The Role of Type I Interferons in Regulating Intestinal Inflammation

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Abstract

Intestinal homeostasis is a delicate balance between suppression of immune responses against innocuous antigens and stimulation of immune responses against pathogens. Type I interferon (IFN-1) cytokines have both immunostimulatory and immunomodulatory effects. Colon mononuclear phagocytes (MP) constitutively produced IFN-1 in a TRIF-dependent manner. We explored the function of endogenous IFN-1 in the colon using the T cell adoptive transfer model of colitis. Transfer of $CD4^{+}CD45RB^{hi}$ naïve T cells from wild type (WT) or IFNAR subunit 1 knockout ($IFNAR1^{-/-}$) mice into $RAG^{-/-}$ hosts resulted in similar onset and severity of colitis. In contrast, $RAG^{-/-}$ x $IFNAR1^{-/-}$ double knockout (DKO) mice developed accelerated severe colitis compared to $RAG^{-/-}$ hosts when transferred WT $CD4^{+}CD45RB^{hi}$ T cells. Although WT or $IFNAR1^{-/-}$ regulatory T (Treg) cells equally prevented disease caused by $CD45RB^{hi}$ naïve T cells, WT Treg cells co-transferred with naïve $CD4^{+}$ T cells into DKO recipients failed to expand or maintain Foxp3 expression and gained effector functions in the colon. IFNAR signaling on host hematopoietic cells inhibited T cell-mediated colitis, but not innate colitis. MPs isolated from the colon lamina propria (cLP) required IFNAR signaling for the production of the anti-inflammatory cytokines, IL-10, IL-27, and IL-1RA, but not for the production of classic pro-inflammatory cytokines. IFN-1-dependent secretion of IL-1RA was particularly important in inhibiting the migration of inflammatory DCs with potent T cell proliferative capacity from the cLP to the mesenteric lymph nodes. Finally, preliminary results suggested that IFN-1 may shape the commensal microbiota, but is not essential for controlling specific colitis-inducing bacteria.

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Abbreviations

ASF	Altered Schaedler flora
BCR	B cell receptor
BM	Bone marrow
BMDM	Bone marrow-derived macrophage
BrdU	5-bromo-2'-deoxyuridine
CD	Crohn's disease
cDC	Conventional dendritic cell
CDK	Cyclin-dependent kinase
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming unit
CSF-1	Colony stimulating factor 1
cLP	Colon lamina propria
CSR	Class switch recombination
DAI	DNA-dependent activator of interferon regulatory factors
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DKO	$RAG^{-/-}/IFNAR1^{-/-}$ Double knockout
dsRNA	Double stranded ribonucleic acid
DSS	Dextran sulfate sodium
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
Foxp3	Forkhead box P3
GBS	Group B streptococcus
GF	Germ-free
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel disease
IFN	Interferon
IFN-1	Type I interferon
IFNAR	Interferon α/β receptor
IL	Interleukin
ILC	Innate lymphoid cell
i.p.	Intraperitoneal
i.v.	Intravenous
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IPS-1	Interferon-β promoter stimulator 1
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-sensitive response element
iTreg	Induced T regulatory
JAK	Janus kinase
JAM-A	Junctional adhesion molecule A
KLH	Key limpet hemocyanin

LCMV	Lymphocytic choriomeningitis virus
LP	Lamina propria
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MDA-5	Melanoma differentiation-associated protein 5
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MOI	Multiplicity of infection
MP	Mononuclear phagocyte
MyD88	Myeloid differentiation primary response gene (88)
NF-κB	Nuclear factor κB
NK	Natural killer
NLR	NOD-like receptor
NLRC4	NLR family CARD domain-containing protein 4
NLRP	Nucleotide-binding domain and leucine rich repeat containing protein
NOD	Nucleotide-binding oligomerization domain
nTreg	Natural T regulatory
OAS	2'-5'-oligoadenylate synthetase
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PD-L1	Programmed death ligand 1
PGE_2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PP	Peyer's patch
PKR	Protein kinase R
PRR	Pathogen recognition receptor
RAG	Recombinase-activating gene
RIG-I	Retinoic acid-inducible gene 1
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RORγt	Receptor tyrosine kinase-like orphan receptor γt
RSV	Respiratory syncytial virus
SCID	Severe combined immunodeficiency
SD	Standard deviation
SFB	Segmented filamentous bacteria
SFV	Semliki forest virus
sIgA	Secretory immunoglobulin A
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	T regulatory

Tyk2	Tyrosine kinase 2
TRIF	Toll/IL-1 receptor domain-containing adapter-inducing interferon-β
UC	Ulcerative colitis
VSV	Vesicular stomatitis virus

Table of Contents

Chapter 1: Introduction

1.1 Overview

In this thesis, I explore the effects of type I interferons (IFN-1) on intestinal immunity. The intestinal tract is home to 100 trillion microbial cells, ten times the number of human cells comprising our bodies (1). These microbes have a mutualistic relationship with the human host by contributing to metabolism and the development of a properly functioning immune system. Thus, tolerance rather than immunity against these commensal microbes provides great benefit to the host (2). The intestinal immune system is specialized for promoting tolerogenic rather than immunogenic responses against the commensal microbiota via mechanisms including modulation of dendritic cell responses; local production of anti-inflammatory mediators such as prostaglandin E_2 (PGE_2), transforming growth factor (TGF)-β, and interleukin (IL)-10; and the maintenance of distance between immune cells and the microbial products that can trigger them (3-6). Breakdown of intestinal tolerance leads to inflammatory bowel diseases (IBD), two of which are Crohn's disease (CD) and ulcerative colitis (UC). Thus, it is of paramount importance to elucidate the mechanisms that induce, maintain and inhibit suppression of immune responses against harmless antigens in the intestine.

IFN-1 are a group of cytokines that have antiviral activity, but also exert contrasting effects of activating or inhibiting immune responses depending on the context. IFN-1 are constitutively produced by small intestinal (7) and large intestinal (this thesis) mononuclear phagocytes, but their role in maintaining intestinal homeostasis remains largely unexplored. In this thesis, I will use multiple mouse models of intestinal inflammation, some dependent on a lack of immune regulation and others dependent on

induced pro-inflammatory responses, to analyze how IFN-1 affects immune responses in the gut. In this section, I give an overview of the biology of IFN-1, including their induction and effects; the general themes governing tolerance and immunity in the intestines; and the currently known roles for IFN-1 in intestinal homeostasis.

1.2 Cells of the immune system

The immune system can be divided into two broad categories: the innate immune system and the adaptive immune system. The innate immune system reacts to antigens in a non-specific way. Cells such as macrophages and dendritic cells (DCs) patrol environmental surfaces including the skin, the lungs and the intestines for potential threats. Upon detection of an antigen, macrophages and DCs are activated to destroy the foreign object by a variety of different mechanisms. Macrophages, especially, are efficient at destroying objects that they have picked up from the environment. Environment objects enter the cell via a membrane-bound phagosome, which fuses with a lysosome. The acidic environment of the lysosome degrades the object. Alternatively, antigen recognition triggers a cascade of chemical reactions known as oxidative burst, which culminates in the production of the antimicrobial compounds, hydrogen peroxide or hypochlorite (8, 9).

These cells do not act alone since activated macrophages, DCs and even non-immune cells that recognize antigen secrete chemokines, proteins which recruit other cells to the location of the antigen, and cytokines, proteins which signal on local and recruited cells to activate their immune response. Examples of recruited cells include monocytes, natural killer (NK) cells, and neutrophils. Monocytes are circulating cells with inflammatory potential. Upon recruitment, they can differentiate into either macrophages or DCs with functions closely tailored to the needs of the local environment (10, 11). NK cells are cytotoxic lymphocytes that target cancerous cells or virus-infected cells (12). They also produce the cytokine, IFN-γ, which further activates macrophages (13, 14). Neutrophils are also phagocytic and possess an enzyme known as myeloperoxidase, which feeds into the oxidative burst cascade. Additionally, neutrophils can extrude their own DNA, which forms a "net" to trap extracellular objects (15). Other innate immune cells include

eosinophils, basophils, and mast cells, which participate in the allergic immune response, and will not be discussed further in this thesis. Finally, a new class of cells termed innate lymphoid cells (ILCs) respond to cues from phagocytes that pick up environmental antigens and are capable of both driving inflammation and enhancing protection against pathogens (16, 17).

These innate immune defenses are often tissue destructive. Thus, the innate immune system, while antigen-non-specific, must be able to discriminate between antigens that pose a danger to the body and innocuous antigens such as ingested food or proteins derived from the body itself. Since macrophages and DCs serve as sentinels of the immune system and orchestrate subsequent responses, they are appropriately equipped with tools to differentiate threats from harmless antigens. Pattern recognition receptors (PRRs) expressed by innate immune cells recognize microbe-associated molecular patterns (MAMPs) and danger-associated molecular patterns (DAMPs) that are either present on foreign organisms or released by cells when normal physiology is stressed. PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and cytosolic nucleic acid sensors. (18, 19). Together, these receptors recognize a wide array of foreign and self antigens including bacteria, viruses, fungi, nucleic acids and necrotic cells that signal impending danger for the body (18, 20). Indeed, full activation of antigen-presenting phagocytes required engagement of PRRs (21). These PRRs will be further discussed in subsequent sections.

The other arm of the immune system, the adaptive immune system, is closely tailored to react against a specific antigen. B cells and T cells are lymphocytes that express B cell receptors (BCRs) and T cell receptors (TCRs), respectively. The genes for BCRs and

TCRs are not typical linear genes. Instead, they are composed of variable (V), diversity (D), and joining (J) gene fragments that are rearranged and spliced together in a process known as V(D)J recombination (22). Each BCR or TCR gene locus contains hundreds of different gene fragments to choose from, making the number of possible unique receptors nearly infinite. Lymphocytes constantly circulate through the blood and reach lymph nodes, dense nexuses of immune cells, where they may encounter a MAMP or DAMP that is specific for their BCR or TCR. Upon antigen recognition, B cells and T cells proliferate to greatly increase the number of cells, which can react against that specific antigen. Some of these cells, the effectors, actively secrete antibodies (B cells), cytokines (B, $CD4^+$ T, and $CD8^+$ T cells) or cytotoxic molecules ($CD8^+$ T cells) to address the imminent threat, but another subset, the memory cells, enter a quiescent state and react more quickly the next time the same antigen is encountered.

A key feature of DCs is their ability to migrate from peripheral tissue to lymph nodes and prime $CD4^+$ and $CD8^+$ T cells responses, thus linking innate and adaptive immunity. DCs process antigens by cleaving proteins into smaller peptide fragments. Peptides derived from intracellular sources, such as self-antigens or viruses, are presented on major histocompatibility complex (MHC) I molecules for presentation to $CD8^+$ T cells, while peptides derived from extracellular, endocytosed antigens are presented on MHC II molecules for presentation to $CD4^+$ T cells (23, 24). Extracellular antigens can also be presented to $CD8^+$ T cells on MHC I molecules via cross-presentation (25).

$CD4^+$ T cell responses are specific, not only due to the TCR expressed, but also due to the T helper cell subset differentiation program initiated. Unlike $CD8^+$ T cells, $CD4^+$ T cells are not, themselves, cytotoxic, but instead secrete cytokines that enhance the immune

activities of other cells, including B cells, $CD8^+$ T cells, macrophages, and DCs. Originally, $CD4^+$ T helper (Th) cell subsets were divided into two groups. Th1 cells produced high amounts of IFN-γ, their signature cytokine, along with interleukin (IL)-2 and lymphotoxin (26). Th2 cells, on the other hand, produced IL-4, along with IL-5, IL-6 and IL-13. IL-3, TNF-α, and GM-CSF were produced by both subsets of $CD4^+$ T cells. The more recently characterized Th17 cells were so named for their ability to produce IL-17A and IL-17F, but they also secrete IL-22 (27-29). The type of $CD4^+$ helper T cell induced depends largely on the type of inflammatory response required to clear the pathogen encountered. For example, Th1 cells are important for the clearance of intracellular pathogens, Th2 cells promote anti-parasitic responses, and Th17 cells enhance immunity against extracellular bacteria and fungi at mucosal surfaces (30). These divisions of labor are not strict, especially since there is much plasticity between $CD4^+$ T cell subsets (31).

Naïve $CD4^+$ T cell subsets are differentiated into Th1, Th2, or Th17 cell lineages depending on the particular transcription factors expressed and activated within the cell. For example, the transcription factor, T-bet, is activated by TCR ligation and directs Th1 cell differentiation (32). T-bet expression enhances IFN-γ and IL-12Rβ2 expression on T cells (32, 33). IL-12 signaling increased the expression of the IL-18R on Th1 cells (34). IFN-γ, IL-12 and IL-18 all further enhance Th1 cell commitment (35-39). In the presence of IL-4, naïve T cells express the transcription factor, GATA-3 and differentiate into Th2 cells (40, 41). Th17 cells are induced in the presence of both TGF-β and IL-6 under the control of the transcription factor, RORγt (42-44), although a recent report suggested that TGF-β was not strictly necessary (45). IL-1, IL-21 and IL-23, all downstream of IL-6

signaling, play critical roles in promoting Th17 cell lineage commitment, cytokine production, and survival (46-50).

Finally, subsets of suppressive T cells exist to counteract the inflammatory responses of effector $CD4^+$ helper T cells. Tr1 cells are IL-10^+IFN-γ^+ $CD4^+$ T cells that are derived from classical IFN-γ-producing Th1 cells (51). This subset was found to be crucial for limiting Th1 mediated immunopathology during toxoplasmosis or colitis (52, 53), suggesting that it is a mechanism by which the immune system limits itself. The transcription factor, Foxp3, defines a second class of suppressive T cells known as T regulatory (Treg) cells (54). Natural Treg (nTreg) cells are differentiated in the thymus upon presentation of self-peptides on MHC II molecules expressed on the surface of the thymic cortical epithelium (55, 56). $CD4^+$ T cells expressing TCRs with a high affinity for self-antigens are either deleted or differentiated into Treg cells to prevent systemic autoimmunity (56). Alternatively, $Foxp3^+$ Treg cells can be induced (iTreg) from $Foxp3^-$ naïve $CD4^+$ T cells in the periphery in the presence of TGF-β (57). Treg cells exert immunosuppressive effects via many mechanisms including direct contact with effector cells (58-60), secretion of anti-inflammatory cytokines (61), and consumption of cytokines required for effector cell survival (62).

The cell types introduced in this section, their responses to IFN-1, and their roles in mucosal immunity and inflammation will be discussed in greater detail in the following section.

1.3 The induction of type I interferons

Since their discovery over fifty years ago, interferons have attracted considerable interest. They were thus named because of their principal function in interfering with viral replication (63). More recently, however, the full breadth of their potential functions have begun to be appreciated. Interferons are classified into three groups: type I, II and III. Type I interferon (IFN-1) is encoded by 30 genes in the human and 24 genes in the mouse and consist of multiple subtypes: α, β, ω, τ, ε, κ, μ, and ζ (64). Amongst these subtypes, IFN-α is the most diverse, which includes 13 members in humans and 14 in mice. Despite this diversity, all IFN-1 signals through a common IFNα/β receptor (IFNAR) that consists of IFNAR1 and IFNAR2 subunits. The type II interferon class is composed solely of IFN-γ while type III interferons consist of three λ subtypes. IFN-1 is produced by cells of both hematopoietic and non-hematopoietic origin, during homeostasis as well as infection (65, 66). IFN-γ is produced by $CD4^+$ and $CD8^+$ T cells and natural killer (NK) cells. Finally, the IFN-λs, also termed IL-28A, IL-28B and IL-29, have only recently been discovered (67, 68). Like IFN-1, they are produced by a wide variety of cell types and initiate similar anti-viral programs, but signal via a unique receptor comprised of IL-10R2 and IFNλR1 (69).

IFN-1 is generally the first class of interferons to be induced after a viral infection and can feed back to potentiate the expression of IFN-1, IFN-II and IFN-III by other cells (70-74). Because of their crucial antiviral properties, the primary stimulus for IFN-1 production has traditionally been thought to be a viral product such as RNA. However, recent work has suggested that IFN-1 induction is not limited to only RNA, viral infection or even nucleic acids. As outlined below, they can be induced by foreign and self-

antigens signaling through cell surface, endosomal, and cytosolic receptors during both homeostasis and infection.

1.3.1 Toll-like Receptors

TLRs are perhaps the best characterized PRRs. In both mouse and human cells, IFN-1 was induced by engagement of TLR3 (75-77), TLR4 (76-79), TLR7 (80-83) and TLR9 (76, 82-86). In humans, single-stranded RNA signaled through TLR8 to induce IFN-1 (81) although a similar induction via murine TLR8 has yet to be shown. Conversely, activation of TLR2 heterodimerized with either TLR1 or TLR6 induced IFN-1 in the mouse (87, 88), but a similar role for human TLR2 remains to be demonstrated. Finally, the TLR5 ligand, flagellin, induced IFN-1 from mouse cells, but a specific role for TLR5 has not been shown (89, 90).

The nucleic acid-sensing TLRs (TLR3, -7, -8, and -9) recognize their ligands within endosomes while TLR2, TLR4 and TLR5 can recognize microbial components directly on the cell surface. Consequently, endosomal TLRs tend to recognize phagocytosed or intracellular ligands. TLR3 recognizes double-stranded RNA (dsRNA), which is characteristic of reovirus, rotavirus, and several single-stranded RNA viruses that produce dsRNA as a replication intermediate (75, 91, 92). Signaling via TLR3 on virus-infected cells of both hematopoietic and non-hematopoietic origin induced a protective IFN-1 response (77, 93-97). Furthermore, IFN-1 augmented TLR3 expression (74) on neighboring cells, which then detected dsRNA from phagocytosed apoptotic or infected cells (91). Unlike other TLRs, TLR3 does not signal through the adapter, myeloid differentiation primary response gene 88 (MyD88), but instead relies exclusively on TIR-

domain-containing adapter-inducing interferon-β (TRIF) for IFN-1 production. TRIF then signals through interferon regulatory factor 3 (IRF3) to activate the promoter for IFN-β transcription (98, 99).

The other endosomal TLRs also recognize nucleic acids: guanosine and uridine rich single-stranded RNA by TLR7 and TLR8 (80, 81) and DNA by TLR9 (100). As opposed to the TRIF/IRF3 axis used by TLR3, TLRs -7, -8 and -9 use MyD88 as an adapter, which ultimately activates IRF7 for IFN-β transcription (101). IRF7 is constitutively produced in a subset of cells known as plasmacytoid dendritic cells (pDC) (102-104). Thus, these cells are poised to potently and rapidly induce IFN-1 during viral infections (105-107). Consequently, pDC-depleted mice infected with mouse cytomegalovirus (MCMV) showed a sharp reduction in early IFN-1 production, but equivalent IFN-1 production at later time points (108). Furthermore, this defect in early production correlated with long-lasting effects on viral loads. Although pDCs are an essential cell type for early IFN-1 production and antiviral immunity, other cell types can compensate at later time points (109).

Although TLR7-mediated induction of IFN-1 had originally been restricted to viruses, bacteria in phagosomes targeted for lysosomal destruction can also be degraded to release bacterial mRNA capable of triggering TLR7 in conventional dendritic cells (cDC) (110). Induction of IFN-1 in these cells was completely MyD88 and IRF1 dependent with a synergistic role for IRF7. Furthermore, TLR7 induction of IFN-1 was only observed when bacteria were restricted to the phagolysosome and not when they were able to escape into the cytosol. Group B Streptococcus (GBS) also induced IFN-1 from cDCs

and macrophages via TLR7 signaling, although they engaged IRF1 and IRF3 in lieu of IRF7 (110).

Although TLR9 does recognize dsDNA viruses (85, 86), it was originally discovered to recognize bacterially derived DNA with unmethylated CpG motifs (100). Because unmethylated CpG motifs are infrequent in the vertebrate genome (111), TLR9 was thought to be able to distinguish between self and non-self nucleic acid. Although IFN-1 was induced upon infection with several pathogenic bacteria (112-115), recent work showed that self DNA can also bind to TLR9, but does not usually do so because of the intracellular endosomal location of TLR9 (116).

The cell surface receptor, TLR4, recognizes the bacterial cell wall component, lipopolysaccharide (LPS), but had to be internalized to induce IFN-1 production (117). After endocytosis, TLR4 interacted with TRIF and activated IRF3 for IFN-β transcription as described above for TLR3. Although LPS is the major TLR4 ligand, other ligands capable of inducing IFN-1 via TLR4 do exist, including viral glycoprotein G (118), the FimH adhesion molecule of enterobacteria (119), endogenous ceramide from cell membranes (120), and prothymosin-α, an antiviral protein produced by $CD8^+$ T cells (121).

Activation of TLR2 can also induce IFN-1. TLR2 heterodimerizes with either TLR1 or TLR6. Both *in vitro* and *in vivo*, ligation of either heterodimer with its corresponding bacterially derived agonist induced IFN-1 via IRF1 and IRF7 signaling intermediates (88). Recently, it was discovered that TLR2 also recognized an unspecified component of DNA viruses to induce IFN-1 in a MyD88, IRF3 and IRF7 dependent manner (87). This

effect was specific to Ly6C^{hi} inflammatory monocytes. Finally, acetylated peptidoglycan from the bacterium *Listeria monocytogenes* also engaged TLR2 and induced IFN-1 (122). However, this induction also required TLR3, TRIF and bacterial nucleic acid. Thus, it is possible that TLR2 recognition of peptidoglycan on the cell surface was necessary for bacterial internalization, which made the bacteria accessible for TLR3 pathways of IFN-1 induction.

1.3.2. Cytosolic Detection of Nucleic Acids

Although it is obvious that TLRs can induce IFN-1 to both viral and bacterial ligands, they remain restricted to endosomal compartments while most viruses replicate in the cytosol. Accordingly, the dsRNA virus, rotavirus, induced IFN-1 in a TLR3-independent manner. Instead, two cytosolic sensors for RNA, melanoma differentiation-association protein 5 (MDA5) and retinoic acid-inducible gene 1 (RIG-I), were required for IFN-1 induction in response to rotavirus (123). MDA5 recognizes long dsRNA while RIG-I is a receptor for the 5' triphosphate moieties of ssRNA genomes and short, blunt ended dsRNA (124-126). Induction of IFN-1 through either of these receptors was dependent on the mitochondrial protein, IFN-β promoter stimulator 1 (IPS-1, also known as MAVS, Cardif or VISA) and IRF3 (127-130).

Two other cytosolic sensors are also capable of detecting dsRNA. In response to dsRNA virus stimulation, IFN-α production, but not IFN-β production was impaired in cells that were deficient for protein kinase R (PKR). Expression of PKR was induced by IFN-β, suggesting that PKR was more important for amplifying the IFN-1 response rather than initiating it (131). Secondly, a complex of three helicases, DDX1, DDX21 and DHX36,

was recently shown to detect cytosolic dsRNA and induce IFN-1 via TRIF, IPS-1 and IRF3 (132).

Induction of IFN-1 by foreign DNA can also occur via cytosolic sensors. The model intracellular pathogen, *Listeria monocytogenes*, produces the pore-forming protein, listeriolysin O, to escape from the phagolysosome into the cytosol. Upon treatment with DNAse but not RNAse, *L. monocytogenes* extracts lost the ability to induce IFN-1, implicating bacterial DNA as the causative agent (133). The first specific cytosolic DNA sensor to be identified was DNA-dependent activator of interferon regulatory factors (DAI, previously called DLM-1 or ZBP1). Overexpression of DAI in mouse embryonic fibroblasts or the L929 mouse connective tissue line resulted in earlier and significantly enhanced IFN-1 expression upon stimulation with B-DNA, Z-DNA, bacterial DNA or mammalian DNA (134). These effects were absolutely dependent upon IRF3 and partially dependent on IRF7. DAI may be a redundant sensor since LRRFIP1 (135), IFI16 (136), and the helicases DDX41, DHX9 and DHX36 (137, 138) were all discovered recently to induce IFN-1 in response to cytosolic DNA.

Two recent reports suggested that in lieu of an additional cytosolic DNA sensor, both mouse and human cells have RNA polymerase (pol) III with the ability to transform A/T-rich dsDNA into dsRNA with a 5' triphosphate moiety, making it an ideal ligand for RIG-I (139, 140). Indeed, *in vitro* studies using human cell lines show that siRNA knockdown of RIG-I or IPS-1 or chemical inhibition of RNA polymerase III significantly decreased the ability of the cells to produce IFN-1 in response to synthetic A/T-rich dsDNA. Additionally, pol III may be required for the IFN-1 response against *Legionella*

pneumophila (140), a bacterium that uses a type IV secretion system to inject DNA into the cytosol (133).

Finally, many species of bacteria produce the dinucleotide, cyclic-di-guanosine monophosphate (c-di-GMP), as a second messenger. c-di-GMP is not produced by eukaryotic cells and is thus an ideal molecule to trigger immunity against pathogens. Accordingly, c-di-GMP induced a IFN-1 response by binding directly to DDX41 and/or stimulator of interferon genes (STING) (141-143), an endoplasmic reticulum-bound protein, which is also a signaling molecule downstream of other cytosolic RNA and DNA sensors (144, 145).

1.3.3. NOD-like Receptors

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), including NOD1 and NOD2, are cytosolic receptors recognizing meso-diaminopimelic (meso-DAP) acid and muramyl dipeptide (MDP) respectively. Both are constituents of peptidoglycan, a component of bacterial cell walls. Thus, the NOD proteins are generally considered regulators of bacterial defense. As mentioned earlier, cytosolic DNA from *L. monocytogenes* can induce IFN-1 (133). Surprisingly, MDP signaling via NOD2 enhanced the IFN-β response to cytosolic *L. monocytogenes* DNA (146), suggesting synergy from different bacterial products in inducing the innate immune response. NOD2 also detected ssRNA from respiratory syncytial virus (RSV) and induced IFN-1 in an IPS-1 and IRF3-dependent manner (147). $NOD2^{-/-}$ mice, which have normal RIG-I and IPS-1 function were unable to induce IFN-β, control RSV infection, or control RSV-mediated lung pathology.

1.3.4 Constitutive IFN-1 Production

As described above, IFN-1 can be induced by both viral and bacterial infections. However, IFN-1 was found at low levels in several healthy tissues (148-150) and unlike microbe-induced IFN-1, constitutive production was independent of IRF3 and IRF7 (151). Instead, constitutive IFN-1 production may be dependent on components of the transcripton factors AP-1 and NF-κB (152, 153). Constitutive IFN-1 serves several physiological purposes in priming the antiviral response (154, 155), patrolling tumor development (156, 157), and maintaining homeostatic immune responses (65). However, the ligands responsible for baseline IFN-1 induction remain elusive. Macrophage colony-stimulating factor 1 (CSF-1) induced release of IFN-1 from bone marrow cells (158). As CSF-1 can be found throughout the body, it is a putative inducer of constitutive IFN-1 systemically. Another hypothesis proposes that a specific inducer of constitutive IFN-1 does not exist. Rather, the promoters for IFN-1 transcription may need to be continuously repressed, but intrinsic leakiness leads to constitutive low-level transcription of IFN-1 genes (155).

