO in altering disease course can be correlated with host gene expression profiles, metabolomic profiling of microbes and metagenomic approaches that may in turn help narrow the microbial factors central to disease pathogenesis

Accumulating studies strongly suggest that endogenously generated CO and exogenous CO gas, inhaled at doses whereby the oxygen-carrying capacity of hemoglobin is not severely compromised (HbCO<20%), elicits protection and beneficial outcomes in multiple organ injury, inflammation, apoptosis, cell proliferation, vasoconstriction and both systemic and pulmonary hypertension. In fact, a therapeutic role for CO is being actively explored in many human diseases. The effects of inhaled CO are being investigated in a phase 2 study in patients receiving renal transplants (14). Similarly, recent studies by Hoetzel et al. reveal that inhaled CO during mechanical ventilation protects against mechanical ventilation injury by reduction in cytokine production and lung recruitment of neutrophils and macrophages via PPARγ (15). Goebel et al. demonstrated that preoperatively inhaled CO inhibited pulmonary inflammation and reduced post-operative

complication rates in cardiopulmonary bypass surgeries (16, 17). Ongoing studies are evaluating the benefit of inhaled CO during endotoxaemia in humans (18).

With the recent development of controlled methods for CO delivery (CORMs) to target these different pathological conditions, the prospect of CO as a therapeutic option is even more of a possibility. Although administration of small amounts of CO gas is likely feasible, gaseous compounds are difficult to deliver directly in an accurate manner. CO liberated from CORMs can be precisely controlled and delivered at given concentrations through all possible routes of administration, unlike CO gas, which can be delivered effectively only by inhalation. Therefore, future studies can be performed to develop other routes through which CORMs may be administered to target colonic inflammation, including oral and intrarectal routes. We used CORM-186, a water soluble compound that results in fast CO release in an aqueous solution. Use of other CORM compounds e.g. CORM-A1, the release of which is slow and strictly pH dependent, may offer exciting possibilities in direct drug delivery to specific inflamed areas of the intestine (19).

Finally, an interesting and well established clinical observation in patients with IBD is that cigarette smoking, while protective in UC, worsens disease course in CD. We have demonstrated a protective effect of CO in ameliorating colitis in IL-10$^{-/-}$ mice. However, cigarette smoke contains a host of other chemicals that may potentiate or counter the beneficial effects of CO. For example, the aryl hydrocarbon receptor (AHR) is a ligand-activated member of Period (Per)/Arnt/Sim family of transcription factors (20). The AHR mediates the biological and toxic responses of a class of environmental pollutants including man-made toxicant aromatic hydrocarbons found abundantly in cigarette smoke (20). AHRs are abundantly expressed on Th17, where its activation leads to production of IL-22 (21).

They are also found on regulatory (Treg) T cell populations (22). However, the role of AHRs on macrophages and in the pathogenesis of IBD remains unexplored.

5.7. Genetics and IBD

We are only beginning to functionally dissect the numerous genetic variants in IBD susceptibility. Recent understanding of genetic variation within the human genome has lead to the development of genome-wide association studies (GWAS) to identify genetic risk factors for these complex polygenic diseases. Both the innate and adaptive immune responses have been implicated by the discovery of genetic associations in IBD. In the innate immune system, the association of CD with polymorphisms in *NOD2* (*CARD15*) and the two autophagy-related genes, *ATG16L1* and *IRGM*, suggest an abnormal recognition and handling of intracellular bacteria in IBD (23). The critical role of the IL-23 pathway in IBD pathogenesis was confirmed by the association of several SNPs throughout the *IL23R* gene that afford protection and susceptibility to CD and UC. Similarly, in a large meta-analysis of genome wide association studies (GWAS) of cohorts of European and North American ancestry, components of the IL-23 signaling pathway (*IL23R, IL12B, STAT3* and *JAK2*) were implicated with the highest level of association with Crohn's disease. Additonally, within a few kb of the UC risk loci on chromosomes 1p36 and 12q15 determined by GWAS was shown to harbor the *ifng* gene (24).