Constitutive IFN-1 could also potentially be triggered by endogenous ligands that signal via the pathogen recognition receptors detailed above. Prothymosin-α produced by $CD8^+$ T cells (121), self nucleic acids (116, 134, 159-161), apoptotic cells carrying self-antigen (162, 163), and the cell membrane structural component, ceramide (120), have all been shown to induce IFN-1. Foreign nucleic acid from non-pathogenic gut-resident bacteria also triggered IFN-1 induction (164), but its role in inducing constitutive IFN-1 is unclear.

In fact, germ-free mice actually have increased transcription of several genes downstream of IFN-1 signaling (165).

In conclusion, viral, bacterial and endogenous ligands have all been shown to induce IFN-1. The mechanisms of induction may be dependent on TLRs, NLRs, RLRs or cytosolic sensors depending on the cell type (Fig 1-1). This diversity allows for recognition of many different pathogens with different routes of entry into a cell. The induction of IFN-1 in response to so many stimuli, both pathogenic and non-pathogenic, suggests it may have many different functions depending on cell-type and context.

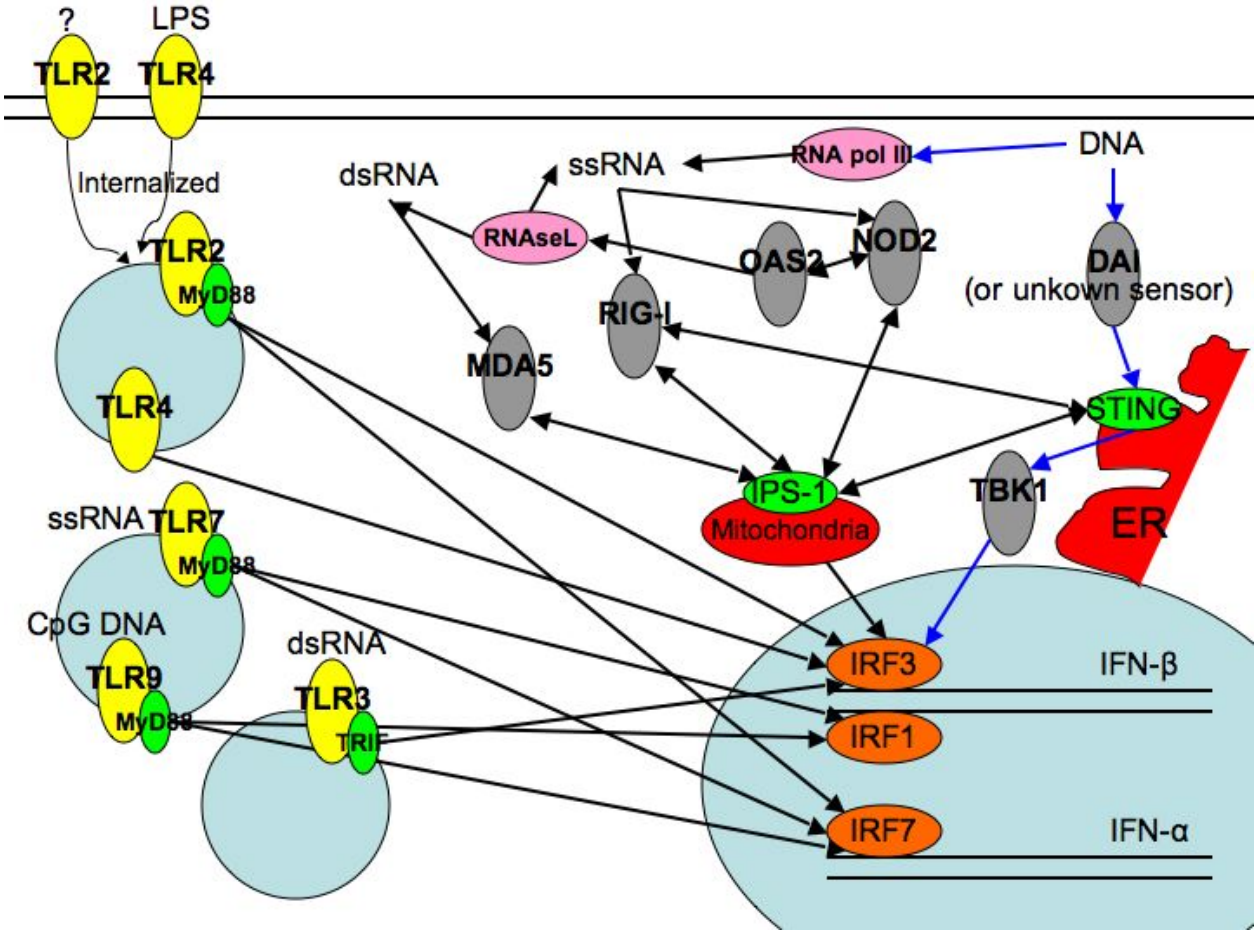


Figure 1-1 The induction of type I interferons

1.4 Type I interferon signaling

Despite the many subtypes of IFN-1 produced by both mouse and human cells, only one receptor, the interferon α/β receptor (IFNAR), is responsible for all IFN-1 signaling. IFNAR is composed of two transmembrane subunits: IFNAR1 and IFNAR2. Both IFNAR1 and IFNAR2 belong to the class II helical cytokine receptor family. IFNAR1 is found in only one isoform, while IFNAR2 can be found in a long transmembrane isoform and a soluble isoform (166). In humans, IFNAR2 can also be found as a short transmembrane isoform, which may act as a dominant negative repressor of IFN-1 responses rather than a signaling subunit (167). For the remainder of this thesis, IFNAR2 will refer to the long transmembrane isoform with cytoplasmic signaling domains. High affinity binding of IFN-1 to the IFNAR1/IFNAR2 heterodimer is necessary for full effectiveness, although signaling via IFNAR2 homodimers can result in suboptimal levels of antiviral activity (168, 169). Additionally, different IFN-1 subtypes bind IFNAR1 and IFNAR2 with different affinities, which may correlate with their ability to effect either anti-proliferative or antiviral responses (170).

IFNAR lacks intrinsic kinase properties and instead relies on Janus-associated kinases (JAKs) for signaling. IFNAR1 is associated with tyrosine kinase 2 (TYK2) while IFNAR2 associates with JAK1 (171). Upon engagement of the receptor, the kinases autophosphorylate and subsequently phosphorylate different signal transducer and activation of trascription (STAT) proteins. The STAT proteins are able to translocate to the nucleus and bind the promoters of different interferon-stimulated genes (ISGs). STAT1 and STAT2 can heterodimerize and associate with IRF9 to form what is known as ISG Factor 3 (ISGF3). ISGF3 then binds to specific regions on promoters for ISGs known as interferon-stimulated response elements (ISREs).

Engagement of IFNAR can result in homodimerization and heterodimerization of various combinations of STAT proteins, which also bind to regions in the promoters of ISGs. STAT1 can homodimerize or heterodimerize with STAT2 in the absence of IRF9 and still initiate transcription of ISGs (172). One way that IFN-1 can have different effects in different cells is via the cell-specific expression of different STAT proteins. For example, IFN-1 activation of STAT1 results in inhibition of cell growth (173) as well as inhibition of IFN-γ production by NK and T cells (174). However, IFN-γ is a crucial part of the antiviral response. In response to lymphocytic choriomeningitis virus (LCMV), antigen-specific $CD8^+$ T cells upregulate their expression of STAT4 while non-specific $CD8^+$ T cells express higher levels of STAT1 (175). STAT4 not only antagonizes STAT1 induction and its downstream anti-proliferative effects, but is also necessary downstream of IFNAR for IFN-γ production during viral infection (71, 175). As another example, IFN-1 activates STAT3 homodimers in $CD8^+$ T cells and neutrophils to promote cell survival (176, 177), but IFN-1-induced STAT3 homodimers in B cells promote apoptosis (178). IFN-1 activates STAT5 in both $CD8^+$ T cells as well as monocytes (176, 179), and STAT6 forms a complex with STAT2 and IRF9 to initiate transcriptional programs exclusively in B cells (180). Once inside the nucleus, STAT dimers can interact with other transcriptional regulators, providing an additional level of complexity in understanding the cell- and context-specific effects of IFN-1 (171).

IFN-1 signaling also activates alternative transcription factors. Via a STAT3 and phosphatidylinositol 3-kinase (PI3K)-dependent mechanism, IFN-1 causes the dissociation of the transcription factor, NF-κB, from its constitutively bound repressor, IκBα (181, 182). In parallel, IFN-1 induces TRAF2 and NF-κB-inducing kinase (NIK)-

dependent processing of NF-κB precursors to their active forms (183). Both of these pathways are necessary for IFN-1-induced activation of NF-κB, which counterintuitively suppresses the antiviral immune response (184). The other major pathway activated by IFNAR signaling is the mitogen-activated protein kinase (MAPK)/p38 signaling cascade (185). Activation of this pathway is also necessary for ISRE-dependent gene transcription. However, MAPK/p38 signaling is not necessary for the phosphorylation of STAT proteins, suggesting that these independent signaling cascades operate in concert to tightly regulate IFN-1-stimulated gene transcription (185).

Thus, these results highlight the complexity of IFN-1 signaling. Depending on the relative expression of various transcription factors within different cell types, IFN-1 can exert pleiotropic effects. Some cell types are only capable of expressing certain transcription factors, but other environmental factors can change the composition of transcription factors in a cell, thus regulating the effect of IFN-1 in a cell- and context-dependent manner (186).

1.5 The effects of type I interferons

1.5.1 Anti-viral activities

The antiviral activities of type I interferons have been known since their discovery in 1957 (63). In this seminal study, influenza A was incubated with pieces of chicken chorio-allantoic membrane. After as early as two hours after incubation, a soluble factor was released into the surrounding media, which interfered with viral replication. The release of this factor, termed interferon, continued for at least 24 hours after infection. Nearly 40 years after the discovery of IFN-1, mice deficient in IFNAR were generated (187). Mice lacking IFNAR succumbed quickly to vesicular stomatitis virus (VSV), reovirus, and Semliki Forest virus and were unable to control titers of vaccinia virus and lymphocytic choriomeningitis virus, proving a generalized necessity for IFN-1 in the response against viruses *in vivo* (187, 188). Additionally, depletion of pDCs capable of producing high levels of IFN-1 significantly impaired the early response against murine cytomegalovirus and VSV (108).

Protein kinase R (PKR) is one of the main antiviral proteins induced by IFN-1 (189). PKR is activated by cytoplasmic dsRNA (189), and activated PKR phosphorylates the protein translation factor, eukaryotic initiation factor (eIF)-2 (190). Phosphorylated eIF-2 is inactive and cannot exchange GDP bound to eIF-2 for GTP, a process that is necessary for eIF-2 to facilitate tRNA binding to ribosomes (191). Viruses rely on host cell machinery for the translation of viral proteins, so by this mechanism PKR shuts down both host and viral protein production in virally infected cells. Activation of PKR is dependent on dsRNA with 5'-triphosphates, which are common in viral DNA, but absent

from mammalian DNA (192). This specificity may have evolved to allow an immediate and drastic response against a pathogen without triggering a shutdown of cellular processes upon detection of self-nucleic acid. PKR provides non-redundant antiviral defense, as mice deficient in PKR are more susceptible to several different viruses (193, 194).

IFN-1 also induces the enzyme, 2',5'-oligoadenylate synthetase (OAS) (195). OAS generates oligoadenylates ranging from 3 to 12 nucleotides in length (196). The oligoadenylates then activate the ribonuclease, RNAse L, which is responsible for degradation of messenger RNA (197), primarily at sites containing uracil (198, 199). Thus, like PKR, the OAS pathway also inhibits the translation of mRNA into protein. The OAS pathway also shows specificity for viral nucleic acids rather than mammalian nucleic acids. First, while OAS is constitutively expressed in many cells (200), its expression is enhanced by up to 100,000 times upon stimulation with IFN-1 induced during an infection (196). Secondly, RNAse L preferentially cleaves mRNA that is linked to dsRNA (201). dsRNA is an intermediate of viral replication, which occurs in the cytosol, while eukaryotic host cell replication occurs in the nucleus and does not involve a dsRNA intermediate. *In vivo*, mice deficient in RNAse L showed impaired resistance to encephalomyocarditis virus (202).

Another key antiviral pathway induced by IFN-1 involves the Mx GTPases (203). MxA in humans and Mx1 in mice are the best-characterized members of this family. MxA localizes to the cytoplasm and binds the nucleocapsid proteins of orthomyxoviruses, preventing their transport into the nucleus, a step necessary for replication of this virus (204). Mx1, on the other hand, is already localized to the nucleus, directly binds viral

polymerase, and prevents primary viral transcription (205). Ectopic expression of Mx proteins in transgenic mice conferred resistance to not only the orthomyxoviruses, influenza and thogotovirus, but also the bunyavirus, La Crosse virus, and the togavirus, Semliki Forest virus (206).

The final IFN-1-inducible pathway discussed in this section is dependent on ISG15. ISG15 is an ubiquitin homologue and like ubiquitin, conjugates itself to many different cellular proteins (207). Unlike ubiquitylation, ISG15 conjugation does not mark proteins for degradation, but rather is postulated to activate them (208). ISG15 acts in concert with other IFN-1-induced antiviral proteins such as PKR and the Mx proteins described above to potentiate the immune response (208, 209). Furthermore, ISG15 can be secreted by virus-infected myeloid and lymphoid cells, allowing it to potentially prime uninfected neighboring cells for an impending viral assault (210).

Although these four pathways were highlighted here, IFN-1 induces thousands of genes (211). Most of these genes have an unidentified function and not all have antiviral effects.

1.5.2 Regulation of cell growth and apoptosis

IFN-1 is also linked to limiting cell growth, both via inhibition of proliferation and induction of apoptosis. RNAse L and PKR, mentioned above, can shut down host cell protein synthesis, although they preferentially target viral nucleic acids. Inhibiting cellular protein synthesis can greatly compromise cell function and may drive cells into apoptosis. Accordingly, mice lacking RNAse L or PKR are resistant to apoptosis (202, 212).

IFN-1 can also exert control over cell growth independent of the classic antiviral proteins. The life cycle of a cell can be divided up into three phases: interphase, in which a cell grows and duplicates its genetic material; mitosis, in which the cell divides into two; and G_0, a resting phase. Interphase is composed of two growth phases, G_1 and G_2, with a DNA synthesis S phase sandwiched in between. Protein conjugates of cyclins bound to cyclin-dependent kinases (cdk) regulate the cell's progression through these phases. IFN-1 has been shown to induce cdk inhibitors that cause cell cycle arrest in primarily the G_0 and G_1 phases, preventing both DNA replication and cell division (213-215). The expression of many cyclins is dependent on the transcription factor, c-myc. IFN-1 reduces both the transcription and translation of c-myc, providing another mechanism of inducing cell cycle arrest (216-218). Finally, the retinoblastoma protein (pRB) is crucial for preventing progression of a cell from G_1 to S. Once ready for replication, cyclin-cdk complexes phosphorylate pRB, inactivating it, and permitting entry into S phase. IFN-1 both increases the intracellular levels of pRB and keeps pRB active by preventing its phosphorylation and inducing its dephosphorylation (219-221).

As mentioned earlier, IFN-1 can drive cells into apoptosis. Many ISGs including Fas, TNF related apotosis-inducing ligand (TRAIL), caspase-4, and caspase-8 are known members of the apoptotic death cascade (211, 222). Fas and TRAIL are cell surface death receptors that activate caspase-8 and a cascade of downstream caspases, and trigger apoptosis. Via this "cell-extrinsic" pathway, IFN-1 can serve as a harbinger of danger and sensitize cells to external pro-apoptotic signals. However, IFN-1 can also initiate a "cell-intrinsic" pathway of apoptosis. In this pathway, internal stress signals lead to the upregulation of pro-apoptotic and downregulation of anti-apoptotic members of the Bcl-2

family. The pro-apoptotic members, including Bax and Bak, form pores in mitochondria, leading to the release of cytochrome c (223). Release of cytochrome c, in turn, stimulates the formation of an "apoptosome" and the activation of executioner caspases (224). IFN-1 feeds into this pathway by promoting the activation of Bax and Bak and downregulating the activation of the anti-apoptotic Bcl-2 members (225, 226). IFN-1 can also lead to Bcl-2 family-independent release of cytochrome c (227). These effects of IFN-1 on cellular proliferation and death have led to the use of IFN-1 as a therapeutic for a wide variety of human cancers of both hematopoietic and non-hematopoietic origin.

The role of IFN-1 in induction of apoptosis is not definitive, however. Depending on the cell-type and context, IFN-1 can have the opposite effect and promote survival of cells. For example, exposure to IFN-1 decreased expression of the anti-apoptotic genes, Bcl-2 and Bcl-x_L, in $CD8\alpha^+$ DCs (228), but increased expression of the same genes in the same cell type when the DCs were loaded with antigen (229). Likewise, IFN-1 signaling inhibited activation-induced cell death of $CD4^+$ and $CD8^+$ T cells *in vitro* (230). *In vivo*, IFN-1 acted directly on both $CD4^+$ and $CD8^+$ T cells to enhance survival during clonal expansion in response to viral infection (231, 232).

1.5.3 Immunomodulation

Because nearly every cell type in the body expresses IFNAR, the effects of IFN-1 on the immune system are as diverse and complex as the immune system itself. Prior to discussing the effects of IFN-1 on each individual cell type, it must be noted that IFN-1 also has global effects by regulating hematopoietic stem cell (HSC) homeostasis. HSCs

are in a default dormant state, but upon inflammation or other injury to the immune system, HSCs proliferated in a IFN-1-dependent manner to re-establish homeostasis (233).

1.5.3.1 Effects on the innate immune system

Dendritic cells develop by a pathway in which HSCs differentiate via several precursors into pDCs, pre-cDCs or monocytes. IFN-1 promoted the differentiation of both pDCs and monocytes into potent inflammatory DCs (234-237). However, when in the presence of GM-CSF and IL-4, IFN-1 inhibited monocyte differentiation into mature DCs (238). Additionally, virus-induced IFN-1 inhibited the progression of DC precursors to DCs (239). IFN-1 has pleiotropic effects depending on the stage of cell development and inflammatory context. Further work must be conducted to determine the specific effects of IFN-1 on each cell-type in various contexts to understand their physiological relevance.

IFN-1 also controls cytokine production from differentiated myeloid cells. IFNAR signaling on monocytes and macrophages suppressed TNF-α (240) and induced a trio of anti-inflammatory cytokines: IL-10, IL-27 and IL-1 receptor antagonist (IL-1RA) (241-243). Two key cytokines produced by DCs during T cell priming are IL-12 and IL-23, both of which were inhibited by IFN-1 (244-246). Although IL-12 enhances Th1 cell differentiation, effects of IFN-1 on Th1 cells were independent of IL-12 and involved induction of several other Th1-polarizing cytokines including IL-15 and IL-18 (235, 237). IL-23 enhances the survival and expansion of Th17 cells (42, 47). Accordingly, IFN-1 treated DCs primed a smaller population of Th17 cells (246). IFN-1 also induced IL-27 from DCs, which further inhibited Th17 cell differentiation via a mechanism involving intracellular osteopontin (247). IFNAR signaling on DCs also enhanced B cell responses

(248). This was in part due to IFN-1 induction of IL-6 secretion from DCs, which promoted the differentiation of T follicular helper cells, a population of $CD4^+$ cells that provide help for B cell antibody responses (249).

IFN-1 plays a key role in regulation of IL-1β secretion. IL-1β is produced in a "pro" form, which must then be cleaved by caspase-1 to produce the active form. Caspase-1 is activated by a multimeric complex termed the inflammasome. Several distinct inflammasome complexes responding to various stimuli have been identified. In response to ATP, alum, or nigericin, for example, the NLRP3 inflammasome activates caspase-1. By reducing both intracellular pro-IL-1β levels as well as pro-IL-1β cleavage, IFN-1 inhibited the NLRP3 inflammasome (250). This inhibitory effect of IFN-1 on NLRP3-induced IL-1β production was shown to be physiologically relevant in *in vivo* models of *Mycobacterium tuberculosis* and *Candida albicans* infection as well as in human multiple sclerosis (250, 251). IFN-1 also inhibited the NLRP1 inflammasome, which recognizes anthrax toxin (250). Curiously, IFN-1 was found to be essential for NLRP3 inflammasome activation in response to gram-negative bacteria such as *Citrobacter rodentium* (252). *C. rodentium* as well as other bacteria such as *E. coli* and *V. cholera* require caspase-11 for NLRP3 activation (253). IFN-1 was found to be necessary for caspase-11 activation (252). Finally, IFN-1 also enhanced activation of the AIM2 inflammasome in response to *Francisella tularensis* infection (254). Thus, IFN-1 has different effects on IL-1β secretion depending on the stimulus. This distinction may have evolved to allow IFN-1 to enhance immunity against select pathogens while restricting sterile inflammation caused by self-derived ligands such as ATP or monosodium urate crystals, both of which activate the caspase-11-independent NLRP3 inflammasome. Other pathogens may have co-evolved to exploit this latter function of IFN-1.

IFN-1 also affects the microbicidal functions of innate immune cells. Blockade of IFN-1 led to a loss of phagocytic potential in macrophages (255). IFNAR signaling on natural killer (NK) cells enhanced their production of IFN-γ (14, 71), and IFN-γ signaling on macrophages enhanced their bactericidal properties as well (14). Despite these immune activating effects, IFN-1 signaling on macrophages downregulated surface expression of the IFN-γ receptor (256), perhaps as a way of balancing the immune response.

1.5.3.2 Effects on the adaptive immune system

Data discussed in the previous section suggested that IFNAR signaling on myeloid cells could regulate lymphocyte responses. In an *in vivo* model of autoimmune encephalitis, IFN-1 decreased MHC II expression on myeloid cells to suppress $CD4^+$ T cell-mediated pathology, but IFNAR signaling on B or T cells had no such effect (257). Also, direct stimulation of DCs by IFN-1 enhanced cross-presentation of internal antigens on MHC I molecules and enhanced IFN-γ production from $CD8^+$ T cells (258).

In addition to these indirect effects, direct effects of IFN-1 on B and T cells have been demonstrated under various circumstances. For example, cytokine production by B cells in response to TLR ligands required cell-intrinsic IFNAR signaling (259). Proliferation of B cells in response to TLR7 stimulation was also dependent on cell-intrinsic IFNAR signaling (259). Moreover, direct activity of IFN-α on naïve B cells triggered IFN-γ secretion via the transcription factors, STAT4 and T-bet (260). B cell effector responses were found to reduce bacteremia and protect mice from mortality in a model of sepsis (261). Thus, transfer of WT B cells rescued septic $RAG^{-/-}$ mice from death, but no such

benefit was observed upon transfer of IFNAR1$^{-/-}$ B cells (261). Direct IFN-1 signaling on B cells enhanced their expression of the activation markers, CD69 and CD25 (262). In addition, B cells can sometimes present antigen and provide co-stimulation to T cells. IFN-1 was found to enhance expression of the co-stimulatory molecule, CD86, and migration of CD86^{hi} B cells to the T cell-B cell border (262, 263).

IFN-1 signaling on B cells also influences antibody production. Naïve B cells are activated in the periphery via recognition of antigen by their B cell receptor. Activated B cells next migrate to germinal centers in secondary lymphoid organs, where they receive CD40L co-stimulation signals from CD4^{+} helper T cells, transition into plasmablasts, undergo class switch recombination (CSR), and differentiate into potent antibody-secreting plasma cells. As mentioned earlier, IFN-1 stimulated differentiation of CD4^{+} T follicular helper cells, which are specialized for providing B cell help (249). IFN-1 also acted on B cells to aid in the transition from activated B cell to plasmablast (264). CSR, a process by which B cells switch the heavy chain of the antibodies they secrete, is important for dictating the tissue- or context-specific effector functions of antibodies. IFN-1 activity on B cells was found to enhance antibody responses by facilitating germinal center formation and CSR (265, 266). *In vivo*, induction of effector plasma cells by IFN-1 was found to exacerbate pathology in lupus-prone mice (267).

IFNAR signaling on CD4^{+} and CD8^{+} T cells can enhance many of their effector functions. In addition to requirements for IFNAR signaling on DCs and B cells for antibody responses, IFN-1 was also essential on CD4^{+} T cells to provide help to B cells (266). On CD4^{+} and CD8^{+} T cells, IFN-1 induced IFN-γ production (71, 268). This was dependent on induction of STAT4 and T-bet as was also observed after IFNAR signaling on NK and

B cells (71, 260, 269). IFN-1 also increased IL-12R on naïve $CD4^+$ T cells, making them more responsive to the Th1-inducing cytokine, IL-12 (270). IFN-1 synergized with IL-12 and IL-18, another Th1-polarizing cytokine, to enhance $CD4^+$ T cell production of IFN-γ (271, 272). Concomitantly, IFN-1 inhibited the development and cytokine secretion of Th2 cells, which normally produce IL-4, IL-5 and IL-13 (273).

IFN-1 induced the activation marker, CD69, on B cells and $CD4^+$ and $CD8^+$ T cells, although this did not always result in enhanced effector function (274, 275). IFN-1-induced CD69 inhibited expression of the receptor, S_1P_1, on lymphocytes (274). S_1P_1 is required for lymphocyte retention in the lymph nodes, which is thought to increase B and T cell interactions with antigen-presenting cells in the lymph node. However, T cells pre-exposed to IFN-1 displayed decreased proliferative responses upon subsequent TCR ligation, despite upregulation of CD69 (275). Furthermore, IFN-1 reduced the colitogenic potential of naïve $CD4^+$ T cells in a CD69-dependent manner in a mouse model of colitis (276, 277). Although T cells expressing STAT4 responded to IFN-1 with increased IFN-γ production (71), T cells with a greater relative expression of STAT1 decreased their production of IFN-γ in response to IFN-1 (174). STAT1 was expressed on resting T cells and was downregulated upon antigen recognition, while STAT4 was enhanced in antigen-responsive T cells (175, 278). Thus, IFN-1 may have inhibitory effects on naïve T cells, while potentiating the immune response in activated T cells already responding to a pathogen.

Finally, IFN-1 also had effects on immunosuppressive T cells. IFN-1 enhanced the differentiation of IL-10^+IFN-γ^+ Tr1 cells in both mice and humans (279, 280). IFN-1 also

expanded the population of Foxp3$^+$ Treg cells, without affecting their initial differentiation (281).

1.5.3.3 Immune cell recruitment

IFN-1 is also able to tune the immune response by controlling chemokine-dependent migration of immune cells. IFN-1 induced CXCL10 from hematopoietic cells and intestinal epithelial cells (282-284). CXCL10 recruits neutrophils, CD8$^+$ T cells and other inflammatory cells to mediate protection against bacteremia and intestinal *Helicobacter pylori* infection. In contrast, IFN-1 inhibited the secretion of two other neutrophil-attracting chemokines, CXCL1 and CXCL2, from colon and lung hematopoietic cells (285, 286). IFN-1 inhibition of neutrophil recruitment led to less severe acute colitis, but an increased susceptibility to post-influenza secondary bacterial infection. IFN-1 also recruited various monocyte/macrophage populations via the induction of CCL2 and CCL5 (285, 287, 288). Because monocytes and macrophages are functionally diverse cells, induction of these chemokines by IFN-1 has physiologically diverse effects. In the colon, recruited monocytes served wound-healing functions to aid in recovery from acute colitis (285). In the spleen and blood, CCL2 stimulated monocyte egress from the bone marrow, which was protective during *Listeria monocytogenes* infection (287). Monocytes recruited by IFN-1 to the lungs, however, served as an intracellular host for *Mycobacterium tuberculosis*. Consequently, *in vivo* induction of IFN-1 in a mouse model led to increased pulmonary bacterial loads and worse pathology (288).

In conclusion, IFN-1 is able to have pleiotropic, often opposing roles on the immune system by triggering different signaling pathways and effector functions in different cells.

The activation status of a cell can change the expression of signaling molecules and transcription factors within the cell, leading to even more diversity in the IFN-1 response.

1.6 Intestinal homeostasis

The intestinal tract forms a significant barrier between the outside environment and the internal anatomy. The intestinal immune system faces the daunting task of determining which antigens are beneficial or harmless and which pose a threat to human health. Ingested food, for example, provides nutrients and induces metabolites that are necessary for physiology, including development of the immune system (289). Commensal bacteria, despite possessing classic pathogen-associated molecular patterns (PAMPs) that can trigger an immune response, are necessary for epithelial cell homeostasis (290). Recognition of commensals via TLRs on epithelial cells induced the expression of several cytokines and heat-shock proteins that protected the intestinal epithelium from injury. However, commensal bacteria that gain access to the tissue beyond the epithelium are still able to induce inflammatory responses (291). The intestine also provides an attractive route of entry for many invasive pathogens. Thus, the intestinal immune system must be able to discriminate between benign and pathogenic antigenic stimulation. In this section, I discuss mechanisms by which this distinction is achieved, the consequences of a failure to induce tolerance, and the known functions of IFN-1 in mucosal immunology.

1.6.1 The mucosal barrier

A physical separation between bacteria in the intestinal lumen and cells of the human body provides the first line of defense. In the colon, a double layer of mucus covers the intestinal epithelium, and the inner of the two layers is devoid of bacteria (292). This physical segregation was dependent on the mucin, Muc2, since bacteria were found to be in physical contact with the epithelium in $Muc2^{-/-}$ mice (292). A similar zone of minimal bacterial colonization in the small intestine was dependent on the antibacterial peptide,

RegIIIγ (293). Detection of bacteria by Paneth cells, a class of secretory epithelial cells, induced the production of RegIIIγ, but also other antimicrobial peptides including RegIIIβ, RELMβ, CRP-ductin, cryptdins and defensins (294). Paneth cells are also activated indirectly by ILC-derived IL-22 (295). Mice in which Paneth cells could not detect bacteria displayed systemic bacterial spread (294). Furthermore, a loss of enteric defensins secreted by Paneth cells resulted in significant alterations to the composition of the microbiota (296).

Underneath the mucus, a single layer of epithelial cells connected by tight junctions and adherens junctions forms a wall to prevent infiltration of commensal bacteria (297). Adherens junctions are necessary for communication between adjacent cells. Tight junctions, composed of claudins, occludins, actins and other membrane proteins, regulate paracellular transport of antigens. At least two distinct pathways exist that allow for transport of solutes of a specific size and charge to pass through. For example, while LPS is able to cross the tight junction, whole bacteria cannot (297).

1.6.2 Mucosal immunity

If barrier defenses are breached, additional mechanisms employed by the immune system exist to prevent further spread of the infiltrating bacteria. For example, in the absence of RegIIIγ, commensal bacteria colonized the intestinal epithelium, leading to inflammatory responses mediated by IgA-secreting B cells and IFN-γ-secreting $CD4^+$ T cells (293). Other studies have similarly shown that bacteria that gain access to the intestinal tissue can elicit an inflammatory immune response (291).

Many bacterial species gain access to the mucosal immune system via microfold (M) cells over Peyer's patches, lymphoid structures interspersed within the small intestinal epithelium (5). In these lymphoid organs, antigen is presented to B and helper T cells, which induces a population of mucosal B cells specialized for the production of secretory IgA (sIgA). sIgA is transient and highly specific to the composition of the commensal microbiota at the time (298), but was nevertheless essential for inhibiting penetration of commensal bacteria to the mesenteric lymph nodes (MLNs) (299). Although it is not entirely clear how sIgA restricts spread of commensal bacteria, sIgA-bound antigens may be more easily trapped in the mucus separating the epithelium from the intestinal lumen, or transcytosis in infected intestinal epithelial cells may expel sIgA-bound bacteria more efficiently (300).

The intestinal epithelium is also interspersed with different subsets of $CD4^+$ and $CD8^+$ T cells. TCRαβ intraepithelial lymphocytes (IEL) are like other T cells in that they recognize foreign antigens presented on MHC class I or class II molecules (301). TCRαβ $CD8\alpha\alpha^+$ T cells, on the other hand, are MHC I-restricted and recognize self-antigens for induction of a tolerogenic response (301). These cells can also be activated by viral antigens, but still respond in a tolerogenic rather than immunogenic manner (302). The antigen-specificity of TCRγδ IELs is currently debated, but these cells do not require their TCR for activation. Instead, they can respond to innate sources of IL-1 and IL-23 to produce IL-17 (303, 304). TCRγδ IELs also secrete antimicrobial peptides within the first few hours of bacterial contact, suggesting that these cells are crucial mediators of the innate immune response against bacteria (305).

In the lamina propria (LP), both macrophages and DCs contribute to immunity against pathogens. Antigens reached the LP either via antigen uptake by CX_3CR1^+ cells extending dendrites into the lumen (306) or via a lumen to LP transport system present in specialized mucus-secreting epithelial cells (307). Via mechanisms that are not currently fully elucidated, antigens in the LP are delivered to $CD103^+$ DCs, which migrate to the MLNs and prime protective T cell responses. $CD11b^+CD103^+$ DCs also responded to flagellin via TLR5 and produced IL-23, which sequentially induced IL-22 and RegIIIγ to enhance mucosal barrier defense (308).

Intestinal macrophages provided innate immune protection against invading pathogens by several diverse mechanisms. The primary form of bacterial killing by macrophages involves phagocytosis and an oxidative burst as mentioned earlier. The oxidative burst cascade produces free radicals, hydrogen peroxide and hypochlorite, which degrade intracellular bacteria, but may also cause tissue damage. MLN and peritoneal macrophages used a TRIF-dependent signaling pathway to secrete IFN-β in response to bacterial recognition (14). IFN-β, in turn, induced IFN-γ from NK cells, which fed back onto macrophages to enhance microbicidal activity. Whether this pathway is physiologically relevant in macrophages isolated from the LP remains to be determined. Second, CX_3CR1, a macrophage surface marker, serves a functional role in innate immune defense as well. Intestinal macrophages supported IL-22 production by ILCs in a CX_3CR1-dependent manner (309). Consequently, CX_3CR1-deficient mice were more susceptible to gastrointestinal pathogens and displayed translocation of bacteria to systemic sites (309, 310). Third, alternatively-activated macrophages in the gut provided immunity against gastrointestinal nematodes in an IL-4 and arginase-1-dependent manner (311). Finally, gut macrophages expressed high levels of pro-IL-1β, the precursor to the

inflammatory cytokine IL-1β (312). Upon recognition of cytoplasmic flagellin or components of bacterial type III secretion system by the NLRC4 inflammasome (313-315), caspase-1 is activated leading to cleavage of pro-IL-1β to its active form. As cytoplasmic flagellin or a type III secretion system are characteristic of invasive bacteria, the NLRC4 inflammasome in intestinal macrophages was able to discriminate between commensal and pathogenic bacteria (312). Thus, these data show that despite hyporesponsiveness to canonical TLR signaling, intestinal macrophages utilize other signaling pathways to provide innate immune defense against invading pathogens.

$CD4^+$ T cell subsets are not directly microbicidal, but enhance innate immune mechanisms. Th1 cells, for example, secrete IFN-γ, which is necessary for activation of macrophage intracellular killing. IFN-γ was necessary for nitric oxide and superoxide production as well as MHC expression on macrophages (316). Consequently, mice with deficiencies in IFN-γ production succumbed to the intracellular pathogen, *Mycobacterium tuberculosis* (317), and mice deficient in Th1 lineage commitment were unable to control growth of intracellular *Leishmania major* (318). Th2 cells secrete IL-4, which alternatively activates macrophages, but also recruits other innate cell populations such as mast cells, eosinophils and basophils for immunity against extracellular nematode infections (319). Th17 cells secrete both IL-17 and IL-22, among other cytokines. As mentioned earlier, IL-22 enhances antimicrobial peptide secretion from Paneth cells. IL-17 production has been associated with protection against gastrointestinal pathogens such as *Helicobacter pylori* (320), *Citrobacter rodentium* (321), and *Salmonella typhimurium* (322). The effects of IL-17 are partially due to induction of chemokines recruiting neutrophils, which also possess phagocytic and microbicidal activity (320, 323).

The MLNs provide the final barrier to systemic spread of gut bacteria. In mice with surgically excised MLNs, live bacteria residing within migratory DCs spread to systemic sites such as the spleen (299). Several populations of innate immune cells residing or recruited to the MLNs were crucial in preventing escape of commensal bacteria from the mucosal immune compartment. ILCs in the MLNs produced IL-22, which induced antimicrobial peptides (17). In addition, antigen-carrying myeloid cells initiated a cascade of antimicrobial effects in the MLNs (324). Inflammasome-dependent IL-18 from myeloid cells induced IFN-γ from innate $CD8^+$ lymphocytes and NK cells. IFN-γ activated macrophages microbicidal activity and recruited phagocytic neutrophils to the MLNs.

1.6.3 Mucosal tolerance

Tolerance in the intestines can be broken up into two distinct groups: oral tolerance to ingested soluble antigens and a finely tuned immune response against commensal bacteria that prevents systemic spread and inflammation, without causing immunopathology. Treg cells have a key role in both types of tolerance.

Soluble protein antigens that enter the body via the intestinal tract are often subjected to a phenomenon known as oral tolerance. Innocuous food antigens, which typically do not contain MAMPs, induce tolerogenic responses in both mucosal and systemic sites. For example, keyhole limpet hemocyanin (KLH), a protein derived from sea snails, induces potent immune responses, but oral administration of KLH to humans reduced T cell proliferation upon subsequent intradermal immunization with KLH (325). Using the model food antigen, ovalbumin (OVA) derived from chicken egg whites, oral tolerance

was found to require transport of the antigen from the intestinal tissue to the MLNs by DCs (326). In the MLNs, antigen-carrying $CD103^+$ DCs induced $Foxp3^+$ Treg cells in a TGF-β- and retinoic acid-dependent manner (4, 6). $Foxp3^+$ Tregs then homed back to the gut, expanded in response to IL-10 secreted by intestinal macrophages, and finally disseminated systemically to suppress T cell responses to orally ingested antigen at extra-intestinal sites (327). OVA feeding also induced $Foxp3^-$ Tr1 cells (53). Furthermore, OVA-specific naïve or effector T cells failed to induce colitis in mice fed OVA in their drinking water (328).

The tolerogenic responses induced by OVA were abrogated when OVA was expressed by *Escherichia coli*, however. Naïve and effector OVA-specific T cells both induced colitis when transferred into mice colonized with *E. coli*-OVA, but not in mice colonized with control *E. coli* (328, 329). These results suggest that when coupled together, signals derived from microbiota inhibit tolerance normally induced against oral antigen. Furthermore, activation of DCs with CpG oligonucleotides, characteristic of microbial DNA, was required for generation of effector T cells and suppressed differentiation of regulatory T cells (21, 330).

Although DNA from some commensal bacteria inhibited Treg cell induction (330), there are many lines of evidence suggesting that specific commensal bacterial also play a key role in Treg cell induction. While CpG-containing DNA inhibited naïve T cell conversion to Treg cells, DNA from *Lactobacillus paracasei* reversed that inhibition (331). In the same study, immunosuppressive *L. paracasei* DNA enhanced peripheral Treg cell conversion during *Toxoplasma gondii*-induced gastrointestinal inflammation. Germ-free (GF) mice, marketed as free of any bacteria, parasites or exogenous viruses, have a

drastic reduction in the frequency of Foxp3$^+$ Treg cells (332). Colonization of GF mice with *Clostridium* species or a defined cocktail of commensal bacteria (altered Schaedler flora; ASF) reversed the absence of Foxp3$^+$ Treg cells (332, 333). *Clostridium* species, especially, were potent inducers of IL-10$^+$Foxp3$^+$ Treg cells, perhaps due to their ability to induce TGF-β, an essential cytokine in peripheral conversion of Foxp3$^-$ to Foxp3$^+$ Treg cells (57, 332). *Helicobacter hepaticus* also induced IL-10$^+$CD4$^+$ T cells, which were essential in preventing pathology due to an otherwise benign bacterial species (334).

The above results suggest that bacteria contribute to intestinal tolerance by promoting induced Treg (iTreg) cells. Accordingly, Foxp3$^+$ Tregs in the colon expressed TCRs specific for commensal bacteria (335). Expression of these unique TCRs on immature T cells did not lead to Foxp3$^+$ Treg cell differentiation upon thymic maturation, suggesting that the majority of these Treg cells were peripherally rather than thymically induced. Furthermore, in a T cell-mediated model of colitis, iTreg cells served non-redundant functions in inhibiting intestinal inflammation (336-338). This was largely due to the fact that iTreg cells had a TCR repertoire distinct from thymically-derived natural Treg (nTreg) cells (336). Incidentally, many Treg cells from the colon share TCRs with Foxp3$^+$ cells in the thymus (339). Upon antibiotic treatment, the overlap of colonic and thymic Treg cell TCRs was diminished, suggesting that many commensal-specific Treg cells in the colon may be thymically derived. How and whether peripheral antigens induce nTreg cells in the thymus is currently under investigation by several groups.

1.6.4 Regulation of tolerance and immunity by intestinal myeloid cells

The above lines of evidence suggest that intestinal homeostasis is a combination of tolerance to innocuous antigens and appropriate inflammatory immune responses to control commensal and pathogenic bacteria. The intestinal LP contains distinct populations of macrophages and dendritic cells that are specialized for promoting either tolerance or immunity.

Macrophages in the mouse intestine, defined by expression of F4/80 and the chemokine receptor, CX_3CR1, have anti-inflammatory properties. These macrophages displayed high phagocytic capacity, constitutive production of the anti-inflammatory cytokines, IL-10 and IL-1RA, and a poor ability to migrate out of the tissue or stimulate $CD4^+$ or $CD8^+$ T cell responses (7, 11, 340). Macrophage production of IL-10 proved to be especially important for maintaining an anti-inflammatory environment. $F4/80^+$ macrophages responded to LPS stimulation with abundant production of IL-10, but negligible production of the inflammatory cytokines, IL-12 and IL-23 (11). Hyporesponsiveness to TLR ligation was due to constitutive IL-10 production, as macrophages isolated from $IL\text{-}10^{-/-}$ mice expressed increased levels of IL-12 and IL-23 both constitutively and upon LPS stimulation (11). Macrophage-derived IL-10 also acted on intestinal DCs to suppress their responsiveness to TLR stimulation (341). LP macrophage IL-10 production was essential for their ability to induce $Foxp3^+$ Treg cells *in vitro* (342), maintain expression of Foxp3 on Treg cells (343), and expand the population of differentiated Treg cells (327).

Intestinal dendritic cells, on the other hand, were characterized by low to intermediate expression of F4/80 and CX_3CR1. These cells could be divided up further into three populations: $CD11b^+CD103^-$, $CD11b^+CD103^+$, and $CD11b^-CD103^+$. All three populations efficiently primed $CD4^+$ T cell proliferation (11). Both $CD11b^+CD103^-$ and

$CD11b^+CD103^+$ DCs migrated from the intestine to the MLNs to prime T cell responses (340, 344, 345). In the steady state, $CD11b^+CD103^-$ DCs primed IFN-γ and IL-17 T cell responses (11) while $CD11b^+CD103^+$ DCs induced differentiation of Treg cells (4, 6, 11). It should be noted that although both CX_3CR1^+ macrophages and $CD103^+$ DCs are capable of differentiating Treg cells *in vitro* (4, 6, 342), only $CD11b^+CD103^+$ DCs are thought to migrate *in vivo* during steady state conditions in the presence of normal commensal microbiota (340). However, this is currently controversial as a recent report showed that antibiotic-induced dysbiosis led to migration of $CD11c^+CX_3CR1^{hi}$ cells (346). Also, $CD11b^+CD103^-$ DCs may migrate under steady state conditions, but prime effector rather than regulatory T cells (344). In the colon, $CD11b^-CD103^+$ DCs were found to reside in isolated lymphoid follicles in close contact with T cells, and were particularly adept at cross-presentation to $CD8^+$ T cells (11).

1.6.5 Clinical manifestations of inflammatory bowel disease

These mechanisms of tolerance and immunity are essential for maintaining intestinal homeostasis. In their absence, humans are predisposed to inflammatory bowel diseases (IBD), which are comprised of ulcerative colitis (UC) and Crohn's disease (CD). UC can affect the entire length of the colon, but inflammation generally starts at the distal colon (rectum) and spreads toward the small bowel. Inflammation is accompanied by mucus and blood in the stool, due to the formation of pathognomonic ulcers in the intestinal wall. Inflammation can extend beyond the intestinal wall, leading to complications such as toxic megacolon. Ulcers, defects in tight junction proteins, and apoptosis of epithelial cells all contributed to decreased epithelial barrier function in UC (347). Inflammation in the LP from UC patients was characterized by T cell production of IL-5 and IL-13 (348,

349). While IL-5 and IL-13 are produced by Th2 cells, the signature Th2 cytokine, IL-4, was decreased in the LP of UC patients (349), suggesting that the source of IL-5 and IL-13 in this disease was not from classical Th2 cells. T cells from UC patients also expressed increased levels of IL-17 (350, 351).

CD, on the other hand, can affect the entire gastrointestinal tract from the oral cavity to the rectum, although lesions are most often found in the ileum and large intestine. Inflammation is not continuous and often present in the form of a "skip lesion," a regional focus of inflammatory infiltrate. Inflammation extending beyond the intestinal wall is more common in CD than in UC. CD also shows the presence of granulomas, a dense collection of macrophages that thickens and hardens the intestinal wall. Granulomas form to wall off foreign objects, but can often facilitate the growth of abscesses as they separate the foreign object from the immune response. Thickened intestinal walls can also lead to stenosis, a narrowing of the intestinal wall, which can be so severe that it obstructs movement of feces through the bowel. CD is strongly correlated with IFN-γ production by Th1 cells, with a minor contribution from Th17 cells producing IL-17 (349-354). Antibodies targeting the p40 subunit shared between IL-12 and IL-23, DC-derived cytokines promoting Th1 and Th17 cell differentiation and survival, have shown promise in the treatment of active CD (355).

Both CD and UC show extraintestinal symptoms. Patients often display pathological weight loss, which may be due to diarrheal water loss, but may also be caused by circulating cachexia-inducing cytokines such as TNF-α (356). TNF-α production by circulating monocytes is elevated in patients with CD (357) and accordingly, antibodies against TNF-α are effective for both the treatment of CD and the maintenance of

remission (358, 359). Anti-TNF-α treatment is also effective for the treatment of UC (360). Other extraintestinal symptoms include inflammation in the skin, the eyes, or the joints. It is not clear whether inflammation initiates in the intestinal tract and spreads systemically or whether systemic inflammation manifests most strongly in the intestinal tract due to the high antigenic load.

1.6.6 Etiologies of IBD

The causes of IBD are multifactorial, including genetic susceptibility, the gut microbiota, environmental triggers, and an aberrant immune response (361). Tolerance and immunity in the gut, as described earlier, are essential for homeostasis, as loss of either leads to IBD.

1.6.6.1 Defects in the epithelial barrier

Many defenses that separate the commensal bacteria from the immune system were found to be defective in IBD. Paneth cells, which secrete antimicrobial proteins such as α-defensins, β-defensins, and RegIIIγ, were abnormal in sections from CD patients (362). Due to a mutation in a critical autophagy gene, ATG16L1, cellular processes were disrupted, resulting in defective exocytosis of antimicrobial peptide-containing granules. A reduction in Paneth cell production of α-defensins during IBD has been reported (363), although similar decreases in β-defensins, RegIIIγ, and other antimicrobial agents have yet to be confirmed. XBP-1, a transcription factor necessary for the endoplasmic reticulum stress response, is necessary for the maintenance of Paneth cells and mucus-secreting goblet cells (364). Mice deficient in XBP-1 showed greater apoptosis of Paneth and goblet cells, had reduced secretion of antimicrobial peptides, and developed a

spontaneous enterocolitis (364). Additionally, hypomorphic variants of XBP-1 were associated with human IBD (364).

Paneth cells are activated either by direct sensing of commensal bacteria (294) or indirectly by ILC-derived IL-22 (295, 365). ILCs can be divided into multiple groups, but IL-22 is produced by $NKp44^+$ "ILC3"s (366). While IFN-γ-producing ILC1s are increased during CD, the frequency of ILC3s decreased in inflamed colon sections from CD patients (367, 368). Thus, a critical source of protective IL-22 may be outcompeted by infiltrating cells during IBD.

Although small intestine epithelium-specific ablation of the tight junction protein, E-cadherin, resulted in spontaneous IBD resembling Crohn's disease (369), defects in epithelial cell tight junctions did not always result in spontaneous disease, despite increased bacterial translocation and significant effector $CD4^+$ T cell accumulation in the LP (370). Mice deficient in the junctional adhesion molecule A (JAM-A) also did not develop spontaneous colitis despite increased barrier permeability (371). It was subsequently found that in mice with a leaky epithelial barrier, IgA-secreting B cells and TGF-β-secreting T cells accumulated in the colon to reduce bacterial invasion of the colon and suppress pro-inflammatory immune responses to commensal bacteria that had breached the barrier (372). Mice with epithelial barrier defects did develop worse experimental colitis (370, 371). Thus, it appears that defects in the epithelial barrier can contribute to colitis, but require additional immune deficiencies to do so. In human IBD patients, the expression of AP-1B, an epithelial cell protein involved in epithelial barrier integrity and preventing bacterial translocation, was decreased (373).

Possibly due to the barrier defects above, ignorance of the intestinal bacteria is also lost during IBD. IgG antibodies to flagellin were found in sera from patients with CD, but not healthy controls (374). LP lymphocytes from affected areas in IBD patients proliferated in response to stimulation with gut bacterial sonicates, while lymphocytes from unaffected areas or from healthy controls did not (375). Increased frequencies of antibodies to *Saccharomyces cerevisiae*, *Alcaligenes* species, *Escherichia coli*, *Pseudomonas fluorescens*, and *Mycobacterium* species have also been reported in the sera of patients with IBD (17, 376, 377). These results suggest that lymphocytes specific for certain microbial antigens, while normally absent at mucosal and systemic sites, emerge during the course of IBD. While it is not clear whether responses against commensal bacteria are the cause or a consequence of IBD, commensal bacteria certainly perpetuate and worsen disease as evidenced by the fact that antibiotic treatment ameliorates symptoms of IBD (378, 379). Furthermore, surgical ileostomies that connect the proximal ileum to the anus or an opening in the skin, thus diverting the fecal stream away from the distal ileum and colon, prevented the recurrence of CD (380).

1.6.6.2 Defects in mucosal immunity

Two PRRs have also been linked to an increased risk for IBD. Polymorphisms in the inflammasome component, NLRP3, correlated with susceptibility to CD (381). The polymorphism was a loss-of-function mutation as cells with the CD-associated mutation in the NLRP3 gene showed decreased expression of the NLRP3 protein and produced less IL-1β in response to microbial stimuli. A defect in NLRP3 function could, thus, lead to impaired immunity and increased risk of colitis-inducing bacteria.

A key intracellular sensor of both bacterial and viral components, nucleotide-binding oligomerization domain-containing protein 2 (NOD2), was also identified as a CD susceptibility gene (382, 383). NOD2 recognizes the bacterial cell wall component, muramyl dipeptide derived from peptidoglycan, as well as viral nucleic acids (147, 383, 384). Cells harboring the CD mutations in NOD2 were unable to activate NF-κB in response to LPS (383). NOD2 also interacted with autophagy proteins to regulate cellular processes involved in antigen presentation and bacterial killing (385). DCs isolated from IBD patients harboring NOD2 mutations were defective in MHC II processing and surface expression, induction of $CD4^+$ T cell responses to bacterial antigens, targeting of intracellular bacteria to lysosomes, and bactericidal activity (385). Furthermore, NOD2 actively suppressed pro-inflammatory TLR2 responses (386). Thus, an impaired ability to ward off bacteria coupled with hyperresponsive TLR signaling to an increased bacterial load may account for the aberrant immune responses seen in IBD patients with NOD2 mutations.

1.6.6.3 Defects in mucosal tolerance

Treg cells are also important for inhibiting IBD. Humans with a genetic defect in FOXP3 develop a fatal inflammatory disease, immunodysregulation polyendocrinopathy enteropathy X- linked syndrome (IPEX), which includes colitis as one of its symptoms (387, 388). Bone marrow transplantation from FOXP3-sufficient human donors has been shown to ameliorate intestinal inflammation in IPEX patients dependent on a reconstitution of $FOXP3^+$ Treg cells (389). In multiple animal models of colitis, Treg cells were able to either prevent or cure inflammation (61, 390, 391). Although the frequency of Treg cells was increased in inflamed sections of colons from CD patients

compared to non-inflamed sections from the same patient or non-inflamed sections from healthy controls, the frequency of Treg cells was significantly lower than the frequency of Treg cells from inflamed colons of patients with diverticulitis, a control gastrointestinal inflammatory disorder (392). Thus, although Treg cell induction was increased in IBD patients, it was restrained compared to a different inflammatory gastrointestinal disease and unable to compensate for the increased level of inflammation in IBD.