We have identified that IFN-γ induced IRF-1 is a negative regulator of IL-23. Interestingly, the IBD5 locus on chromosome 5q31 contains the IRF-1 gene. Similarly, haplotype analysis revealed a putative functional HO-1 promoter polymorphisms, one (S/A haplotype) conferring a strong protective effect whereas the other (L/A haplotype) showed

the opposite tendency in patients with rheumatoid arthritis (25). Ongoing studies can search for more complete genotype–phenotype correlations and for independent evidence of the functional consequences of sequence alteration within the *Hmox1*, *ifng* and *irf1* gene loci.

5.8 Conclusion

In conclusion, altered macrophage function is central to the pathogeneisis of IBD. This has been confirmed with identification of multiple genetic susceptibility genes for IBD that belong to specific innate immune pathways. IL-23 is a critical mediator of chronic intestinal inflammation. Indeed, intestinal macrophages in the inflamed mucosa of IBD patients differ significantly in phenotype from the resident intestinal macrophage under physiological conditions. This reveals a critical failure of macrophages as gatekeepers that drive immune responses to either tolerance induction or initiation of inflammatory reactions.

We identify three macrophage specific homeostatic checkpoints that are critical in regulating mucosal immune responses to the enteric-microbiota. Heme oxygenase-1 (HO-1) and carbon monoxide (CO) act as important homeostatic checkpoints in regulating pro-inflammatory immune responses in macrophages through IL-10 or by regulating the ability of macrophages to eradicate intracellular bacteria. IFN-γ inhibits T-helper (T_H) 17 cells proliferation through inhibition of TLR induced *Il23a* macrophages. Mechanistically, IFN-γ inhibits IL-23 in macrophages through alterations in NF-κB and IRF recruitment to the *Il23a* promoter (Figure 5).

Further studies should characterize detailed mechanisms of how local macrophages functionally differentiate and participate in the pathogenesis of IBD. This may allow for designing specific strategies to target the induction of dysregulated immune responses and

excessive inflammation. IL-12 family members, IL-12 and IL-23 along with anti-inflammatory molecules like HO-1 may play an important role in the plasticity of these macrophages and require further study.

5.9 References

1. Mosser, D.M., and J.P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958-969.

2. Carmody, R.J., Q. Ruan, H.C. Liou, and Y.H. Chen. 2007. Essential roles of c-Rel in TLR-induced IL-23 p19 gene expression in dendritic cells. *J Immunol* 178:186-191.

3. Erdman, S., J.G. Fox, C.A. Dangler, D. Feldman, and B.H. Horwitz. 2001. Typhlocolitis in NF-kappa B-deficient mice. *J Immunol* 166:1443-1447.

4. Kullberg, M.C., D. Jankovic, C.G. Feng, S. Hue, P.L. Gorelick, B.S. McKenzie, D.J. Cua, F. Powrie, A.W. Cheever, K.J. Maloy, and A. Sher. 2006. IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. *J Exp Med* 203:2485-2494.

5. Uhlig, H.H., B.S. McKenzie, S. Hue, C. Thompson, B. Joyce-Shaikh, R. Stepankova, N. Robinson, S. Buonocore, H. Tlaskalova-Hogenova, D.J. Cua, and F. Powrie. 2006. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* 25:309-318.

6. Carmody, R.J., Q. Ruan, S. Palmer, B. Hilliard, and Y.H. Chen. 2007. Negative regulation of toll-like receptor signaling by NF-kappaB p50 ubiquitination blockade. *Science* 317:675-678.

7. Natoli, G., S. Saccani, D. Bosisio, and I. Marazzi. 2005. Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time. *Nat Immunol* 6:439-445.

8. Zhou, L., A.A. Nazarian, and S.T. Smale. 2004. Interleukin-10 inhibits interleukin-12 p40 gene transcription by targeting a late event in the activation pathway. *Mol Cell Biol* 24:2385-2396.