Three of the dominant cytokines found in lesions from CD patients, IL-12, IL-23 and IFN-γ, have each been found to restrict differentiation of $Foxp3^+$ Treg cells from naïve T cells (393-395). During experimental colitis, $CD11b^+CD103^+$ DCs lost their ability to induce Treg cell differentiation and instead primed IFN-γ and IL-17 from $CD4^+$ T cells (396). Additionally, in the presence of IL-15, another pro-inflammatory cytokine, retinoic acid no longer promoted Treg cell induction, but inhibited it (397).

IL-12 also affects the function and maintenance of differentiated Treg cells. IL-12-treated Treg cells downregulated surface expression of the IL-2R (395). IL-2 was necessary for survival of $Foxp3^+$ Treg cells (398), providing another explanation for how the inflammatory context during IBD can restrict Treg cell accumulation. Furthermore, IL-12 was shown to induce IFN-γ from $Foxp3^+$ Treg cells during colitis (399), suggesting that depending on inflammatory context, Treg cells may both suppress and contribute to colitis.

1.6.6.4 Alterations in myeloid cell populations

Myeloid cell dysfunction is also characteristic of IBD. As in the mouse, human intestinal macrophages and DCs displayed a profound anergy to TLR stimulation (400). This was due to environmental conditioning by stroma-derived cytokines (400) as well as a lack of CD14 expression on the cell surface (401, 402). CD14 is required for recognition of LPS by TLR4 (403), explaining why colon resident myeloid cells were hyporesponsive to LPS stimulation. During IBD, however, $CD14^+$ monocytes were recruited to the inflamed colon, where they differentiated into pro-inflammatory myeloid cells (404, 405). In response to commensal gut bacteria, $CD14^+$ monocyte-derived cells from CD patients produced significantly higher amounts of IL-23 and TNF-α than cells from healthy controls (404). $CD14^+$ cells from UC patients showed a predilection for IL-6 production. Surprisingly, IL-23 in the CD colonic environment promoted T cell production of IFN-γ rather than IL-17 (351, 404). In a positive feedback loop, IFN-γ promoted the production of IL-23 from intestinal myeloid cells. IL-23 in the UC environment promoted T cell production of IL-17 (351). IL-23 plays a central role in the pathogenesis of IBD, as mutations in its receptor, IL23R, were associated with protection against both UC and CD (406). Myeloid cells in IBD also produced increased levels of other pro-inflammatory cytokines, including IL-12 and IL-18 (352, 407-410).

A key function of intestinal myeloid cells, especially dendritic cells, is to process and present antigens to stimulate lymphocyte responses. Antigen-presenting cells express the co-stimulatory molecules, CD80 (B7-1) and CD86 (B7-2) on their cell surface. These molecules interact with both CD28 and CTLA-4 on T cells, which in conjunction with MHC-TCR interactions stimulate T cell proliferation (411-417). CD80 and CD86 are important for not only initial T cell priming, but also secondary expansion in the colon tissue (418). In healthy mucosa, some myeloid cells expressed CD86, but in inflamed

mucosa from IBD patients, subsets of cells expressing high levels of CD80 and CD86 accumulated (419). These cells associated with lymphoid aggregates *in situ* (419). Antigen-presenting cells isolated from the colonic mucosa or peripheral blood of IBD patients showed a greater T cell proliferative capacity *in vitro* (420, 421).

CD40, another molecule on the surface of antigen-presenting cells, interacts with CD40 ligand (CD40L) on the surface of T cells. Rather than impart activation signals onto T cells, myeloid cells expressing CD40 receive activation signals from T cells, enhancing their ability to produce IL-12 (422-424). Myeloid cells isolated from mucosa from IBD patients showed increased expression of CD40 (410). Conversely, T cells isolated form IBD patients showed increased and prolonged expression of CD40L compared to healthy controls (425). CD40-CD40L interactions *in vitro* stimulated IL-12 and TNF-α production (425). Blockade of TNF-α reduced CD40 expression (410, 426), and blockade of either CD40 or CD40L inhibited IL-12 and TNF-α secretion (425), suggesting that myeloid cell-T cell interactions participate in a self-perpetuating loop of inflammation. Blockade of TNF-α has been effective in the treatment of IBD (359, 360) and blockade of CD40-CD40L interactions could be a promising new therapeutic for IBD.

Production of the anti-inflammatory cytokine, IL-10, is a crucial mechanism of tolerance in the intestines. IL-$10^{-/-}$ mice developed a spontaneous colitis that is at least partially mediated by Th1 cells as transfer of Th1 cells from colitic IL-$10^{-/-}$ mice into WT mice induced colitis (427, 428). In humans, mutations affecting the IL-10R gene led to early-onset pediatric IBD (429) and IL-10 was identified as a susceptibility gene for UC (430). Treatment of LP myeloid cells isolated from IBD patients with IL-10 reduced their production of the pro-inflammatory cytokines, IL-1β and TNF-α, and induced the anti-

inflammatory cytokine, IL-1RA (431). IBD patients treated with recombinant IL-10 saw modest improvement in disease activity (432-434). In these clinical trials, IL-10 was delivered either intravenously or subcutaneously. Mucosal delivery of IL-10 may prove to be more efficacious.

In summary, mechanisms of both tolerance and immunity maintain a delicate balance in the intestines, allowing for appropriate responses against pathogens and suppressing responses against commensal bacteria and dietary antigens. A breakdown of either tolerance or immunity disrupts homeostasis and triggers the development of IBD. Indeed, evidence from human IBD patients shows defects in several of these mechanisms including epithelial barrier dysfunction, unbalanced T cell responses, loss of anergy in myeloid cell populations, defective defense against commensal and pathogenic bacteria, and mutations in IL-10.

1.6.7 Animal models of IBD

Further understanding of the etiologies of IBD has been gained through the use of mouse models of disease. A commonly used model, the T cell adoptive transfer model of colitis (391), recapitulates many features of CD. In this model, naïve $CD4^+$ T cells, identified by high expression of the surface marker, CD45RB, are transferred into lymphopenic $RAG^{-/-}$ or SCID hosts. Lymphopenia drives two different kinds of T cell proliferation: a slow homeostatic proliferation dependent on IL-7 and self peptide-HC complexes and a rapid clonal expansion dependent on foreign antigen presentation by DCs (435). T cell proliferation is especially evident in the MLNs due to the high level of bacterial antigens from the intestinal lumen presented there by migrating DCs. Microbiota from the gut

induced IL-6 from DCs, which also drove $CD4^+$ T cell proliferation, but in an antigen-non-specific manner (329). $CD4^+$ T cell accumulation and development of colitis in this model were dependent on both antigen-independent and antigen-dependent mechanisms of lymphopenia-driven T cell proliferation (329). $CD4^+$ T cells reacting against gut bacteria caused colitis within four to eight weeks with histological features similar to human IBD, such as lymphoid and myeloid cell infiltration and transmural inflammation (436). $RAG^{-/-}$ recipients of naïve $CD4^+$ T cells also showed higher transcription of many genes that were also upregulated in the intestinal mucosa of IBD patients (437).

T cell adoptive transfer colitis was a better model of CD than UC, as it is dominated and driven by a Th1 response (438). Inhibition of Th1 responses via transfer of Treg cells or administration of recombinant IL-10 prevented or cured inflammation (390, 391, 438). As in human disease, a monocyte-derived population of cells was recruited from the blood during T cell transfer colitis (11, 439). These cells expressed high levels of various TLRs, produced pro-inflammatory cytokines including IL-6, TNF-α, IL-12 and IL-23, and induced robust $CD4^+$ T cell proliferation *in vitro* (11, 439). Importantly, IL-23 played an essential role in driving disease in this mouse model, corroborating genetic studies on human populations. When $RAG^{-/-}$ hosts were crossed to IL-23-deficient mice, transfer of naïve $CD4^+$ T cells no longer induced disease (440). Likewise, transfer of IL-23R-deficient naïve $CD4^+$ T cells failed to induce disease (441). The importance of DC-T cell interactions was further demonstrated by blocking CD40 or CD40L to inhibit disease (442, 443).

The anti-CD40 model of colitis was developed to specifically examine the role of myeloid cells in driving pathology. A single dose of agonistic anti-CD40 antibody into

$RAG^{-/-}$ mice leads to a rapid, systemic wasting disease and development of inflammation in the proximal colon (444). Systemic wasting was dependent on IL-12 while mucosal inflammation was dependent on IL-23. Both cytokines were secreted by myeloid cells after CD40 ligation. Rather than acting on $CD4^{+}$ T cells, IL-23 in this model acted on ILCs to induce pro-inflammatory cytokine secretion (16).

Colonization of mice with specific bacteria can also trigger colitis. *Helicobacter hepaticus* is a commensal bacterium found in many animal facilities (445). However, in $IL\text{-}10^{-/-}$ mice or upon treatment with an IL-10R neutralizing antibody, *H. hepaticus* induced an IL-23-dependent Th1-mediated chronic inflammation (446, 447). Histological features in this model are very similar to those in T cell transfer colitis and thus, human IBD. However, the *H. hepaticus* model of colitis has the advantage of using a lymphoreplete host, thus better representing humans with IBD whose disease may involve B cells, NKT cells and other lymphocytes.

Finally, *Citrobacter rodentium* represents a classic attaching/effacing gastroenteritis-inducing pathogen. Infection causes a self-limiting acute colitis that is cleared in an antibody-dependent manner in approximately three weeks (448). Bacteria attach to the epithelial layer and efface the brush border. Epithelial cell hyperplasia is the main histological feature. During infection, mice may experience bloody stools, but weight loss, bacterial translocation, morbidity and mortality are minimal. Defects in immunity, however, lead to uncontrolled growth of the bacteria and spread to extraintestinal sites. Rather than play a pathogenic role in this model, IL-23 enhanced immunity against *C. rodentium* and thus prevented bacterially induced pathology (47, 449). IL-23 was originally thought to promote immunity by supporting Th17 cells, but recent evidence

suggests a more important role for IL-23-responsive ILCs in protection against *C. rodentium* early after infection (365). One week post-infection, however, $CD4^+$ T cells became the source of protective IL-22 (450). Inflammasomes provided another non-redundant mechanism of protection (451). Thus, *C. rodentium* infection does not reflect the symptoms or immunopathology of chronic IBD, but better models gastroenteritis caused by the human pathogens, enteropathogenic *E. coli* and enterohemorrhagic *E. coli*.

1.6.8 IFN-1 in intestinal homeostasis

IFN-1 has varying roles in different inflammatory disorders, either driving disease as in systemic lupus erythematosus or inhibiting it in multiple sclerosis. The role of IFN-1 in IBD is less clear. Treatment of UC patients with IFN-β has shown promise, partly through the inhibition of IL-13 (452, 453). However, IFN-α/β was only marginally effective in CD (454, 455). This may be due to the finding that mononuclear cells isolated from the intestines of CD patients were hyporesponsive to IFN-1 (456). The genetic locus containing IFNAR1 and IFNAR2 has been weakly associated with IBD (457), although no specific gain-of-function or loss-of-function polymorphisms have been identified.

In the murine T cell transfer model of colitis, however, poly (I:C)-induced IFN-1 ameliorated disease (276). Suppression of pathology was dependent on direct signaling of IFN-1 on $CD4^+$ T cells. Treatment of naïve $CD4^+$ T cells with IFN-1 *in vitro* prior to injection into lymphopenic hosts also attenuated their colitogenic potential (277). Treatment of Treg cells with IFN-1 enhanced their expansion (281). $IFNAR1^{-/-}$ $CD4^+CD25^+$ Treg cells were unable to protect mice from T cell-mediated colitis due to poor expansion of Treg cells and greater expansion of contaminating effector

$CD4^+CD25^+$ T cells. Treatment of mice with exogenous IFN-1 in a system where only Treg cells were able to respond to IFN-1 enhanced the relative expansion of Treg cells.

In the dextran sulfate sodium (DSS)-induced model of colitis, IFN-1 inhibited colitis in a T cell-independent manner. DSS chemically disrupts the epithelial barrier, leading to an acute colitis that resolves within two weeks. When treated with the TLR9 ligand, CpG oligodeoxynucleotide, however, $RAG^{-/-}$ mice were protected from disease (458). Protection was dependent on IFN-1 since antibodies to IFNAR abrogated the anti-inflammatory effects of CpG, and recombinant IFN-1 recapitulated protection. Adoptive transfer of WT bone marrow-derived macrophages (BMDM), but not $IFNAR1^{-/-}$ BMDMs into DSS-fed mice given a subcutaneous injection of CpG lessened the severity of colitis (458). Conventional $CD11c^+$ DCs were the source of CpG-induced IFN-1, which acted on tissue macrophages to inhibit secretion of inflammatory cytokines including TNF-α, IL-1β, and IL-6 and the neutrophil-attracting chemokine, CXCL1 (285). IFN-1 enhanced production of CCL5, which recruited a monocyte/macrophage population with tissue-regenerative properties (285).

Despite consistently suppressing inflammation in mouse models of IBD, IFN-1 worsened intestinal pathology in multiple non-IBD models of intestinal inflammation. In a graft vs. host reaction (GvHR) model, in which splenocytes are transferred into genetically mismatched mice, poly (I:C) treatment exacerbated intestinal pathology in a IFN-1-dependent manner (459). IFN-1 enhanced NK cell cytotoxicity, leading to increased villous blunting and crypt cell hyperplasia. Experiments using a human gut explant culture showed that addition of IFN-α to the culture in conjunction with α-CD3 also

resulted in villous atrophy, epithelial cell hyperplasia, and increased T cell secretion of IFN-γ, while treatment with α-CD3 alone did not cause such pathological changes (460).

As discussed earlier, IFN-1 has pleiotropic effects dependent on cell-type and context. This is no different in the intestinal environment, as IFN-1 can exacerbate intestinal pathology via activity on NK cells and inhibit it via signaling on T cells or gut-resident macrophages. Contrasting effects on different cell-types may explain the poor response of IBD patients to treatment with recombinant IFN-1.

1.7 Aims of this thesis

IFN-1 is produced by immunomodulatory macrophages in the intestines (7). The function of constitutive IFN-1 in the intestines is currently poorly understood. We hypothesized that because IFN-1 was present outside of an infectious context, it served a homeostatic function other than providing antiviral immunity.

Using the T cell adoptive transfer model of colitis, my first aim was to examine the effects of IFN-1 on regulating $CD4^+$ T cell expansion. By using mice that were deficient in IFNAR signaling on either the transferred naïve $CD4^+$ T cells or hematopoietic or non-hematopoietic cells in the recipient, I was able to examine the effects of IFN-1 on different cellular compartments. By transferring both naïve and $CD25^+$ Treg cells, I was able to test the effects of IFN-1 on different T cell subsets.

As intestinal homeostasis involves tight regulation of myeloid cells, my second aim was to analyze the effects of IFN-1 on mononuclear phagocytes in the colon. To accomplish this, I analyzed the function and phenotype of isolated WT and $IFNAR1^{-/-}$ myeloid cells *ex vivo* as well as *in vivo* using the anti-CD40 model of innate colitis.

Finally, interactions with the microbiota are a key feature of intestinal homeostasis. The final aim of this thesis was to determine whether IFN-1 affected the composition of the intestinal microbiota or the immune response against it.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Mice

WT C57BL/6 (CD45.2) mice were purchased from National Cancer Institute, Frederick, MD, USA. SJL (CD45.1), IFNAR1$^{-/-}$ (187) and RAG1$^{-/-}$ mice, all on a C57BL/6 background were obtained from Taconic Farms bred on contract with National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID, NIH). Foxp3-GFP mice were originally from V. Kuchroo (43) and were bred in-house at NIH. TRIF$^{-/-}$, MyD88$^{-/-}$, and TRIF$^{-/-}$/MyD88$^{-/-}$ mice were originally from A. Sher and were bred in-house at NIH. WT C57/BL/6 controls for these three strains were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). RAG2$^{-/-}$/IFNAR1$^{-/-}$ double knockout (DKO) mice were generated by crossing RAG2$^{-/-}$ mice with IFNAR1$^{-/-}$ mice in-house. IFNAR1$^{-/-}$ mice on the 129S7 background (187) were purchased from B&K Universal (Hull, United Kingdom). 129S7 IFNAR1$^{-/-}$ mice were crossed to 129SvEv RAG1$^{-/-}$ mice maintained in-house at the University of Oxford to generate RAG1$^{-/-}$/IFNAR1$^{-/-}$ double knockout mice on the 129 background. All mouse experimental protocols were approved by the NIAID Animal Care and Use Committee or the United Kingdom Animals Scientific Procedures Act (1986).

In vivo protocols conducted in this thesis including animal handling, genotyping, intraperitoneal (i.p.) and intravenous (i.v.) injections, oral gavage, administration of BrdU water, euthanasia and the dissection of tissues were conducted primarily by the author with occasional help from lab members or the animal facility staff. Animal breeding, ear

punching for genotyping, tail vein bleeds and maintenance of antibiotic water was conducted primarily by the animal facility staff, but occasionally by the author himself.

2.1.2 Antibodies

Antibodies used for staining macrophage and dendritic cell subsets were PE-Cy5-CD19 (eBio1D3), PE-Cy5-TCRβ (H57-597), PE-Cy5-TCRγδ (eBioGL3), eFluor 450-MHC Class II IA/IE (M5/114.15.2), APC-CD11c (N418), PE-Cy7-F4/80 (BM8), APC-Cy7-CD11b (M1/70), and PE-CD103 (2E7).

Antibodies used for staining T cells extracellularly were PE-Cy5 or APC-Cy7-CD4 (RM4-5), PE-Cy5-TCRβ (H57-597), PE-CD45RB (C363.16A), PE-CD25 (PC61.5), FITC or PE-Cy7-CD62L (MEL-14), and APC-CD44 (IM7). For Treg co-transfer and bone marrow chimera experiments, PE-Cy7-CD45.1 (A20) and APC-CD45.2 (104) antibodies were added. Antibodies used for staining T cells intracellularly were FITC-Foxp3 (FJK-16s), PE-IL-17A (eBio17B7), and APC-IFN-γ (XMG1.2). Appropriate isotype controls were used for intracellular antigens.

Antibodies used for genotyping cells in the blood were FITC-CD11b (M1/70) and PE-IFNAR1 (MAR1-5A3).

Fc block (anti-CD16/32; clone 93) was used to block non-specific antibody binding.

All antibodies were purchased from eBioscience (San Diego, CA, USA) with the exception of F4/80 and IFNAR1, which were purchased from Biolegend (San Diego, CA,

USA). All antibodies were used at a 1:100 dilution with the exception of PE-IL-17A, which was used at 1:200.

For the innate anti-CD40-induced acute colitis model, anti-CD40 (FGK4.5) was purchased from Bio X Cell (West Lebanon, NH, USA).

For the *Helicobacter hepaticus*-induced chronic colitis model, anti-IL-10R (1B1.2) was purified from the supernatants from hybridomas grown in-house at the University of Oxford.

2.1.3 Biologicals for *in vitro* cell culture

Macrophages and dendritic cells were stimulated with IFN-αA (PBL Interferon Source, Piscataway, NJ, USA), recombinant IL-27 (R&D Systems, Minneapolis, MN, USA), lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO, USA), FSL-1 (Invivogen) or flagellin from *Salmonella typhimurium* (Invivogen). Anti-IL-27 (R&D Systems) and anti-IFNAR1 (Biolegend) were also used in some culture conditions.

$CD4^+$ T cells were stimulated with phorbol myristate acetate (PMA), ionomycin, Golgi Stop (BD, San Jose, CA, USA) and/or anti-CD3 (eBioscience).

Reagents used in T cell differentiation assays were anti-CD3, anti-IFN-γ, anti-IL-4, IL-6, TGF-β, and IL-2. Antibodies were purchased from eBioscience while cytokines were purchased from R&D Systems.

2.1.4 Cell buffers and media

Cell buffers used for tissue digestion:

Buffer A: HBSS containing 5% Fetal Calf Serum (FCS), 25 mM HEPES

Buffer B: HBSS containing 2mM EDTA, 25 mM HEPES

Buffer C: HBSS containing 5% FCS, 5mM EDTA, 15 mM HEPES, 1 mM dithiothreitol (DTT)

Complete Iscove's Modified Dulbecco's Medium (IMDM): 500 mL IMDM + 50 mL FCS + 5 mL Pen/Strep + 7.5 mL of 1M HEPES + 25 mL of NCTC-109 + 0.5 mL 0.05M 2-mercaptoethanol + 5 mL 200 mM glutamine

Digestion Buffer: Complete IMDM containing 0.17 mg/mL Liberase TL (Roche, Indianapolis, IN, USA; Catalog #05401020001), 30 μg/mL DNAse I (Roche; Catalog #10104159001).

Sorting Buffer: PBS containing 10% FCS, 2mM EDTA

Complete RPMI: same as complete IMDM, except added to 500 mL RPMI instead of IMDM.

L-cell conditioned medium (LCM): L929 fibroblasts were grown in 50 mL of DMEM + 10% FCS in several 175 mL flasks for ten days, centrifuged at 3000 RPM for 10 minutes, passed through a 0.45 μm filter, and frozen in 50 mL aliquots until use.

R10 Media: RPMI containing 10% FCS, 10 mM HEPES, Penicillin (100 U/mL), Streptomycin (100 μg/mL), 2 mM L-glutamine

2.1.5 Growth of bacteria

Helicobacter hepaticus was purchased from the American Type Culture Collection (NCI-

Frederick isolate 1A strain 51449). *H. hepaticus* was grown from a frozen stock on blood agar plates containing the antibiotics, trimethoprim, vancomycin, and polymyxin B (TVP, Oxoid) at 37°C in a closed chamber with a gas mix composed of a N_2 base, 10% CO_2, 10% H_2, and residual O_2 (microaerophilic conditions). Bacteria grown on plates were moved to a Tryptone Soya Broth (TSB, Oxoid, Cambridge, UK) liquid culture containing TVP and 10% FCS. Bacteria were inoculated at 0.05 optical density $(OD)_{600nm}$ and cultured in a 37°C shaker set at 150rpm. Bacteria were passaged in liquid culture by diluting the culture to 0.05-0.10 OD_{600nm} daily. OD was converted to CFU using the formula $CFU = ODx10^8$.

Citrobacter rodentium (isolate ICC169 containing a nalidixic acid resistance gene) was a gift from G. Frankel. The bacteria was streaked across a Luria broth and nalidixic acid (LB/NA) agar plate and grown overnight at 37°C. A single colony was picked off the plate, placed in liquid LB/NA, and incubated at 37°C with shaking. Prior to use, OD of the liquid culture was measured and diluted to 0.2 in LB/NA. The bacteria were grown for an additional four hours prior to feeding or 2.5 hours prior to use in *in vitro* cultures. OD was converted to CFU using the formula $CFU = (ODx8x10^8)-10^8$.

2.2 Cell isolation, enrichment and sorting

2.2.1 Isolation of cells from spleen and lymph nodes

Spleens were injected with Digestion Buffer (see 2.1.4) and both spleens and lymph nodes were minced. Both were placed in pre-warmed Digestion Buffer for 30 minutes with horizontal shaking in a 37°C water bath. Tissue pieces were washed (unless

otherwise noted, washes indicate centrifugation at 1500 RPM for 7 minutes) and resuspended in 10 ml of pre-warmed HBSS/EDTA solution (9 ml HBSS, 1 ml FCS, 100 ul EDTA, 250 ul HEPES) and incubated for 5 minutes at 37°C. Tissue pieces were then mashed through a 100 micron filter and washed with HBSS. The pellet was then resuspended in PBS and passed through a 40 micron filter. If dendritic cells were not desired from the spleen or lymph nodes, the initial incubation in Digestion Buffer and subsequent incubation in HBSS/EDTA solution was omitted.

2.2.2 Isolation of cells from the small and large intestines

From small intestines, Peyer's patches were excised and the lumen was flushed with Buffer A. Both small and large intestines were then cut into 3-4 cm pieces and manually shaken in Buffer A. Media including intestinal pieces were poured over a tea strainer (Target Stores, USA). Tissue pieces were picked off of the tea strainer and manually shaken in Buffer B. Media including intestinal pieces were again poured over a tea strainer. Tissue pieces were picked off of the tea strainer, placed in pre-warmed Buffer C, and shaken horizontally in a 37°C water bath for 15 minutes. Tissue pieces were next manually shaken and poured over the tea strainer. Tissue pieces were then placed in cold Buffer A, manually shaken and poured over the tea strainer. This last step was repeated five times or until the supernatant was clear. Tissue pieces were placed in Buffer A and washed. The pellet was resuspended in pre-warmed Digestion Buffer (30 mL for up to two small intestines and up to ten large intestines) and shaken horizontally in a 37°C water bath for one hour. After digestion, tissue pieces were pelleted by centrifugation (1500 RPM for 7 minutes) and resuspended in Buffer A. Tissue pieces were then mashed through a 100 micron filter, washed, and resuspended in Buffer B. Single cells in Buffer

B were passed through a 40 micron filter. If dendritic cells were not desired, then manual shaking could be replaced by vortexing.

2.2.3 Enrichment of dendritic cells from the spleen

Single cells from the spleen were resuspended in MACS buffer (Miltenyi Biotec, Auburn, CA, USA) at a concentration of $2.5x10^8$ cells/mL. 100 µL of CD11c MicroBeads (Miltenyi Biotec Product #130-052-001) were added per 10^8 cells and incubated for 15 minutes at 4°C. Additional antibodies to stain cells were added for an extra 10 minutes. Cells were washed and resuspended in MACS buffer at a concentration of $2x10^8$ cells/mL. Cells were enriched in an AutoMACS Pro using a positive selection program.

2.2.4 Enrichment of $CD4^+$ T cells from the spleen or lymph nodes

Single cells were resuspended in MACS buffer at a concentration of $5x10^8$ cells/mL. 100 µL of Biotin-antibody cocktail (part of Miltenyi Biotec $CD4^+$ T cell Isolation Kit II; Product #130-095-248) were added per 10^8 cells and incubated for 10 minutes at 4°C. Then, an additional 300 µL of MACS buffer and 200 µL of anti-biotin MicroBeads were added per 10^8 cells and incubated for 15 minutes at 4°C. Cells were washed and resuspended in MACS buffer at a concentration of $2x10^8$ cells/mL. Cells were enriched in an AutoMACS Pro using a negative selection program.

2.2.5 Enrichment of mononuclear phagocytes from the small and large intestine

Single cells were resuspended in Nycodenz (Accurate Chemical, Westbury, NY, USA) containing 2mM EDTA at a concentration of 10^7 cells/mL and placed in a 15 mL conical

tube. 2 mL of complete RPMI + 2mM EDTA without FCS was layered on top of the cells. Cells were centrifuged at 1600xg for 15 minutes at 10°C with no brake. Cells were collected from the Nycodenz and RPMI interface and washed with complete RPMI.

2.2.6 Enrichment of $CD4^+$ T cells from the small and large intestine

P100 solution was prepared by combining 1 part 10X PBS with 9 parts Percoll (GE Healthcare, Piscataway, NJ, USA). P70 solution was prepared by combining 7 parts P100 with 3 parts PBS + 1% Bovine Serum Albumin (BSA). P40 solution was prepared by combining 4 parts P100 with 6 parts complete RPMI. 4 mL of P40 was layered over 3 mL of P70 in a 15 mL conical tube. Cells isolated from small or large intestinal preparations were resuspended in 3 mL of P30 (3 parts P100 combined with 7 parts PBS + 1% BSA) and layered over P40. Cells were centrifuged at 1800 RPM for 20 minutes at 20°C with no brake. Cells were collected from the P70 and P40 interface and washed with complete RPMI.

2.2.7 Cell sorting

If not already stained during Miltenyi MACS enrichment, cells were stained with antibodies to identify cell populations for sorting. After incubation with antibodies for at least 15 minutes at 4°C, cells were washed and resuspended in sorting buffer at a concentration of $4x10^7$ cells/mL. Cells were sorted on a FACSAria II (BD) using the sorting parameters indicated in the descriptions of each individual experiment.

2.3 *In vivo* models

2.3.1 T cell adoptive transfer model of colitis

Naïve T cells were sorted as PE-Cy5-$CD4^+$, APC-$CD25^-$, and PE-$CD45RB^{hi}$. $CD45RB^{hi}$ was defined as being the 30% of $CD4^+CD25^-$ cells that expressed the highest levels of CD45RB. Sorted cells were washed and resuspended in sterile PBS. $3x10^5$ naïve T cells were injected intraperitoneally (i.p.) into age- and sex-matched $RAG^{-/-}$ or DKO mice. For Treg co-transfer studies, Tregs were sorted from Foxp3-GFP mice (43) as PE-Cy5-$CD4^+$, APC-$CD25^-$, and GFP-$Foxp3^+$. In experiments where Foxp3-GFP mice were not used, Tregs were sorted as PE-Cy5-$CD4^+$ and APC-$CD25^-$, which were >95% $Foxp3^+$. $4x10^6$ naïve T cells were co-transferred with $1x10^6$ Tregs by i.p. injection. In CFSE dilution studies, $1x10^6$ T cells were labeled with CFSE (Invitrogen, 10 μM) according to the manufacturer's protocol prior to intraperitoneal injection. In some experiments, mice were treated with anakinra (human recombinant IL-1RA; purchased from Division of Veterinary Resources, NIH, Bethesda, MD, USA; 1 mg daily) or recombinant IL-10 (0.5 μg daily) by i.p. injection. Mice were weighed twice a week to monitor body weight loss. Mice were also monitored continuously through the study for development of clinical symptoms such as scruffy coat, hunched appearance, lethargy, and diarrhea. At the study endpoint, sections were collected from the proximal, mid-, and distal colon for histological and RT-PCR analysis.

2.3.2 Anti-CD40-mediated innate model of colitis

$RAG^{-/-}$ and DKO mice were injected i.p. with 40 μg of anti-CD40. Mice were weighed daily and monitored for development of clinical symptoms such as scruffy coat, hunched appearance, lethargy, and diarrhea. After seven days, sections were collected from the proximal colon for histological and RT-PCR analysis.

2.3.3 *Helicobacter hepaticus*-induced colitis

WT and IFNAR1$^{-/-}$ mice on the 129 background were gavaged orally on three consecutive days with 1x10^{8} CFU of *H. hepaticus*. Mice received 1 mg of anti-IL-10R once per week by i.p. injection. Mice were monitored for approximately eight weeks or until loose stools were observed in the cage.

2.3.4 *Citrobacter rodentium*-induced colitis

Mice were gavaged orally with 10^{8}-10^{9} CFU *C. rodentium*. Exact dose of feeding was measured by plating feeding suspension on LB/NA plates at various dilutions and counting colonies. Mice were weighed daily and fecal pellets were collected daily. CFU of *C. rodentium* were quantified in the feces daily by homogenizing the stool in sterile PBS, plating on LB/NA plates, and counting colonies the following morning.

2.3.5 Histological scoring

Intestinal pieces were preserved in 10% formal saline. Pieces were then embedded in paraffin, sectioned and stained with hematoxylin and eosin by either Histoserv, Inc. (Germantown, MD, USA) or R. Stillion at University of Oxford. Sections were examined under a light microscope and scored according to an established protocol (393), which is reproduced in Table 2.1. Each section of intestine was given a score from 0-3 in four categories: epithelium, inflammation in lamina propria, area affected and markers of severe inflammation. Scores from each category were added for a combined score of 0-12 per intestinal piece. Scores of multiple pieces from a single mouse were averaged.

Epithelium	**Hyperplasia**	**Goblet Cell Depletion**
0	None	None
1	Mild (1.5X)	Mild (<25%)
2	Moderate (2-3X)	Marked (25-50%)
3	Severe (>3X)	Substantial (>50%)
Inflammation in Lamina Propria		
0	None – Few leukocytes	
1	Mild – Some increase in leukocytes at tips of crypts or many lymphoid follicles	
2	Moderate – Marked infiltrate (notable broadening of crypt)	
3	Severe – Dense infiltrate throughout	
Area Affected (% of section)		
0	None	
1	<25%	
2	25-50%	
3	>50%	
Markers of Severe Inflammation		
0	None	
1	Submucosal inflammation OR few crypt abscesses (<5)	
2	Submucosal inflammation AND few crypt abscesses (<5)	
2	Many crypt abscesses (>5) OR extensive submucosal inflammation OR crypt branching (for *H. hepaticus* colitis only)	

3	Many crypt abscesses (>5) AND extensive submucosal inflammation or crypt branching (for *H. hepaticus* colitis only)
3	Ulceration or extensive fibrosis

Table 2.1 Histological scoring parameters

For *Citrobacter rodentium* experiments, submucosal inflammation was separated from other markers of severe inflammation for a combined score of 0-15.

2.3.6 *In vivo* regulatory T cell induction

Naïve $CD4^+CD25^-CD45RB^{hi}$ cells were transferred into $RAG^{-/-}$ or DKO mice by i.p. injection. Cells isolated from the MLNs ten days later were stained for Treg markers.

Alternatively, $CD4^+CD25^-$ cells were isolated from the spleens and lymph nodes of $RAG^{-/-}$/OT-II transgenic mice that expressed the congenic marker CD45.1. 10^6 $CD45.1^+CD4^+CD25^-$ T cells were transferred intravenously (i.v.) into WT or $IFNAR1^{-/-}$ mice on the B6 (CD45.2) background. Mice were fed 1.5% ovalbumin (Sigma-Aldrich) in the drinking water for five days and then resumed on normal drinking water for two days. On day seven post-transfer, cells isolated from MLNs and Peyer's patches of WT or $IFNAR1^{-/-}$ hosts were stained for Treg markers.

2.3.7 Generation of bone marrow chimeras

Femurs and tibias isolated from donor mice were sterilized in 70% ethanol. Epiphyses were cut off and marrow was flushed from the diaphysis with sterile PBS + 2mM EDTA in a 26 gauge needle. Additional marrow was obtained by mincing and rinsing the

epiphyses. All marrow was washed in cold sterile PBS prior to use.

$RAG^{-/-}$ mice were irradiated with one dose of 900 rads of γ-radiation. Immediately after irradiation, mice were reconstituted with $3x10^6$ bone marrow cells from donor mice by tail vein injection. Eight weeks later, mice were bled to check for depletion of host cells and reconstitution with donor cells. Reconstitution efficiency was checked either by using congenic $RAG^{-/-}$ recipients (CD45.1) or by staining for IFNAR on blood cells.

2.3.8. Assessment of *in vivo* cell turnover

WT and $IFNAR1^{-/-}$ mice on the B6 background were injected i.p. with 0.8 mg of 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) dissolved in 200 μL of PBS. Mice were subsequently administered BrdU in their drinking water (0.8 mg/mL) for up to 14 days. BrdU water was prepared fresh daily, protected from light, and supplemented with 1% sucrose. At study endpoint, cells were isolated from colons and MLNs and stained for intracellular BrdU using a FITC anti-BrdU kit (BD Biosciences) according to the manufacturer's instructions. Briefly, cells were stained for extracellular markers, fixed, permeabilized, treated with DNAse I for one hour and stained with FITC anti-BrdU at room temperature.

2.4 *In vitro* assays and procedures

2.4.1 Stimulation of intestinal mononuclear phagocytes

Mononuclear phagocytes (MPs) from the colon were sorted as eFluor 450-MHC II IA/IE^{hi} and PE-Cy5-$lineage^-$. Within that gate, any cells that were either APC-$CD11c^+$ or

PE-Cy7-F4/80^{+} were collected. Lineage markers used were CD19, TCRβ, and TCRγδ. DCs from the MLNs were sorted as eFluor 450-MHC II IA/IE^{hi}, PE-Cy5-lineage^{-}, and APC-CD11c^{+}. Sorted cells were resuspended in complete RPMI at a concentration of 10^6 cells/mL. Cells were left unstimulated or stimulated with FSL-1 (500 ng/mL), flagellin (1 μg/mL), or LPS (10 μg/mL) in 96 well flat bottom plates (Costar) in a 37°C incubator. In some experiments, anti-IFNAR1 (5 μg/mL), IFN-αA (500 U/mL), recombinant IL-27 (20 ng/mL), or anti-IL-27 (10 μg/mL) were added to the cultures. After 24 hours of culture, plates were placed in a -20°C freezer.

Supernatants and cell lysates were thawed and sent to Aushon Biosystems (Billerica, MA, USA) for multiplex cytokine analysis. For some experiments, samples were analyzed by commercially purchased DuoSet ELISAs (R&D Systems) to detect IL-10 and IL-1 receptor antagonist (IL-1RA).

2.4.2 Stimulation of lymphocytes for cytokine production

Non-enriched cells from the intestines of steady state and colitic mice were resuspended in complete RPMI at a concentration of 10^6 cells/mL. Cells were stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) in a 6-well flat bottom plate (Costar). After two hours, Golgi Stop (BD) was added and left in culture for an additional three hours before analysis by flow cytometry.

2.4.3 Flow cytometry

Isolated cells were washed in PBS and placed in V-bottom plates (Nunc; $5x10^6$ cells/well). Washes in V-bottom plates were conducted at 2000 RPM for two minutes. Cells were

stained with LiveDead Fixable Blue or Violet Dead Cell Staining kits (Invitrogen; product #L23105 or L34955) for 20 minutes at 4°C. At this time, Fc block and antibodies against extracellular antigens were added. Intracellular antigens were stained using the Foxp3 intracellular staining kit (eBioscience; product #00-5523-00). Briefly, cells were washed twice with PBS and resuspended in fixation/permeabilization buffer for up to 18 hours. Cells were washed in permeabilization buffer, blocked with Fc block in permeabilization buffer and stained with antibodies against intracellular antigens for up to one hour. Cells were washed twice more with permeabilization buffer. Stained cells were analyzed on a BD LSR II or Dako Cyan flow cytometer. Compensation was achieved using anti-rat and anti-hamster Ig compensation beads (BD; product #552845).

2.4.4 DC-T cell co-cultures

Splenic DCs were sorted from WT and IFNAR1$^{-/-}$ mice on the B6 background as eFluor 450-MHC II IA/IE^{hi}, PE-Cy5-lineage^{-}, APC-CD11c^{+}. Lineage markers used were CD19, TCRβ, and TCRγδ. Naïve CD4^{+} T cells were sorted from WT B6 mice as PE-CD4^{+}, PE-Cy5-TCRβ^{+}, FITC-CD62L^{hi}, and APC-CD44^{lo}. Cells were mixed at a ratio of 1 dendritic cell to 5 naïve T cells at a combined concentration of 10^{6} cells/mL. Cells were cultured either in 96-well round-bottom plates (200 μL/well) or 48-well flat-bottom plates (500 μL/well).

For T cell polarization assays, cells were cultured with anti-CD3 (1 μg/mL), ovalbumin peptide (1 μM), TGF-β (5 ng/mL), IL-6 (20 ng/mL), IL-1β (10 ng/mL), anti-IFN-γ (10 μg/mL), anti-IL-4 (10 μg/mL), and/or IFN-α (100 U/mL) for five days. For Treg polarization assays, cultures were administered IL-2 (5 ng/mL) on 24 and 72 hours after

the start of the culture. At the end of five days, PMA, ionomycin and Golgi Stop were added to the cultures for five hours as in 2.4.2 and cells were analyzed for cytokine production by flow cytometry. For IL-10 measurement, cells were restimulated without Golgi Stop and measured by ELISA (R&D Systems).

For T cell proliferation assays, cells were cultured with anti-CD3 and/or IFN-α for three days. On the third day, 1 μCi H^3-labeled thymidine was added to the cultures. Seven hours later, the plate was frozen at -20°C. Upon thawing, lysed cells were harvested onto a filter paper membrane using a Mach II Manual Harvester (TOMTEC, Hamden, CT, USA) and H^3 per well was measured by a Wallac MicroBeta Trilux Luminescence Counter (PerkinElmer; product #1450-024).

2.4.5 Generation of bone marrow derived macrophages (BMDM)

Bone marrow was isolated as in 2.3.7. Marrow from up to two tibias and two femurs was resuspended in 15 mL R10 media + 15% LCM (R10-LCM). 5 mL of bone marrow cells were added to 20 mL of R10-LCM in 15 cm bacterial petri dishes (Falcon) and incubated in a 37°C/5% CO_2 incubator. 15 mL of R10-LCM was added three days later. On day 6, the media was replaced with 25 mL of fresh R10-LCM. When macrophages were ready to use (between days 7 and 9), they were lifted using PBS + 10 mM EDTA + 4 mg/mL lidocaine.

2.4.6 Inflammasome activation

BMDM suspended in R10 media were aliquoted onto a 24-well plate (10^6 cells/well) and incubated overnight at 37°C in the presence or absence of IFN-β (500 U/mL or 10,000

U/mL). Supernatants were taken off the wells, leaving adherent BMDM on the plate bottom. Wells were washed three times with room temperature PBS. LPS (20-100 ng/mL) diluted in R10 was added to specified wells for four hours. Supernatant was removed and wells were again washed three times with PBS. Nigericin (5 μM) diluted in Opti-MEM (Invitrogen) was added for up to one hour in the presence or absence of IFN-β (500 U/mL or 10,000 U/mL).

For stimulation with *Citrobacter rodentium*, $2x10^7$ CFU (Multiplicity of infection, MOI of 20) diluted in Opti-MEM were added per well for 90 minutes in the presence or absence of IFN-β (500 U/mL or 10,000 U/mL). Wells were washed three times with room temperature PBS and administered gentamicin (100 μg/mL) diluted in Opti-MEM for 5.5 hours in the presence or absence of IFN-β (500 U/mL or 10,000 U/mL).

For stimulation with *Helicobacter hepaticus*, cells that were pre-treated overnight with IFN-β were washed three times with room temperature PBS and incubated with $1.5x10^7$ CFU *H. hepaticus* (MOI of 15) overnight.

All incubations were conducted at 37°C. After the final incubation for each condition, supernatants were aspirated and saved in Eppendorf tubes. IL-1β was measured by sandwich ELISA using anti-mouse/rat IL-1β (eBioscience, product #14-7012-85, 1 μg/mL) as a coating antibody, biotin-anti-mouse IL-1β (eBioscience, product #13-7112-85, 5 μg/mL) as a detection antibody and recombinant mouse IL-1β (PeproTech, Rocky Hill, NJ, USA, product #211-11b) as a standard. A top standard of 2000 pg/mL with seven 1:3 dilutions was used to generate a standard curve. IL-10 was measured using a commercially available DuoSet (R&D Systems).

2.4.7 Genotyping

Ear punches were digested in 50 μL lysis buffer (84 mL water, 2ml 5M NaCl, 10ml 1M Tris pH 8.0, 2ml 10%SDS, and 2ml 0.5M EDTA) + 2 μL proteinase K at 55°C for two hours or overnight. 450 μL of water was added after digestion and samples were centrifuged in a benchtop centrifuge (Eppendorf) at 7600 RPM for 6 minutes. 1 μL of DNA was used in each PCR reaction.

PCR reactions were set up using HotStarTaq polymerase and associated protocol (Qiagen, product #203601). $RAG2^{-/-}$ mice were genotyped by including the following primers: RagA: GGG AGG ACA CTC ACT TGC CAG TA; RagB: AGT CAG GAG TCT CCA TCT CAC TGA; and RagNeo: CGG CCG GAG AAC CTG CGT GCA A. Denaturation was achieved at 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 60 seconds. The PCR reaction ended with 10 minutes at 72°C and storage at 4°C. The reaction created a 300 bp WT band and a 400 bp $RAG2^{-/-}$ band.

$IFNAR1^{-/-}$ mice were genotyped by including the following primers: UM4: AAG ATG TGC TGT TCC CTT CCT CTG CTC TGA; UM5: ATT ATT AAA AGA AAA GAC GAG GCG AAG TGG. Denaturation was achieved at 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds. The PCR reaction ended with 10 minutes at 72°C and storage at 4°C. The reaction created a 150 bp WT band and a 1,300 bp $IFNAR1^{-/-}$ band.

Alternatively, blood was collected from the mice and stained for PE-Cy5-TCRβ, APC-

CD4, FITC-CD11b, and/or PE-IFNAR1 and examined by flow cytometry for the presence of T cells in the blood and IFNAR1 expression on blood $CD11b^+$ cells.

2.4.8 Quantitative RT-PCR

RNA was extracted using RNEasy Mini Kit (Qiagen, product #74104), converted to cDNA using a qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD, USA, product #95048) and used in a PCR reaction with PerfeCTa qPCR FastMix (Quanta Bioscience, product #95077), FAM-labeled probes and primers for genes of interest. FAM-labeled probe and primer sets were purchased from Applied Biosystems (product #Mm00439552_s1 for *Ifnb1* and product #Mm99999915_g1 for *Gapdh*). PCR reactions were run on a 7900 Real-time PCR machine (Applied Biosystems). Quantitative expression was normalized to transcript levels of GAPDH using the formula $1/2^{\Delta CT}$.

2.4.9 Analysis of fecal bacterial DNA

DNA was extracted from one or two fecal pellets per mouse using the QIAmp DNA Stool Mini Kit (Qiagen, product #51504). Segmented filamentous bacteria were quantified by RT-PCR using PerfeCTa SYBR Green FastMix (Quanta Biosciences, product #95072) with the forward primer GACGCTGAGGCATGAGAGCAT and the reverse primer GACGGCACGGATTGTTATTCA. Levels were normalized to Eubacteria measured with the forward primer ACTCCTACGGGAGGCAGCAGT and reverse primer ATTACCGCGGCTGCTGGC. Primers were previously reported (461).

Levels of *Helicobacter hepaticus* in stool were measured by amplifying the *cdtB* gene in stool DNA with the forward primer CCGCAAATTGCAGCAATACTT, the reverse

primer TCGTCCAAAATGCACAGGTG, and the FAM-labeled probe AATATACGCGCACACCTCTCATCTGACCAT as previously reported (462). A standard curve was generated using purified *H. hepaticus* DNA of known concentrations.

Microbiome sequencing was conducted in collaboration with A. Dzutsev and G. Trinchieri. Conserved sequences of 16s rDNA were used to amplify variable regions of 16s rDNA. Samples were barcoded and the variable regions were sequenced using a 454 machine (Roche). $1x10^4$-$1.5x10^4$ reads were obtained per sample. After removal of low quality reads, high quality reads were clustered into operational taxonomic units (OTUs), which were classified using Mothur (www.mothur.org, Ann Arbor, MI) software. Alpha and beta diversities as well as Unifrac distances were also calculated using Mothur. Downstream statistical analysis was performed using Partek 6.0 software.

2.5 Statistics

Body weight curves were analyzed using one-way ANOVA and Bonferroni post-test corrections. *In vivo* studies using individual mice were analyzed using the non-parametric Mann-Whitney test, as these data are not normally distributed. *In vitro* data using cells pooled from several mice were analyzed using a student's t-test. Analysis was performed using Prism 5 software (GraphPad). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Chapter 3: The role of type I interferons on T cell-mediated colitis

3.1 Introduction

Although the innate immune system is vital in driving IBD, characterization of the mucosa from IBD patients showed a prominent role for $CD4^+$ T cell-driven immunopathology. Compared to healthy controls, intestinal sections from IBD patients showed marked infiltration of T cells (463, 464). Early studies reported that in ulcerative colitis (UC) patients, intestinal T cells produced mainly the Th2 cytokines, IL-5 and IL-13 (349, 465), while intestinal T cells from Crohn's disease (CD) patients showed a predominance toward IFN-γ production (349, 351, 352). In addition, the Th17 cell signature cytokine, IL-17, could also be found in the mucosa of both UC and CD patients in higher quantities than healthy controls (350, 354).

Lymphocytes from CD patients were also more likely to express IL-12Rβ2 and IL-18R (466, 467), components of the receptors for IL-12 and IL-18, respectively. Accordingly, IL-12 and IL-18 were also produced in greater quantities by mucosal mononuclear cells from CD patients (352, 408, 409). Both IL-12 and IL-18 are cytokines known to promote IFN-γ production from $CD4^+$ T cells (35, 39) and can synergize to further augment IFN-γ secretion (35). Studies investigating IL-12, however, were further confounded by the fact that IL-12 shares a subunit with IL-23 (468). Thus, many pathogenic roles attributed to IL-12 were actually caused by IL-23. A genome-wide association study (GWAS) identified the IL-23R as a susceptibility gene for CD (406) and subsequent studies identified a crucial role for IL-23R signaling on T cells in driving colitis in mice (441). IL-23 was originally identified as a cytokine that promoted the differentiation of Th17

cells (469), but it can also drive intestinal inflammation independently of IL-17 by inducing Th1 cells and inhibiting the induction of regulatory T cells (393, 441). However, the increased expression of IL-12Rβ2, which is a component of the IL-12R and not the IL-23R, in human CD samples (466, 467) suggests that both IL-12 and IL-23 signaling play roles in driving intestinal inflammation.

IFN-1 can signal directly onto human $CD4^+$ T cells and replace IL-12 in polarization toward a Th1 phenotype (271, 470). Addition of IFN-1 into T cell activation assays enhanced the production of IFN-γ by ten-fold (268). Although able to act independently of IL-12, IFN-1 also acted synergistically with both IL-12 and IL-18 to enhance IFN-γ production from $CD4^+$ T cells (271, 272), in part by inducing the expression of IL-12Rβ1 and IL-12Rβ2 on T cells (270, 471).

In addition to signaling directly onto $CD4^+$ T cells, IFN-1 also regulates T cell differentiation via signaling on dendritic cells. However, IFN-1 has different effects on DCs depending on the differentiation and maturation protocols used. When used in conjunction with GM-CSF during DC differentiation, IFN-1 enhanced the Th1 polarization capacity of the resultant DCs compared to DCs differentiated in the presence of the standard combination of GM-CSF and IL-4 (237). However, when DCs are differentiated with both GM-CSF and IL-4, addition of IFN-1 to the cultures inhibits their production of IL-12 (238). Addition of IFN-1 to T cells co-cultured with DCs (differentiated *in vitro* with GM-CSF and IL-4 and matured with TNF-α) inhibited IL-12 production and Th1 differentiation (245). As mentioned above, IL-12 shares the p40 subunit with the Th17-inducing cytokine, IL-23. Analysis of the IL-23-specific subunit, IL-23p19, showed that IFN-1 treatment of human DCs inhibited their ability to produce

IL-23, and $CD4^+$ T cells cultured with IFN-1-treated DCs were less likely to differentiate into Th17 cells (246). IFN-1 also induced IL-27 from DCs, which could inhibit Th17 differentiation both in vitro and in vivo (246, 247, 472).

Because IFN-1 has opposing effects on DCs and T cells, it has been difficult to decipher the role of IFN-1 in colitis. Levels of IFN-1 were observed to be increased in the guts of patients of celiac disease (473), an inflammatory disorder in which aberrant immune responses are generated against the wheat protein, gliadin. However, intestinal mononuclear cells from IBD patients were found to be unresponsive to IFN-1 (456) and polymorphisms in the genetic locus that includes *IFNAR1* and *IFNAR2* have recently been identified to increase the risk for developing IBD (457). These results suggest that IFN-1 is involved in the inflammatory process during intestinal inflammation, but whether they drive inflammation or are induced as a compensatory mechanism to suppress inflammation remains to be determined. It is possible that IFN-1 acts on different cell types *in vivo* to exert both pro- and anti-inflammatory effects, which would explain the limited and inconsistent response of IBD patients to treatment with IFN-1 in clinical trials (453, 454).

In contrast to *in vitro* human studies where direct IFN-1 signaling on $CD4^+$ T cells enhanced their production of IFN-γ, $CD4^+$ T cells exposed to exogenous or high levels of induced IFN-1 became less inflammatory and produced less IFN-γ in murine models of colitis (276, 277). To better define the role of IFN-1 signaling in intestinal inflammation, we employed the T cell adoptive transfer model of colitis (391), a murine model of chronic colitis that reproduces many of the pathological and genetic changes seen in patients with IBD (436, 437). By either transferring WT or $IFNAR1^{-/-}$ naïve $CD4^+$ T cells

into $RAG^{-/-}$ hosts, or by transferring WT naïve $CD4^+$ T cells into $RAG^{-/-}$ or $RAG^{-/-}$ x $IFNAR1^{-/-}$ double knockout (DKO) hosts, we were able to determine the distinct contributions of IFN-1 signaling on T and non-T cells.

Pathogenic IFN-γ^+ T cells can be suppressed by various subsets of immunosuppressive T cells, including $CD4^+CD25^+Foxp3^+$ Treg cells (53, 474). In human IBD, the proportion of Treg cells as a percentage of all T cells is decreased in the active areas of inflammation in IBD patients compared to unaffected areas (392). Furthermore, disease in the T cell adoptive transfer model of colitis can be prevented or cured by the co-transfer of $CD4^+CD25^+$ Treg cells (390, 391, 475), further proving the importance of Treg cells in suppressing intestinal inflamation. By assessing the ability of co-transferred WT or $IFNAR1^{-/-}$ Treg cells to suppress colitogenic naïve T cells in $RAG^{-/-}$ or DKO hosts, we were also able to examine the contribution of IFN-1 in regulating the function of this cell population vital to intestinal homeostasis.