9. Ivanova, E.P., Y.V. Alexeeva, D.K. Pham, J.P. Wright, and D.V. Nicolau. 2006. ATP level variations in heterotrophic bacteria during attachment on hydrophilic and hydrophobic surfaces. *Int Microbiol* 9:37-46.

10. Atarashi, K., J. Nishimura, T. Shima, Y. Umesaki, M. Yamamoto, M. Onoue, H. Yagita, N. Ishii, R. Evans, K. Honda, and K. Takeda. 2008. ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455:808-812.

11. Soares, M.P., and F.H. Bach. 2009. Heme oxygenase-1: from biology to therapeutic potential. *Trends Mol Med* 15:50-58.

12. Rioux, J.D., R.J. Xavier, K.D. Taylor, M.S. Silverberg, P. Goyette, A. Huett, T. Green, P. Kuballa, M.M. Barmada, L.W. Datta, Y.Y. Shugart, A.M. Griffiths, S.R. Targan, A.F. Ippoliti, E.J. Bernard, L. Mei, D.L. Nicolae, M. Regueiro, L.P. Schumm, A.H. Steinhart, J.I. Rotter, R.H. Duerr, J.H. Cho, M.J. Daly, and S.R. Brant. 2007. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 39:596-604.

13. Xavier, R.J., and D.K. Podolsky. 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448:427-434.

14. 2008. Carbon monoxide now orphan drug for transplantations. *Inpharma. 2008;1:21.*

15. Hoetzel, A., T. Dolinay, S. Vallbracht, Y. Zhang, H.P. Kim, E. Ifedigbo, S. Alber, A.M. Kaynar, R. Schmidt, S.W. Ryter, and A.M. Choi. 2008. Carbon monoxide protects against ventilator-induced lung injury via PPAR-gamma and inhibition of Egr-1. *Am J Respir Crit Care Med* 177:1223-1232.

16. Goebel, U., M. Siepe, A. Mecklenburg, T. Doenst, F. Beyersdorf, T. Loop, and C. Schlensak. 2008. Reduced pulmonary inflammatory response during cardiopulmonary bypass: effects of combined pulmonary perfusion and carbon monoxide inhalation. *Eur J Cardiothorac Surg* 34:1165-1172.

17. Goebel, U., M. Siepe, A. Mecklenburg, P. Stein, M. Roesslein, C.I. Schwer, R. Schmidt, T. Doenst, K.K. Geiger, H.L. Pahl, C. Schlensak, and T. Loop. 2008. Carbon monoxide inhalation reduces pulmonary inflammatory response during cardiopulmonary bypass in pigs. *Anesthesiology* 108:1025-1036.

18. Bathoorn, E., D.J. Slebos, D.S. Postma, G.H. Koeter, A.J. van Oosterhout, M. van der Toorn, H.M. Boezen, and H.A. Kerstjens. 2007. Anti-inflammatory effects of inhaled carbon monoxide in patients with COPD: a pilot study. *Eur Respir J* 30:1131-1137.

19. Foresti, R., M.G. Bani-Hani, and R. Motterlini. 2008. Use of carbon monoxide as a therapeutic agent: promises and challenges. *Intensive Care Med* 34:649-658.

20. Esser, C., A. Rannug, and B. Stockinger. 2009. The aryl hydrocarbon receptor in immunity. *Trends Immunol* 30:447-454.

21. Veldhoen, M., K. Hirota, A.M. Westendorf, J. Buer, L. Dumoutier, J.C. Renauld, and B. Stockinger. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453:106-109.

22. Quintana, F.J., A.S. Basso, A.H. Iglesias, T. Korn, M.F. Farez, E. Bettelli, M. Caccamo, M. Oukka, and H.L. Weiner. 2008. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453:65-71.