3.2 Results

3.2.1 IFNAR signaling on host hematopoietic cells attenuates T cell-mediated colitis

We transferred $CD45RB^{hi}$ (naïve) $CD4^+$ T cells from WT or $IFNAR1^{-/-}$ mice into $RAG^{-/-}$ hosts and followed the disease course for seven weeks. Transfer of either WT or $IFNAR1^{-/-}$ naïve $CD4^+$ T cells induced equivalent wasting disease (Fig 3-1A). Both types of naïve $CD4^+$ T cells also induced equivalent colon pathology as measured by histological scoring (Fig 3-1B), expanded, and infiltrated the colon lamina propria (cLP) to a similar degree (Fig 3-1C). Isolated cLP cells were restimulated with PMA and ionomycin and

subsequently stained for intracellular cytokine production. Both WT and IFNAR1$^{-/-}$ naïve CD4^{+} T cells gave rise to equivalent frequencies of IFN-γ and IL-17-secreting T cells (Fig 3-1D). Thus, IFNAR1 signaling in naïve CD4^{+} T cells was not required for colitogenic activity.

To assess whether IFN-1 signaling in non-T cells regulated colitis, we transferred WT naïve CD4^{+} T cells into RAG$^{-/-}$ x IFNAR1$^{-/-}$ DKO mice. Compared to RAG$^{-/-}$ mice, DKO mice developed a more severe and rapid wasting disease (Fig 3-2A). Typically, RAG$^{-/-}$ mice show symptoms of colitis six to eight weeks after T cell transfer, but DKO mice began losing weight and showed severe colon histopathology by three weeks (Fig 3-2B). DKO recipients also had a significant increase in the number of CD4^{+} T cells that accumulated in the cLP compared to RAG$^{-/-}$ recipients (Fig 3-2C). In addition, while the proportions of colonic CD4^{+} T cells producing IFN-γ in RAG$^{-/-}$ or DKO recipients were similar, the proportions producing IL-17 (either alone or in conjunction with IFN-γ) were significantly higher among colonic CD4^{+} T cells isolated from DKO recipients (Fig 3-3A,B).

Although most cells in the body express IFNAR, including both hematopoietic and non-hematopoietic cells, we hypothesized that the enhanced accumulation of CD4^{+} T cells in the cLP of DKO recipients was due to an enhancement in the activation and stimulatory signals T cells receive from hematopoietic cells. To determine the cell types responsible for the indirect effects of IFN-1 signaling on T cell expansion and colitis development, we reconstituted lethally irradiated RAG$^{-/-}$ mice with bone marrow (BM) from RAG$^{-/-}$ or DKO mice. Following BM reconstitution, the chimeras were adoptively transferred with WT naïve CD4^{+} T cells. Two weeks after the transfer of WT naïve CD4^{+} T cells, DKO

BM chimeric mice exhibited rapid weight loss (Fig 3-4A), significantly exacerbated colitis (Fig 3-4B) and a significantly greater accumulation of $CD4^+$ T cells in the colon than $RAG^{-/-}$ BM chimeric mice (Fig 3-4C), echoing the phenotype observed in DKO mice. Also similar to results found in DKO mice, while no differences were observed in the proportions of cLP $CD4^+$ T cells producing IFN-γ between DKO and $RAG^{-/-}$ BM chimeric recipients, the percentages producing IL-17 or IL-17 and IFN-γ were significantly higher in DKO BM chimeric recipients than in $RAG^{-/-}$ BM chimeric recipients (Fig 3-4D). Together, these results suggest that both the increased expansion of T cells as well as the enhanced IL-17 production by T cells can be indirectly suppressed by IFNAR signaling on non-T hematopoietic cells.

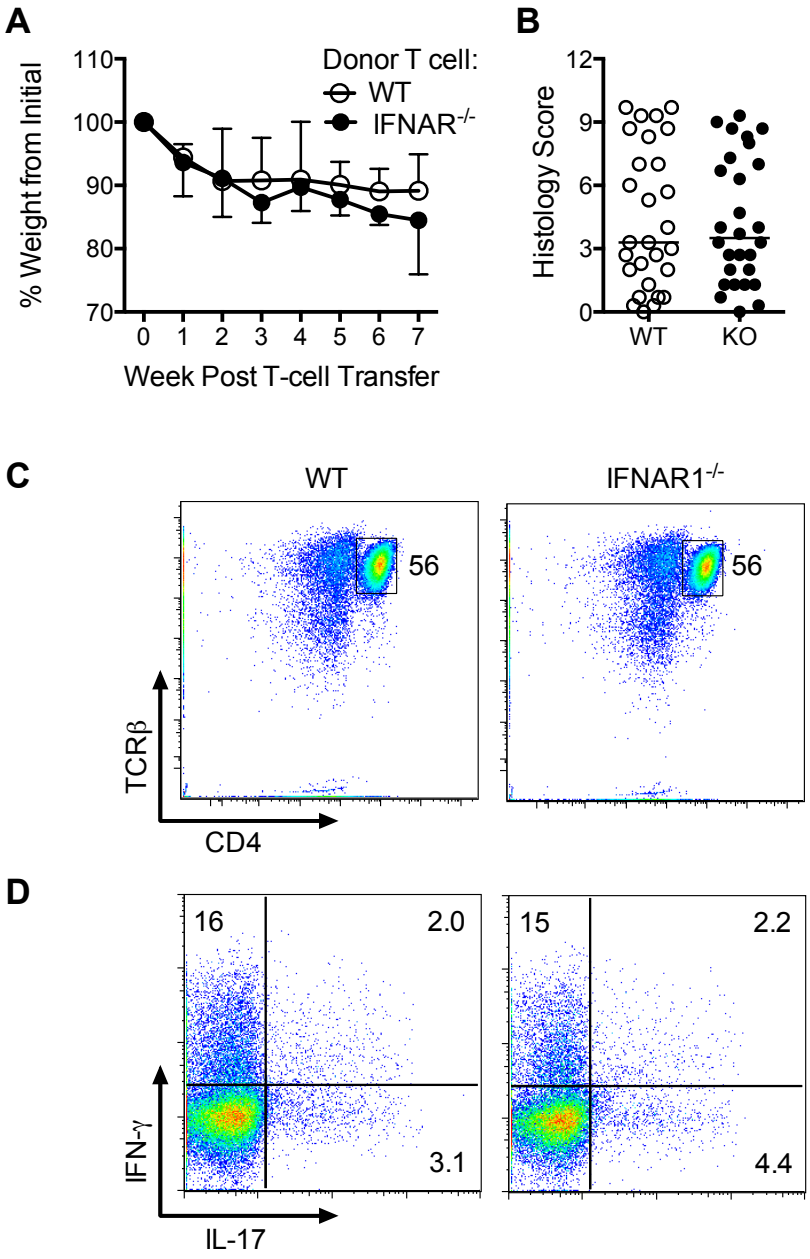


Figure 3-1. WT and IFNAR1$^{-/-}$ naive CD4^{+} T cells induce equivalent colitis. 3x10^{5} naïve CD4^{+} T cells from WT or IFNAR1$^{-/-}$ spleens were transferred into RAG$^{-/-}$ hosts. (A) Mean body weight +/- SD from one experiment representative of four independent experiments is shown (n=5-7 mice per group). (B) Colon histological scores from four independent experiments with similar results were compiled (n=4-12 per experiment, per group). Each symbol represents an individual mouse and horizontal bars represent the median. (C) Cells were isolated from the cLP and stained for TCRβ and CD4. One representative FACS plot pre-gated on live cells is shown. (D) cLP cells were restimulated *in vitro* for five hours with PMA and ionomycin and stained for intracellular cytokines. One representative FACS plot pre-gated on live CD4^{+} T cells is shown. Plots in (C) and (D) are from one experiment representative of four independent experiments with similar results, and display cells pooled from at least four mice per group. Numbers represent the percentage of cells in each quadrant or gate.

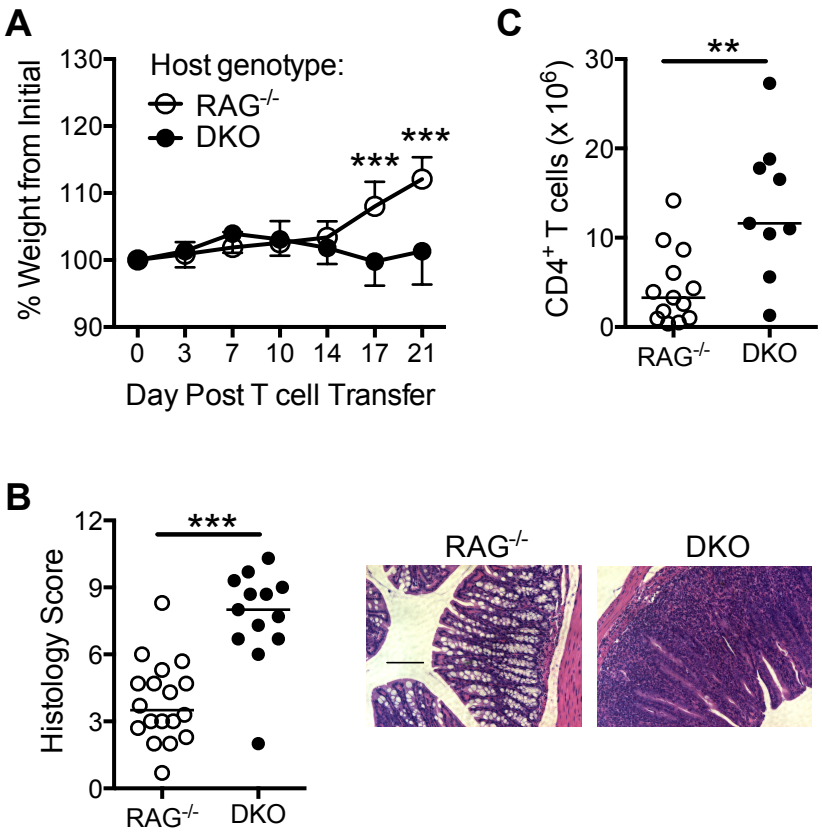


Figure 3-2. WT naïve $CD4^+$ T cells cause more severe and accelerated colitis in DKO mice. $3x10^5$ WT naïve $CD4^+$ T cells were transferred into $RAG^{-/-}$ or DKO hosts. Mean body weight +/- SD throughout the experiment (A); colon histological scores (B) and quantification of the $CD4^+$ T cells in the cLP (C) at three weeks post-transfer are shown. (B) Representative phtotomicrographs of the cLP three weeks post-T cell transfer are shown. Bar, 100 µm. Data shown is compiled from five (A, B) or three (C) independent experiments, each with at least three mice per group. Each symbol in (B) and (C) represents an individual mouse and horizontal bars represent the median. Statistics were calculated using a two-way ANOVA with Bonferroni post-test corrections (A) or Mann-Whitney U-test (B, C). **p<0.01; ***p<0.001.

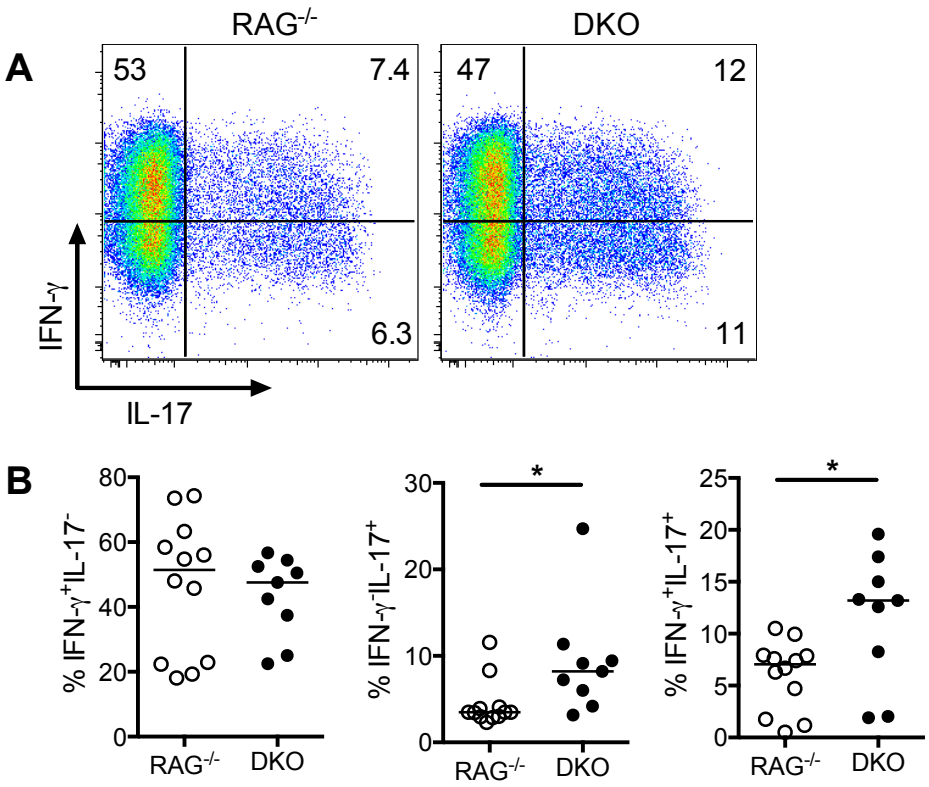


Figure 3-3. DKO recipients have increased frequencies of IL-17-producing T cells during colitis. cLP cells were isolated from RAG$^{-/-}$ or DKO recipients of WT naïve CD4^{+} T cells, restimulated *in vitro* for five hours (10^6 cells/mL) with PMA (50 ng/mL) and ionomycin (500 ng/mL) and stained for intracellular cytokines. (A) One representative FACS plot pre-gated on CD4^{+} T cells is shown. The FACS plot displays cells pooled from five mice per group from one experiment representative of three independent experiments with similar results. Numbers represent the percentage of cells in each quadrant. (B) Percentages of CD4^{+} T cells producing different combinations of cytokines are shown. Data is compiled from three independent experiments with similar results, each with at least three mice per group. Each symbol represents an individual mouse and horizontal bars represent the median. Statistics were calculated using the Mann-Whitney U-test. *$p<0.05$

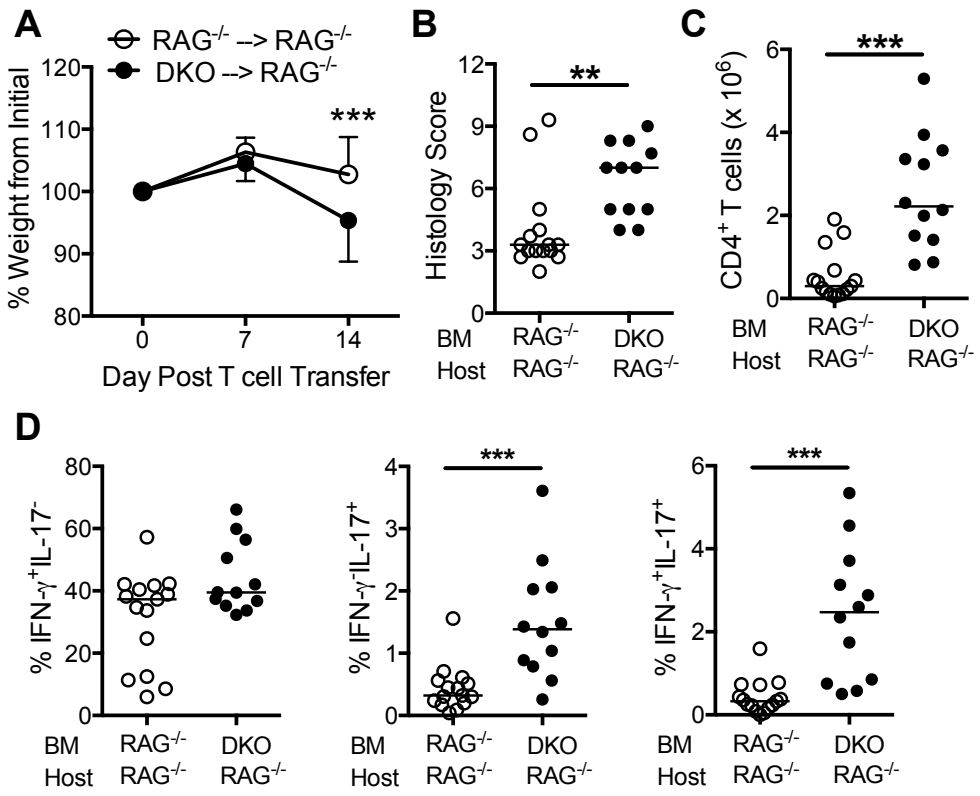


Figure 3-4. Suppression of T cell accumulation and IL-17 production in the colon is dependent on IFNAR signaling on host hematopoietic cells. $3x10^5$ naïve T cells from WT spleens were transferred into $RAG^{-/-}$ mice that had previously been irradiated and reconstituted with BM from $RAG^{-/-}$ or DKO mice. Mean body weight +/- SD (A), colon histological scores (B), and quantification of the $CD4^+$ T cells in the cLP (C) are shown at two weeks post-transfer. (D) cLP cells were restimulated for five hours (10^6 cells/mL) with PMA (50 ng/mL) and ionomycin (500 ng/mL) and stained for intracellular cytokines. Percentages of $CD4^+$ T cells producing different combinations of cytokines are shown. Data shown is pooled from three independent experiments, each containing a minimum of three mice per group. Each symbol in (B)-(D) represents an individual mouse and horizontal bars represent the median. Statistics were calculated using a two-way ANOVA with Bonferroni post-test corrections (A) or Mann-Whitney U-test (B-D). **p<0.01; ***p<0.001.

3.2.2 Type I interferon signaling on dendritic cells inhibits T cell proliferation and Th17 cell differentiation *in vitro*.

To confirm that the enhanced T cell accumulation and Th17 differentiation we observed in DKO mice was due to a lack of IFNAR signaling on non-T hematopoietic cells, we moved to an *in vitro* system. WT naïve $CD4^+$ T cells were co-cultured with WT or $IFNAR1^{-/-}$ DCs and anti-CD3 in the presence or absence of IFN-α. After five days of culture, we found that the proportion of live $CD4^+$ T cells was increased when IFN-α was added to the cultures and this effect was dependent on IFNAR signaling in DCs (Fig 3-5A). Anti-CD3 generally activates T cells and induces cytokine production, but can paradoxically also induce apoptosis in T cells (476). To determine whether the increased proportion of live $CD4^+$ T cells in this context was due to protection from apoptosis or increased proliferation, we assayed T cell proliferation by measuring incorporation of H^3-labeled thymidine into actively dividing cells on the third day of culture. However, despite the increased T cells viability, IFNAR signaling on DCs did not enhance T cell proliferation, but rather inhibited it (Fig 3-5B). Thus, these results suggest that IFN-1 signal via DCs to enhance T cell survival while simultaneously inhibiting T cell proliferation.

We next used a similar co-culture assay to analyze the effects of IFNAR signaling on $CD4^+$ T cell polarization. We sorted naïve $CD4^+$ T cells from OTII transgenic mice with a TCR specific for a peptide of ovalbumin (OVA) and cultured them with DCs from WT or $IFNAR1^{-/-}$ mice plus OVA peptide, in the presence or absence of IFN-α. In the absence of polarizing cytokines, TCR-engaged naïve T cells differentiate into IFN-γ-producing Th1 cells by default (247). Indeed, we found that when presented by either WT or $IFNAR1^{-/-}$ DCs, OVA peptide robustly induced Th1 cells and that addition of exogenous IFN-α did

not alter Th1 cell differentiation (Fig 3-6A). In the presence of IL-6 and TGFβ, stimulated naïve $CD4^+$ T cells differentiate into Th17 cells (42, 43). Consistently, we found that in the presence of these polarizing cytokines, Th1 differentiation was completely inhibited and a large proportion of the $CD4^+$ T cells expressed IL-17 (Fig 3-6B). Furthermore, consistent with previously published data (247), we observed that addition of exogenous IFN-α inhibited Th17 differentiation and this inhibition required IFNAR signaling on DCs (Fig 3-6B). However, when we added additional Th17-skewing cytokines (IL-1β and TNF-α) to the cocktail, and concomitantly blocked Th1 and Th2 cytokines, we observed enhanced differentiation of Th17 cells, which could not be inhibited by the addition of IFN-α (Fig 3-6B). Thus, exogenous supplementation with factors mimicking a Th17-polarizing inflammatory environment can overcome the inhibitory effects of IFN-α on the ability of DCs to prime Th17 cell responses.

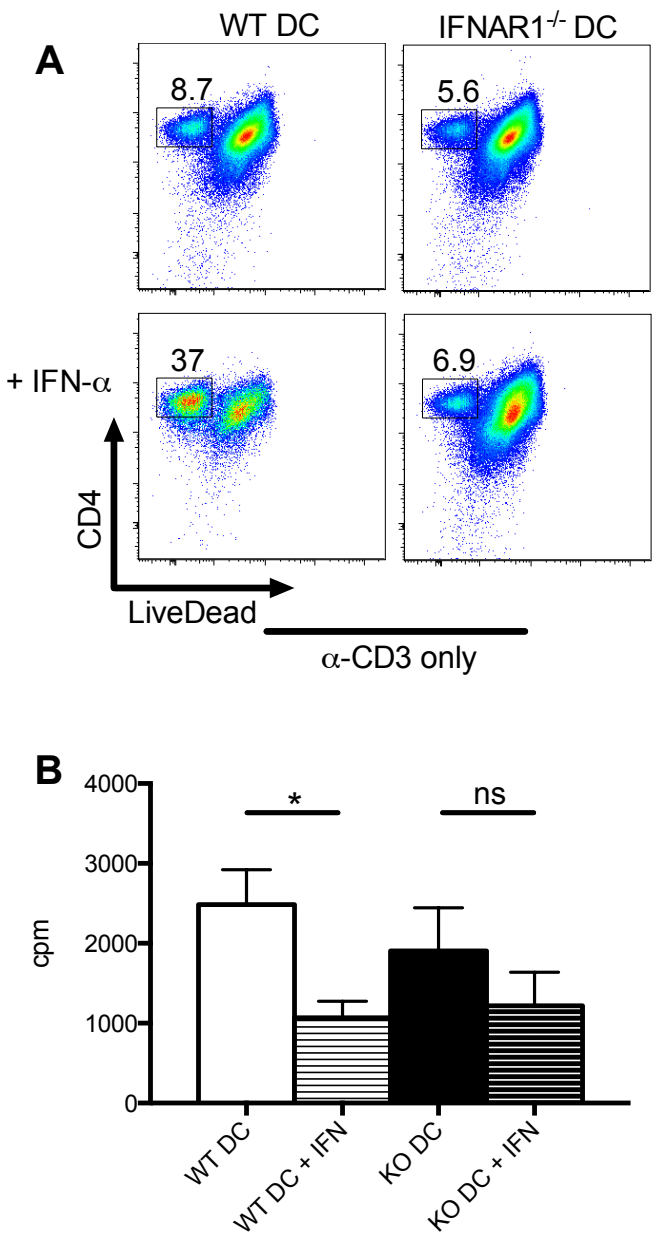


Figure 3-5. IFNAR signaling on DCs inhibits T cell proliferation, but enhances T cell viability *in vitro*. WT splenic $CD62L^{hi}CD44^{lo}$ (naïve) $CD4^+$ T cells were co-cultured with splenic $CD11c^+$ DCs from WT or $IFNAR1^{-/-}$ mice at a ratio of 5:1 with anti-CD3 (1 μg/mL) in the presence or absence of IFN-α (100 U/mL). (A) After five days, cells were taken out of culture and stained for CD4 and the viability dye, LiveDead. FACS plots are from one experiment representative of five independent experiments with similar results. Numbers represent the percentage of total cells that are live $CD4^+$. (B) After three days, H^3-labeled thymidine was added to the cultures for seven hours and uptake was measured as counts per minute (cpm) on a β-radiation counter. Data shown are the means +/- SD of three replicates per condition from one experiment. Statistics were calculated using a student's t-test. *$p<0.05$.

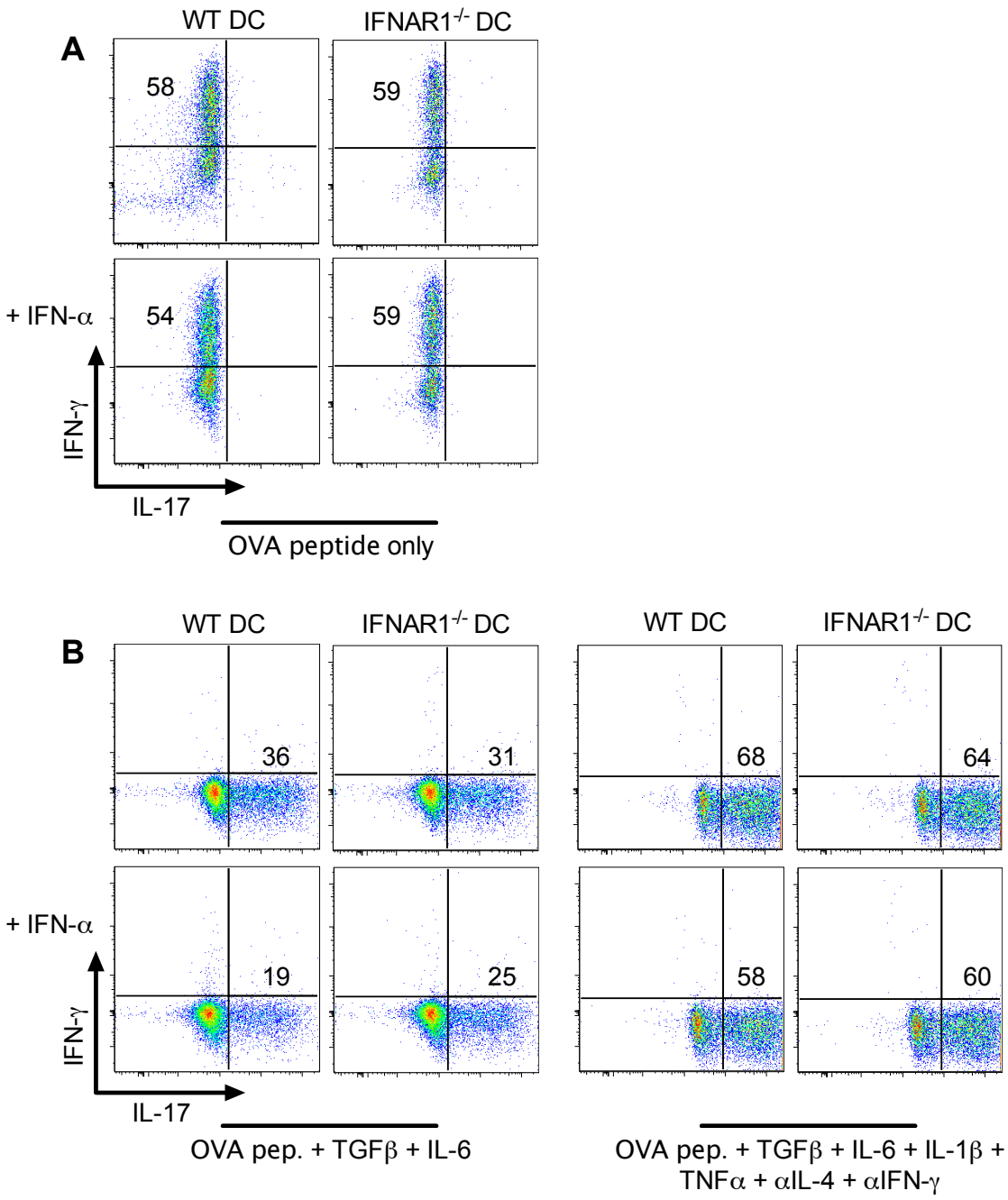


Figure 3-6. IFN-1 inhibits Th17 cell differentiation *in vitro* via signaling on DCs. RAG$^{-/-}$/OT-II transgenic splenic CD62L^{hi}CD44^{lo} (naïve) CD4^{+} T cells were co-cultured with splenic CD11c^{+} DCs from WT or IFNAR1$^{-/-}$ mice at a ratio of 5:1 with OVA peptide (1 μM) in the presence of TGF-β (5 ng/mL), IL-6 (20 ng/mL), IL-1β (10 ng/mL), anti-IFN-γ (10 μg/mL), anti-IL-4 (10 μg/mL), and/or IFN-α (100 U/mL). After five days, cells were restimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) for five hours and stained for intracellular cytokine production. Data shown is from one experiment representative of five similar experiments. FACS plots are pre-gated on live CD4^{+} T cells and numbers represent the percentage of CD4^{+} T cells producing the indicated cytokine.

3.2.3 IL-1-dependent T cell proliferation in the MLNs drives T cell accumulation in the colon.

The increased accumulation of $CD4^+$ T cells we observed in DKO mice could be due to either increased proliferation of T cells, an increased migration of T cells to the colon, or enhanced survival of T cells in the colon microenvironment. Our *in vitro* data suggested that IFN-1 might inhibit T cell proliferation via signaling on DCs (Figure 3-5). To test this hypothesis *in vivo*, we labeled naïve $CD4^+$ T cells with carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye that labels cells for long periods of time. When a cell divides, the dye is divided evenly among the daughter cells. Thus, the level of CFSE detected in a cell indicates its proliferative history. Seven days after injection into $RAG^{-/-}$ or DKO hosts, we isolated $CD4^+$ T cells from the MLNs, the draining lymph node for the colon and a major site for the induction of intestinal T cell responses (5, 477). We found increased frequencies of $CD4^+$ T cells in the MLNs of DKO mice, even at this early time point (Fig 3-7A). In contrast, very few $CD4^+$ T cells could be detected in the colon at this early time point, even in DKO recipients (Fig 3-8), suggesting that the accumulation of $CD4^+$ T cells observed in DKO mice later during colitis (Fig 3-2) occurred following expansion in the MLNs.

We next looked at the levels of CFSE in the $CD4^+$ T cells recovered from the MLNs. The majority of $CD4^+$ T cells from the MLNs of DKO recipients had undergone extensive proliferation to the point that CFSE could no longer be detected in the divided cells (Fig 3-7B). In contrast, $CD4^+$ T cells from the MLNs of $RAG^{-/-}$ recipients had either retained all of their CFSE signal (44%) or lost CFSE in two discrete peaks (55%), indicating they had divided 0-3 times (Fig 3-7B). Furthermore, the slowly proliferating $CD4^+$ T cells recovered from $RAG^{-/-}$ recipients expressed CD62L, a marker of naïve T cells and of T

cells undergoing homeostatic proliferation, while the rapidly dividing T cells from DKO recipients expressed low levels of CD62L and high levels of CD44 (Fig 3-7C,D), a marker of effector and memory T cells (478). It is important to note, however, that we cannot determine the antigen-specificity of the enhanced T cell proliferation in DKO mice from this experiment.

IL-1 has been previously implicated in driving antigen-specific T cell proliferation in the lymph nodes (479). Thus, we next asked whether the increased T cell proliferation could be blocked with anakinra, an antagonist of the human IL-1 receptor (IL-1RA) with cross-reactivity in mice (480, 481). We found that anakinra treatment did indeed inhibit $CD4^+$ T cell accumulation in the MLNs of DKO recipients, without having any effect on $CD4^+$ T cell accumulation in the MLNs of $RAG^{-/-}$ recipients (Fig 3-9A,B). These data suggest that DKO mice have increased T cell proliferation in the MLNs due at least partially to an inability to regulate the IL-1 axis.

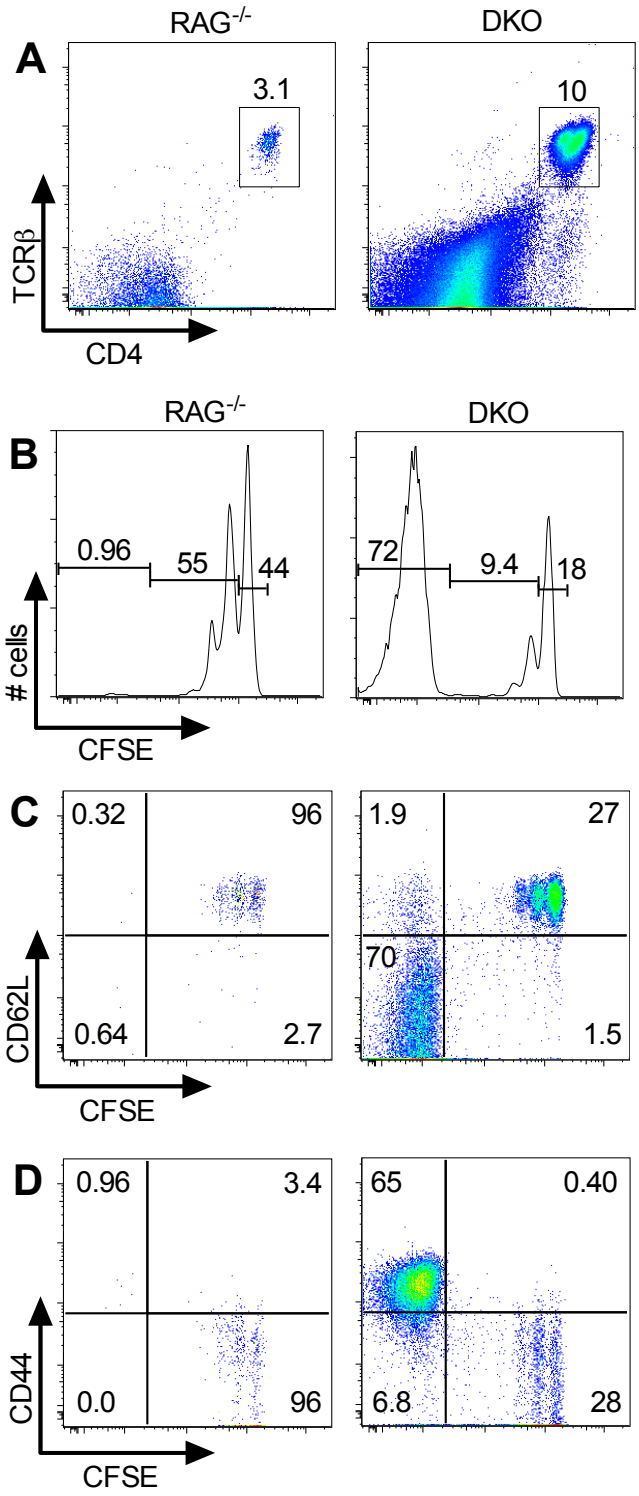


Figure 3-7. Increased CD4$^+$ T cell accumulation in DKO mice correlates with rapid proliferation in the MLNs. 1x10^6 CFSE-labeled naïve CD4$^+$ T cells were transferred into RAG$^{-/-}$ or DKO mice. Seven days later, cells were isolated from the MLN and stained directly. Live cells were gated on CD4$^+$ T cells (A) and analyzed for CFSE dilution (B), CD62L expression (C), and CD44 expression (D). Data shown is from one experiment representative of two independent experiments with similar results, each with at least three mice per group. Plots shown are composed of cells pooled from three mice per group.

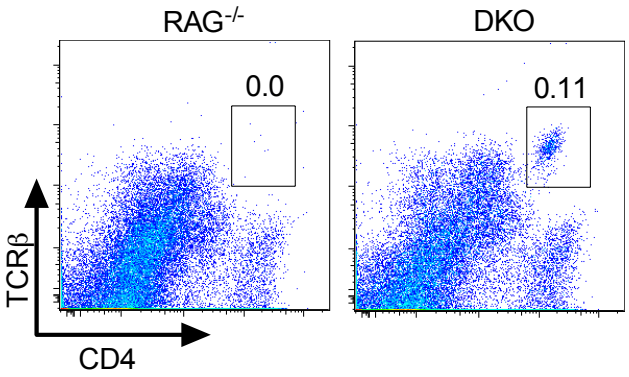


Figure 3-8. Early T cell accumulation in the colon in DKO mice is limited. $1x10^6$ CFSE-labeled naïve $CD4^+$ T cells were transferred into $RAG^{-/-}$ or DKO mice. Seven days later, cLP cells were isolated and stained directly. FACS plots shown are pre-gated on live cells and are representative of two independent experiments with similar results, each with at least three mice per group. Plots shown are composed of cells pooled from three mice per group.

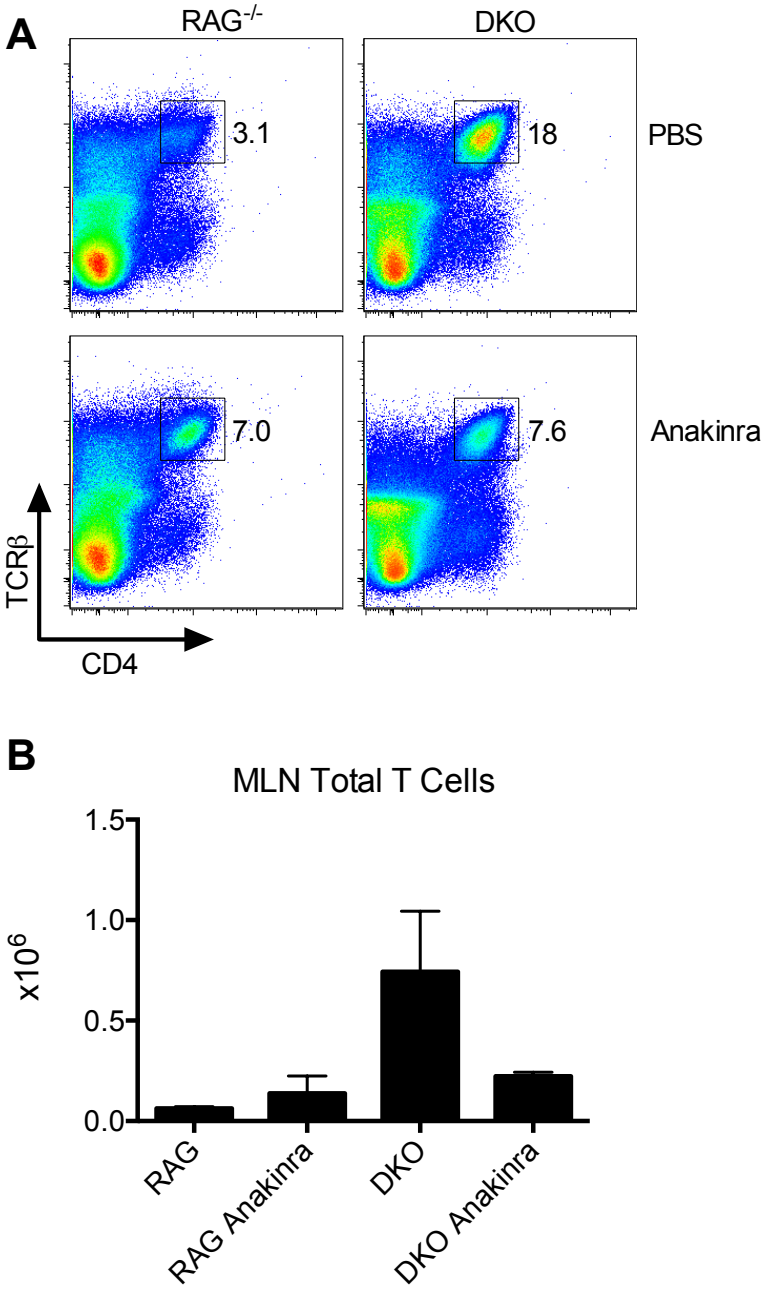


Figure 3-9. IL-1RA inhibits accelerated T cell proliferation in the MLNs. $3x10^5$ naïve $CD4^+$ T cells were transferred into $RAG^{-/-}$ or DKO mice with or without treatment with 1 mg of anakinra (IL-1RA) daily. Ten days later, cells were isolated from the MLNs and $CD4^+$ T cells were counted. (A) FACS plots pre-gated on live cells from one experiment representative of two independent experiments with similar results are shown. Plots shown are composed of cells pooled from five mice per group. Numbers represent the percentage of live cells that are $CD4^+$ T cells. (B) Absolute counts of $CD4^+$ T cells in the MLNs. Data shown is the mean +/- SD of two independent experiments with similar results, each with cells pooled from at least five mice per group.

3.2.4 IFNAR signaling is not required for Foxp3$^+$ Treg cell induction.

Foxp3$^+$ Treg cells are essential for intestinal homeostasis (482). They can both prevent the development of colitis as well as cure established colitis (390, 391). Thus, we next investigated whether IFN-1 could regulate the size of the regulatory CD4$^+$ T cell population as it did with effector T cells. In the steady state colon, the proportion of Foxp3$^+$ Treg cells was equivalent in WT and IFNAR1$^{-/-}$ mice (Fig 3-10). We next looked specifically at Treg cell induction using an *in vitro* system. TGF-β is essential for the conversion of naïve peripheral T cells to Foxp3$^+$ Treg cells (57). In the absence of this cytokine, WT OTII naïve CD4$^+$ T cells did not convert to Foxp3$^+$ Treg cells whether WT or IFNAR1$^{-/-}$ DCs were used to provide co-stimulation (data not shown). However, with the addition of TGF-β, we observed modest Foxp3$^+$ Treg cell conversion using either WT or IFNAR1$^{-/-}$ DCs and that this was not affected by addition of IFN-α (Fig 3-11). IFNAR1$^{-/-}$ DCs in fact appeared to convert naïve CD4$^+$ T cells to Foxp3$^+$ Treg cells with slightly greater efficiency (Fig 3-11).

We next looked for *de novo* conversion of naïve CD4$^+$ T cells to Treg cells in two different *in vivo* models. First, we transferred congenically labeled CD45.1$^+$ naïve CD4$^+$ T cells from OT-II transgenic mice into WT or IFNAR1$^{-/-}$ mice, in which all endogenous T cells were CD45.2$^+$. After feeding OVA, dendritic cells convert OVA-specific T cells into Foxp3$^+$ Treg cells, through a retinoic acid-dependent process that occurs in both the MLNs and the Peyer's patches (PP) (6). Indeed, we found that, following oral administration of OVA, a small population of Foxp3$^+$CD45.1$^+$CD4$^+$ T cells emerged from the transferred OTII naïve CD4$^+$ T cell population in both WT and IFNAR1$^{-/-}$ recipients (Fig 3-12). Furthermore, the naïve to Foxp3$^+$ T cell conversion rates observed in both the

MLNs and PPs were similar between the two groups of recipient mice and were comparable to previously published data (6).

Secondly, we looked for the conversion of naïve $CD4^+$ T cells to $Foxp3^+$ Treg cells after transfer into lymphopenic hosts. Following transfer of WT naïve $CD4^+$ T cells into either $RAG^{-/-}$ or DKO recipients, we observed the differentiation of a small population of $Foxp3^+$ Treg cells that was of equivalent magnitude in the MLNs or cLP of either recipient group (Fig 3-13A,B). As a confirmatory experiment, we also assayed $Foxp3^+$ Treg cell induction in BM chimeric mice in which irradiated $RAG^{-/-}$ hosts were reconstituted with bone marrow from either $RAG^{-/-}$ or DKO mice and then injected with WT naïve $CD4^+$ T cells as described above. Again, we found equivalent $Foxp3^+$ Treg cell induction in both recipient groups (Fig 3-13C), confirming that IFNAR signaling was not required for $Foxp3^+$ Treg cell conversion.

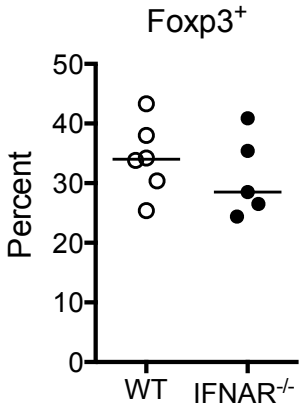


Figure 3-10. WT and IFNAR1$^{-/-}$ mice have similar proportions of Foxp3^{+} Treg cells. cLP cells were isolated from the colons of WT or IFNAR1$^{-/-}$ mice and stained for intracellular Foxp3 expression prior to analysis by flow cytometry. Each symbol represents an individual mouse and horizontal bars represent the median percentage of CD4^{+} T cells expressing Foxp3. Data is compiled from two independent experiments with similar results.

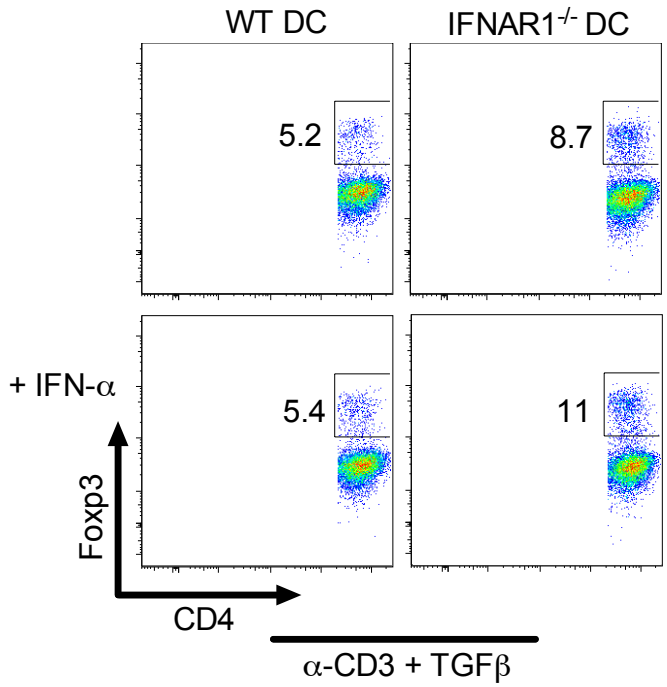


Figure 3-11. IFNAR1$^{-/-}$ DCs are not defective in Foxp3^{+} Treg cell induction *in vitro*. RAG$^{-/-}$/OT-II transgenic splenic CD62L^{hi}CD44^{lo} (naïve) CD4^{+} T cells were co-cultured with splenic CD11c^{+} DCs from WT or IFNAR1$^{-/-}$ mice at a ratio of 5:1 in the presence of anti-CD3 (1 μg/mL) and TGF-β (5 ng/mL). IL-2 (5 ng/mL) was added to the cultures 24 and 72 hours after the start of the culture. Five days later, cells were harvested and stained for intracellular Foxp3. FACS plots shown are pre-gated on live CD4^{+} T cells and are from one experiment representative of five similar experiments. Numbers represent the percentage of CD4^{+} T cells expressing Foxp3.

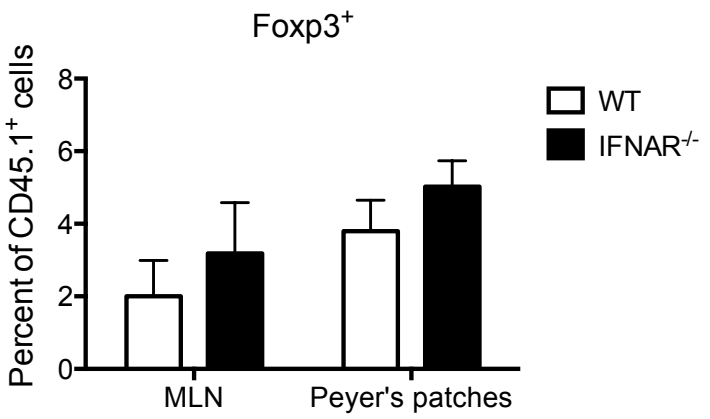


Figure 3-12. Equivalent *de novo* induction of antigen-specific Foxp3$^+$ Treg cells in WT and IFNAR1$^{-/-}$ mice. 1x10^6 CD45.1$^+$RAG$^{-/-}$/OT-II transgenic CD4$^+$ T cells were injected into CD45.2$^+$ WT or IFNAR1$^{-/-}$ mice. Recipient mice were fed ovalbumin in the drinking water for five days. On day 7, cells were isolated from the MLNs and Peyer's patches and stained for surface markers and intracellular Foxp3. Data shown is the mean +/- SD from one experiment with two (IFNAR1$^{-/-}$) or three (WT) mice per group.

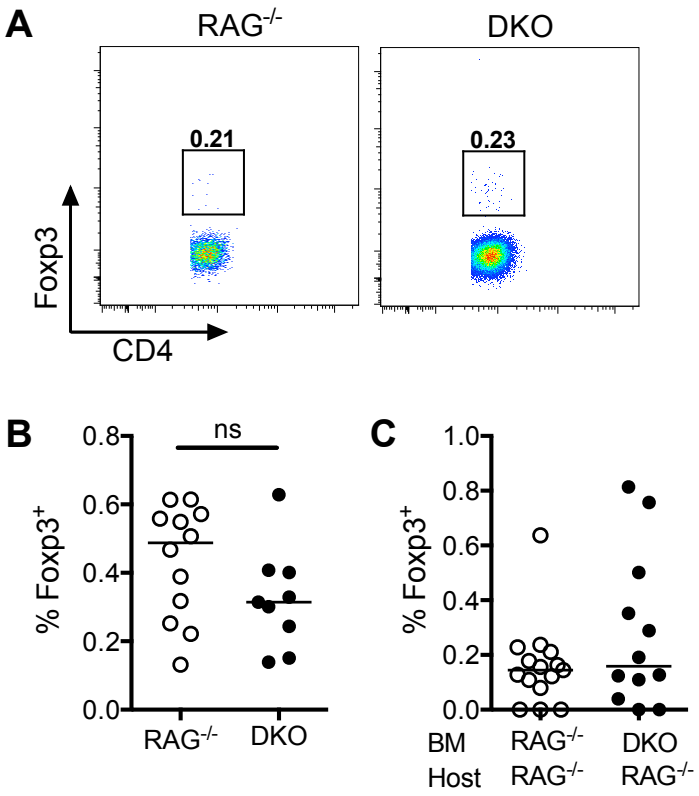


Figure 3-13. Equivalent conversion of naïve CD4$^+$ T cells into Foxp3$^+$ Treg cells in WT and IFNAR1$^{-/-}$ lymphopenic hosts. 3x10^5 naïve CD4$^+$ T cells were transferred into either RAG$^{-/-}$ or DKO mice (A, B), or irradiated RAG$^{-/-}$ mice reconstituted with bone marrow from RAG$^{-/-}$ or DKO mice (C). Ten days (A), two weeks (C), or three weeks (B) later, MLN (A) or cLP (B, C) cells were isolated and stained for intracellular expression of Foxp3. (A) FACS plots are from one experiment representative of three independent experiments with similar results, each with MLNs pooled from five mice per group. Numbers represent the percentage of live CD4$^+$ T cells expressing Foxp3. (B, C) Each symbol represents an individual mouse and horizontal bars represent the medians. Data shown in (B) and (C) are each compiled from three independent experiments with similar results, each with at least three mice per group. Statistics were calculated using a Mann-Whitney U-test.

3.2.5 IFNAR signaling on host cells is required for maintenance of the Foxp3$^+$ Treg cell population during colitis.

Although the induction of Treg cells was unaffected by IFNAR signaling, we hypothesized that IFN-1 could still influence the function or maintenance of Treg cells. To test this, we first co-transferred WT naïve CD4$^+$ T cells into RAG$^{-/-}$ mice with CD4$^+$CD25$^+$ Treg cells isolated from WT or IFNAR1$^{-/-}$ mice. We found that both WT and IFNAR1$^{-/-}$ Treg cells were efficiently able to suppress weight loss, intestinal pathology and the accumulation of CD4$^+$ T cells in the cLP (Fig 3-14A-C). Consistent with suppression of intestinal inflammation, both WT and IFNAR1$^{-/-}$ Treg cells also potently inhibited the accumulation of IFN-γ-producing T cells in the colon (Fig 3-14D). In contrast, co-transfer of WT or IFNAR1$^{-/-}$ Treg cells was associated with an increased frequency of IL-17-producing T cells in the colon (Fig 3-14D), consistent with previously published data showing that Treg cell consumption of IL-2 inhibits Th17 cell polarization (483, 484). Finally, recent data suggested that IFNAR1$^{-/-}$ Treg cells are unstable in inflammatory settings because Treg cells require IFN-1 for maintenance of Foxp3 expression (485). However, we found that both WT and IFNAR1$^{-/-}$ Foxp3$^+$ cells could be recovered from the cLP and constituted around 25% of all CD4$^+$ T cells, which is very similar to the initial ratio of Treg cells transferred (Fig 3-14E). Treg cell conversion from the CD45RB^{hi} population is minimal (Fig 3-13), so it is likely that the vast majority of these Treg cells represent stability of the transferred CD4$^+$CD25$^+$ Treg cell population and not *de novo* conversion.

We next asked whether IFNAR signaling on a host cell could indirectly regulate the Treg cell population, as was the case for the expansion of effector T cells (Fig 3-2). Thus, we co-transferred CD45.1$^+$ naïve T cells with congenic CD45.2$^+$CD4$^+$CD25$^+$Foxp3^{GFP+} Treg

cells into either RAG$^{-/-}$ or DKO recipients. We found that, following co-transfer, Foxp3^{+} Treg cells constituted a smaller proportion of total CD4^{+} T cells in the inflamed colons of DKO recipients relative to that found in RAG$^{-/-}$ recipients (Fig 3-15A). Thus, the mechanisms that led to greater expansion of effector T cells in DKO mice did not proportionally expand regulatory T cells as well. Furthermore, although almost all of the CD45.2^{+} Treg cells maintained expression of Foxp3 when co-transferred into RAG$^{-/-}$ recipients, approximately 50% of co-transferred CD45.2^{+} Treg cells lost expression of Foxp3 in DKO recipients (Fig 3-15B). Additionally, the instability of the transferred Foxp3^{+} Treg cell population was not restricted to the site of inflammation. We found that CD45.2^{+} cells constituted a smaller percentage of CD4^{+} T cells and lost Foxp3 expression in the MLNs as well (Fig 3-15C,D). Consistent with continued Foxp3 expression being crucial for maintaining Treg cell suppressor phenotype (486), we observed that a subset of the co-transferred CD45.2^{+} Foxp3^{+} T cells that had lost Foxp3 had gained the ability to produce IFN-γ and IL-17 and that these populations were significantly elevated in the cLP of DKO recipients (Fig 3-16A,B).