23. Cho, J.H. 2008. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 8:458-466.

24. Silverberg, M.S., J.H. Cho, J.D. Rioux, D.P. McGovern, J. Wu, V. Annese, J.P. Achkar, P. Goyette, R. Scott, W. Xu, M.M. Barmada, L. Klei, M.J. Daly, C. Abraham, T.M. Bayless, F. Bossa, A.M. Griffiths, A.F. Ippoliti, R.G. Lahaie, A. Latiano, P. Pare, D.D. Proctor, M.D. Regueiro, A.H. Steinhart, S.R. Targan, L.P. Schumm, E.O. Kistner, A.T. Lee, P.K. Gregersen, J.I. Rotter, S.R. Brant, K.D. Taylor, K. Roeder, and R.H. Duerr. 2009. Ulcerative colitis-risk loci on chromosomes 1p36 and 12q15 found by genome-wide association study. *Nat Genet* 41:216-220.

25. Rueda, B., J. Oliver, G. Robledo, M.A. Lopez-Nevot, A. Balsa, D. Pascual-Salcedo, M.A. Gonzalez-Gay, M.F. Gonzalez-Escribano, and J. Martin. 2007. HO-1 promoter polymorphism associated with rheumatoid arthritis. *Arthritis Rheum* 56:3953-3958.

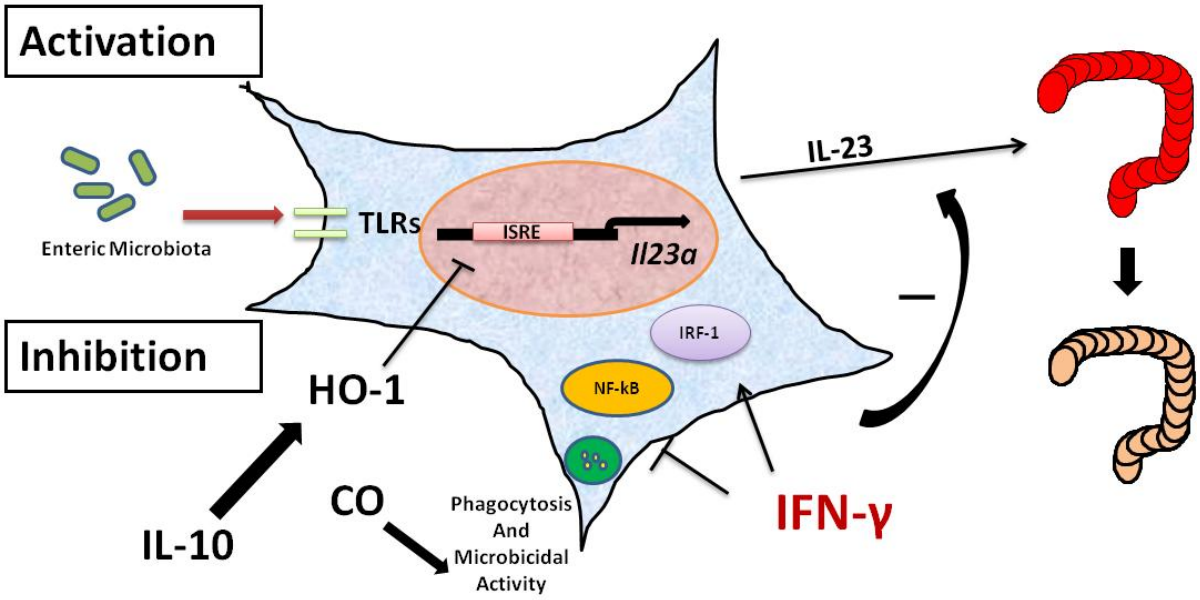


Figure 5. A schematic representation of the IL-23 regulation in macrophages. The enteric-microbiota represents an important environmental trigger for mucosal immune responses. We identify heme oxygenase-1 (HO-1) and carbon monoxide (CO) as important homeostatic checkpoints in regulating pro-inflammatory immune responses in macrophages. Both HO-1 and CO mediate their protective effects in murine experimental colitis through regulation of IL-10 or by regulating the ability of macrophages to eradicate intracellular bacteria. IFN-γ inhibits T-helper (T_H) 17 cells proliferation through inhibition of TLR induced *Il23a* macrophages. Mechanistically, IFN-γ inhibits IL-23 in macrophages through alterations in NF-κB and IRF recruitment to the *Il23a* promoter.