It must be noted that the emergence of CD45.2^{+}Foxp3^{-} effector T cells could have been due to preferential expansion of a contaminating population of Foxp3^{-} cells within the sorted Treg cells. However, this was unlikely as the CD4^{+}Foxp3^{GFP+} Treg cells sorted as in Fig 3-17A were 98.8% Foxp3^{+} (Fig 3-17B). Furthermore, when transferring only sorted CD4^{+}Foxp3^{GFP+} Treg cells into RAG$^{-/-}$ or DKO mice, the small population of contaminating Foxp3^{-} cells failed to expand and accumulate in the cLP (Fig 3-18). Conversely, the sorted naïve CD4^{+} T cells contained no detectable contamination of Foxp3^{+} cells (Fig 3-17C,D).

Treg cell expansion and maintenance have both been linked to IL-10 produced by myeloid cells in the cLP (327, 343). Thus, we asked whether we could enhance expansion of CD45.2^{+}Foxp3^{GFP+} Treg cells and prevent the loss of Foxp3 by administering exogenous IL-10 throughout the course of colitis development. However, we found that upon co-transfer into DKO recipients, Treg cells could not inhibit the accumulation of effector CD4^{+} T cells in the cLP with or without IL-10 treatment (Fig 3-19A). In addition, recombinant IL-10 treatment had negligible effects on Foxp3^{+} Treg cell expansion (Fig 3-19B). Based on preliminary results, it is possible that IL-10 treatment could protect Foxp3^{+} Treg cells from loss of Foxp3 (Fig 3-19C), but additional experiments with more mice will need to be done in order to confirm this conclusion.

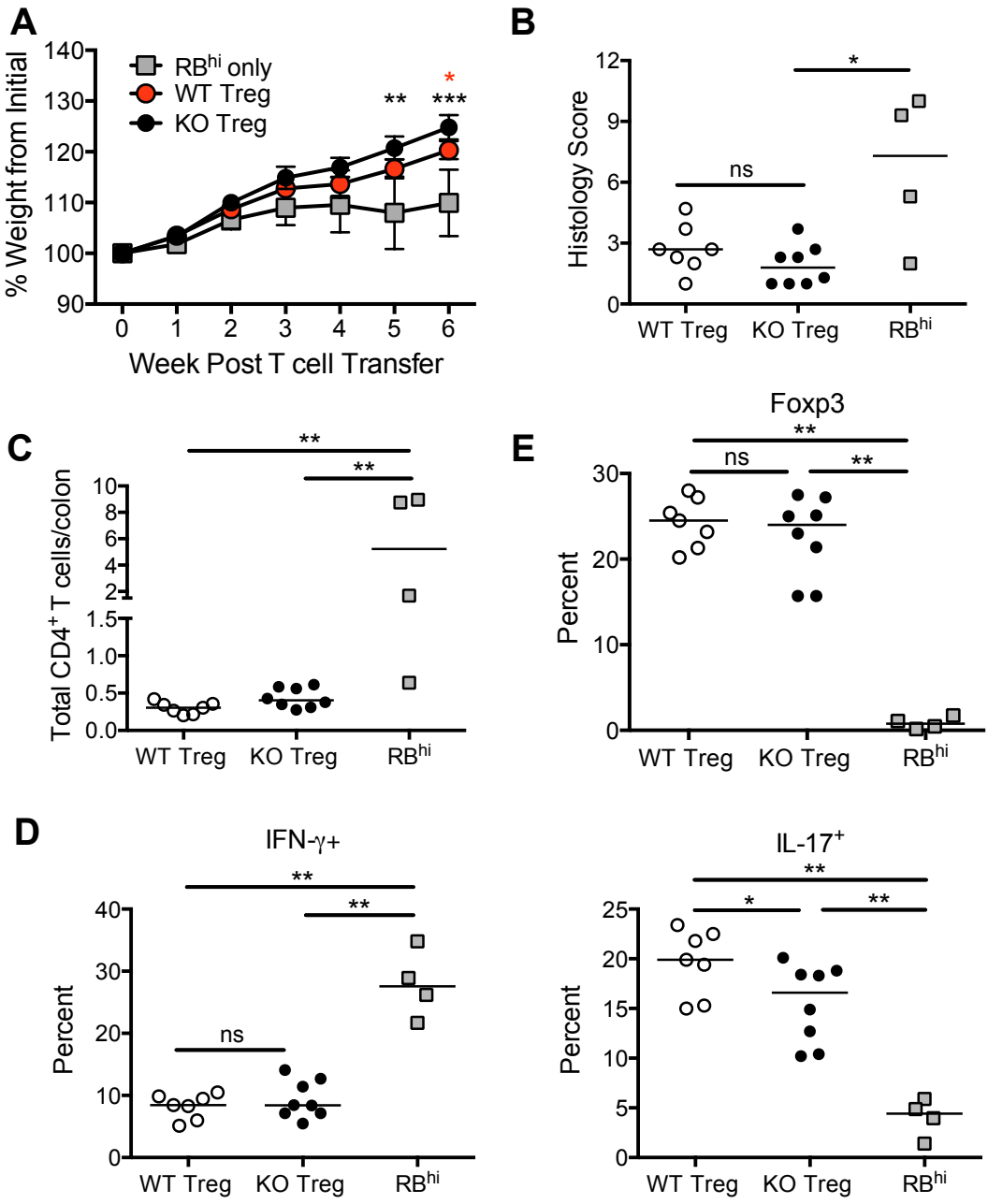


Figure 3-14. WT and IFNAR1$^{-/-}$ Treg cells inhibit T cell transfer colitis. WT RAG$^{-/-}$ recipients were adoptively transferred with WT naïve CD4^{+} T cells with or without CD4^{+}CD25^{+} Treg cells from WT or IFNAR$^{-/-}$ mice at a 4:1 ratio. Weight loss (A), histopathology (B), counts of cLP CD4^{+} T cells (C), cytokine production from restimulated cLP CD4^{+} T cells (D), and Foxp3 expression in cLP CD4^{+} T cells (E) are shown. Data shown is from one experiment representative of two independent experiments with similar results. Means +/- SD of individual mice are shown for weight curves. In all other plots, each symbol represents an individual mouse and horizontal bars represent the medians. Statistics were calculated using a two-way ANOVA with Bonferroni post-test corrections (A) or Mann-Whitney U-test (B-E). Asterisks in (A) representing statistical significance are color-coded to represent WT Treg cells (red) or KO Treg cells (black) compared to RB^{hi} only. *p<0.05, **p<0.01.

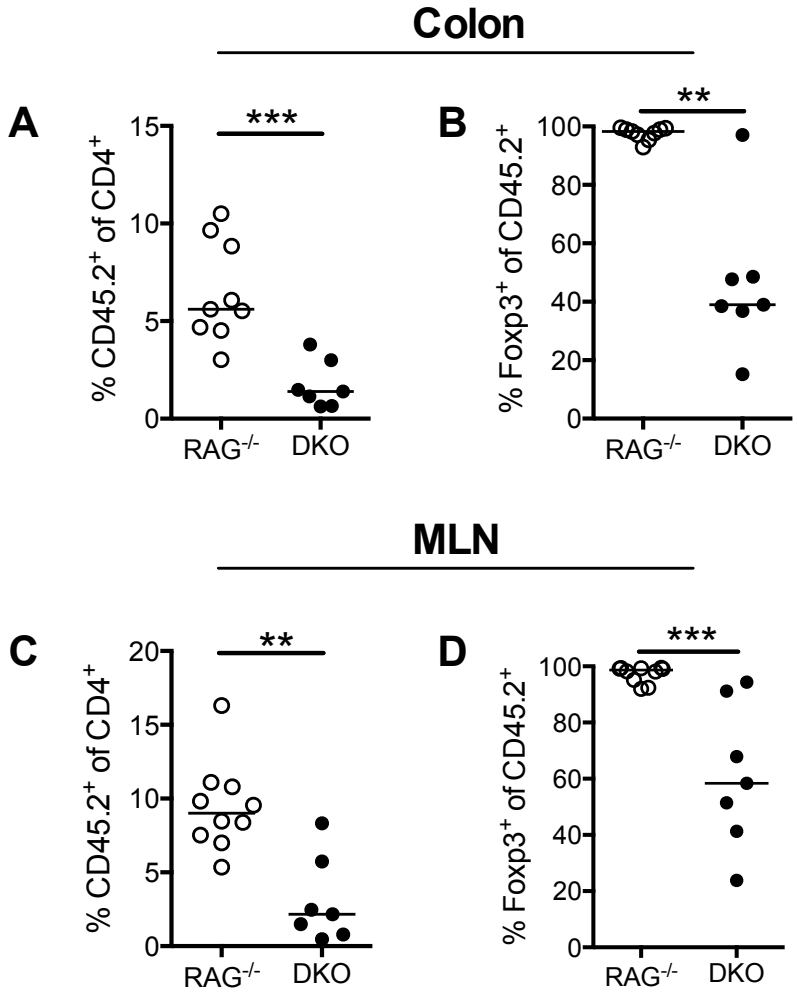


Figure 3-15. DKO mice are unable to maintain or expand the transferred Treg cell population. WT naïve CD45.1^{+}CD4^{+} T cells were co-transferred with WT CD45.2^{+} Foxp3^{GFP+} Treg cells into RAG$^{-/-}$ or DKO hosts at a 4:1 ratio. Four weeks later, cells were isolated from cLP and stained for extracellular markers. Median percentages of CD4^{+} T cells expressing CD45.2 (A) and CD45.2^{+} cells expressing Foxp3 (B) are shown. Data is compiled from two experiments with similar results. Each symbol represents an individual mouse and horizontal bars represent the medians. Statistics were calculated using the Mann-Whitney U-test. **p<0.01; ***p<0.001.

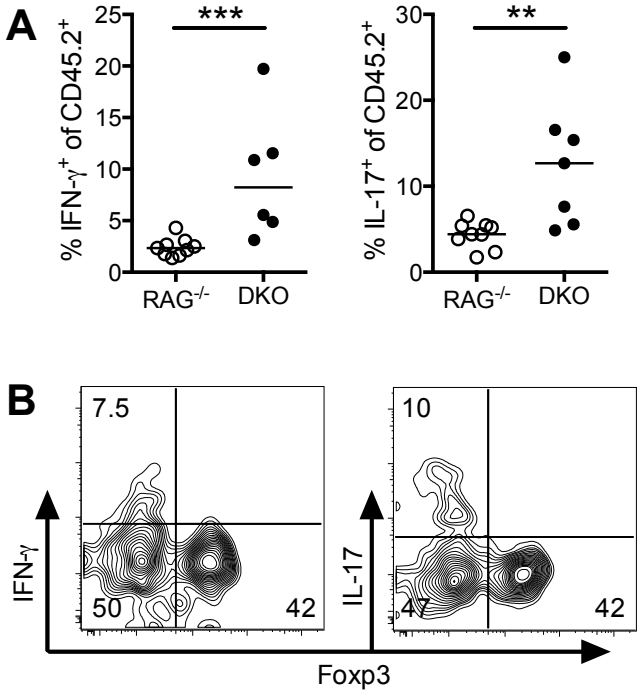


Figure 3-16. A subset of Treg cells that have lost Foxp3 produce pro-inflammatory cytokines. WT naïve $CD45.1^{+}CD4^{+}$ T cells were co-transferred with WT $CD45.2^{+}Foxp3^{GFP+}$ Treg cells into $RAG^{-/-}$ or DKO hosts at a 4:1 ratio. Four weeks later, cells were isolated from cLP, restimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL), and stained for extracellular markers. (A) Percentages of $CD45.2^{+}$ cells producing the indicated cytokine are shown. Each symbol represents an individual mouse and horizontal bars represent the medians. Data shown is compiled from two independent experiments with similar results. (B) One representative FACS plot pre-gated on $CD45.2^{+}$ cells and representative of mice in two independent experiments is shown. Numbers represent the percentage of cells in the quadrants. Statistics were calculated using the Mann-Whitney U-test. **p<0.01; ***p<0.001.

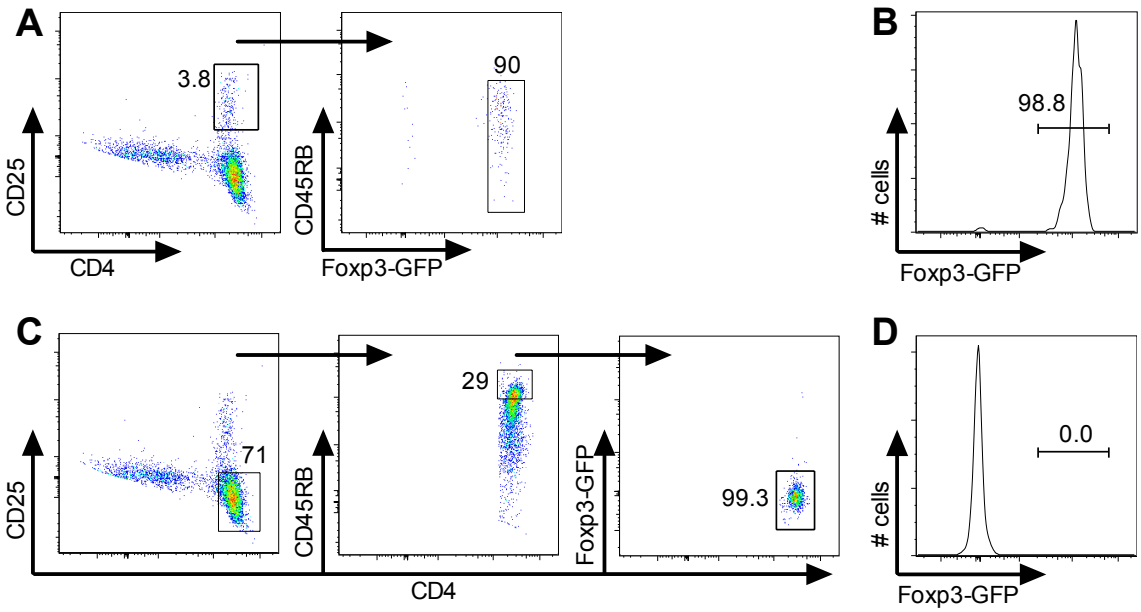


Figure 3-17. Minimal contamination of Foxp3$^+$ and Foxp3$^-$ T cell sorts. Splenic CD4$^+$ T cells from Foxp3^{GFP} mice were sorted into CD4$^+$CD25$^+$Foxp3^{GFP+} Treg cells (A) and CD25$^-$CD45RB^{hi}CD4$^+$ naïve T cells (C) based on the gates shown. The purified populations were analyzed post-sort for expression of Foxp3-GFP (B, D). Numbers represent the percentage of cells in the gated regions. FACS plots are representative of all experiments in which naïve or regulatory T cells were sorted.

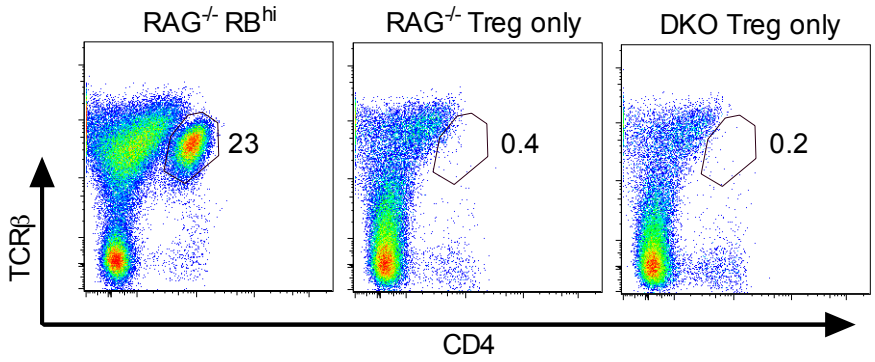


Figure 3-18. Foxp3^{+} Treg cells do not cause colitis in DKO mice. 1.5x10^{5} naïve CD45RB^{hi}CD4^{+} T cells or CD4^{+}CD25^{+}Foxp3^{GFP+} Treg cells were injected into RAG$^{-/-}$ or DKO recipients. Four weeks later, cLP cells were isolated and stained for CD4 and TCRβ. FACS plots shown are from one experiment and are composed of cells pooled from three to five mice per condition. Numbers represent the percentage of live cells expressing CD4 and TCRβ.

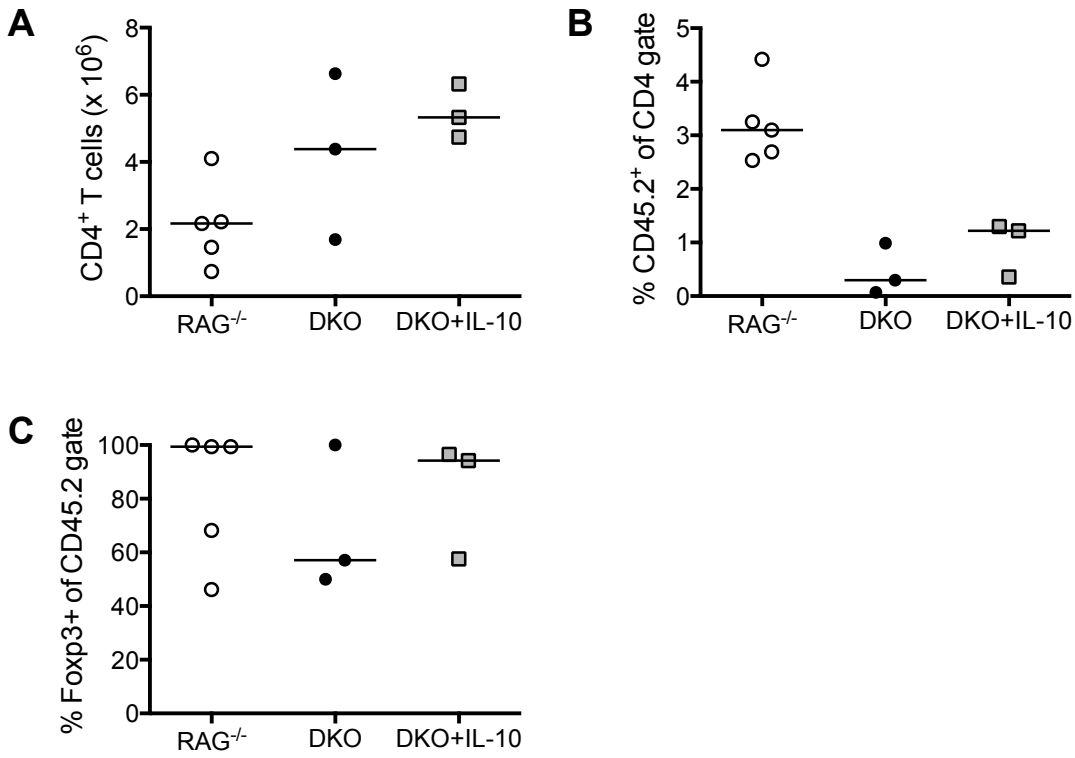


Figure 3-19. Recombinant IL-10 does not reverse Treg cell dysfunction in DKO mice. WT naïve CD45.1^{+}CD4^{+} T cells were transferred with WT CD45.2^{+} Foxp3^{GFP+} Treg cells into RAG$^{-/-}$ or DKO hosts at a 4:1 ratio. Mice were either treated with rIL-10 (0.5 μg daily) or left untreated. Four weeks later, cells were isolated from cLP and stained for extracellular markers. Median percentages of live cells expressing CD4 (A), CD4^{+} T cells expressing CD45.2 (B) and CD45.2^{+} cells expressing Foxp3 (C) are shown. Each symbol represents an individual mouse and horizontal bars represent the medians. Data shown is from one experiment.

3.3 Discussion

Despite strong evidence that IFN-1 signaling directly on T cells can enhance their survival (230), proliferation (487), and differentiation into Th1 cells (268, 270-272, 470, 471), we found that IFNAR1$^{-/-}$ naïve CD4^{+} T cells were able to expand, produce IFN-γ, and drive colitis in RAG$^{-/-}$ recipients in an equivalent manner to WT CD4^{+} T cells (Fig 3-1). We may not have seen an effect of direct IFN-1 signaling because most studies that linked IFN-1 to enhanced T cell responses looked at an acute response to IFN-1. The T cell adoptive transfer model of colitis, however, induces chronic inflammation and may not provide a strong enough dose of IFN-1 to enact changes in CD4^{+} T cell phenotypes directly. Furthermore, although CD4^{+} T cells required IFNAR signaling in order to expand during a viral infection, IFN-1 was dispensable for clonal expansion during a bacterial infection (231). In fact, during *Listeria monocytogenes* infection, IFN-1 had the opposite effect by sensitizing T cells to apoptosis (488). As T cell transfer colitis is driven by bacterial signals (329), perhaps it is unsurprising that IFN-1 does not affect T cell expansion via direct signaling on CD4^{+} T cells *in vivo*. Furthermore, when CD4^{+} naïve T cells were treated with IFN-1 prior to transfer into lymphopenic hosts (277) or when high levels of IFN-1 were induced by *in vivo* poly (I:C) administration (276), IFNAR signaling directly on CD4^{+} T cells attenuated the severity of colitis. Thus, the effects of IFN-1 are highly dependent on the stimulus inducing IFN-1, the dose of IFN-1 induced, and the characteristics of the inflammation present in the experimental model used.

We did find, however, that IFN-1 controlled the size of the T cell niche indirectly via effects on other hematopoietic cells (Fig 3-2, Fig 3-4). In the absence of IFNAR signaling on host innate immune cells in DKO recipients, CD4^{+} T cells underwent rapid proliferation in the MLNs (Fig 3-7B). There are several types of proliferation T cells can

undergo in lymphopenic conditions: homeostatic proliferation driven by self ligands, IL-7 and IL-15; "spontaneous" proliferation driven by an overabundance of DC- and T cell-derived proliferative cytokines; and antigen-specific clonal expansion (435). T cells undergoing homeostatic proliferation divide slowly and retain a naïve phenotype. Spontaneous proliferation is rapid, but not dependent on recognition of cognate antigen. These T cells adopt either effector or memory cell phenotypes. Finally, upon antigen presentation by DCs in the lymph nodes, T cells undergo rapid antigen-specific proliferation and adopt an effector phenotype. The development of T cell transfer colitis is dependent on both spontaneous as well as foreign antigen-driven proliferation (329). The rapidly dividing $CD4^+$ T cells in the MLNs of DKO mice adopted a $CD44^{hi}CD62L^{lo}$ effector phenotype (Fig 3-7C,D), suggesting that spontaneous proliferation and/or antigen-driven proliferation was enhanced in DKO hosts. Transfer of CFSE-labeled flagellin-specific $CD4^+$ T cells into $RAG^{-/-}$ or DKO mice raised under germ-free or conventional husbandry would allow us to determine whether the enhanced $CD4^+$ T cell proliferation observed was antigen-specific or driven primarily by lymphopenia.

There are several mechanisms by which IFNAR signaling on DCs could control the rate of T cell proliferation. First, DCs could downregulate their expression of various costimulatory molecules in response to IFN-1. IFN-1 could also decrease DC production of proliferation-promoting cytokines such as IL-6, IL-1 or IL-15 (329, 435, 479). Finally, IFN-1 has been shown to inhibit CCR7-dependent DC migration to the lymph nodes (489), which could also impair priming of naïve T cells. Whether IFN-1 activity on DCs regulates T cell proliferation via any of these mechanisms will be explored more fully in the following chapter.

In figure 3-9, we specifically targeted IL-1 as a possible cytokine that could drive $CD4^+$ T cell proliferation in DKO recipients. Previous studies have shown that IFN-1 can inhibit IL-1 secretion both *in vitro* and *in vivo* (250, 251), and IL-1R signaling directly on $CD4^+$ T cells has been shown to drive antigen-specific expansion (479). We blocked the IL-1 axis using anakinra, a human IL-1RA, and found that it could inhibit the accelerated $CD4^+$ T cell proliferation in DKO recipients of naïve CD4+ T cells. In contrast, $CD4^+$ T cell accumulation in $RAG^{-/-}$ recipients was unaffected by anakinra treatment, suggesting that anakinra inhibited a T cell proliferative mechanism that was specifically enhanced in DKO mice. Recent data from our lab has shown that IL-1 signaling on $CD4^+$ T cells is necessary for T cell accumulation in the colon, but is dispensable for their initial proliferation in the MLNs (49). Thus, it is possible that IL-1RA may suppress $CD4^+$ T cell proliferation indirectly via activity on an intermediate cell type. For example, IL-1 stimulates migration of DCs from the tissue to draining lymph nodes (490, 491) and enhances T cell priming. The effects of IL-1RA on DCs will be explored further in the next chapter.

Although the main phenotype we observed in DKO recipients was an increase in the accumulation of all $CD4^+$ T cells, we also found enhanced Th17 cell differentiation in the absence of IFNAR signaling on hematopoietic cells (Fig 3-3, Fig 3-4). These results are consistent with *in vitro* data showing a role for IFNAR signaling on DCs in suppressing Th17 cell differentiation (Fig 3-6B) and *in vivo* data showing a role for IFNAR signaling on innate immune cells in suppressing Th17 cell-mediated experimental autoimmune encephalitis (247, 472). In these studies, IFN-1 suppressed Th17 cell differentiation by inducing IL-27 from DCs. IFN-1 inhibition of IL-1 secretion may also play a role since IL-1, though not strictly necessary for Th17 cell differentiation, promotes the survival of

Th17 cells (49). Furthermore, IL-1 synergizes with other Th17 cell polarizing cytokines to increase expression of RORγt , the key transcription factor in Th17 cell differentiation (50). When we added IL-1β along with other polarizing cytokines to *in vitro* cultures, IFN-1 was no longer able to suppress Th17 cell differentiation (Fig 3-6B). Thus, it is possible that inhibition of IL-1β secretion from DCs may be an additional mechanism by which IFN-1 suppresses Th17 cell differentiation.

The role of Th17 cells in colitis is debated. Although Th17 cells and IL-17 are upregulated in the mucosa of patients with IBD (350, 351, 354), IFN-γ-producing Th1 cells are the predominant cell type in CD (349). In our studies, we found that a larger population of IFN-γ^+IL-17^+ $CD4^+$ T cells emerged in DKO recipients of naïve $CD4^+$ T cells (Fig 3-3B, Fig 3-4D). This population, termed "double producers," has been correlated with intestinal pathology (441) and recently been shown to be T cells transitioning from Th17 cells to Th1 cells (492). It appears that Th17 cells may control their own fate since the production of IL-17 by T cells induces IL-12 and IL-23 from innate cells to promote the conversion of Th17 cells into Th1 cells (493). Thus, IFN-1 suppresses the accumulation of a pathogenic IL-17-producing $CD4^+$ T cell subset in the colon, regardless of whether IL-17 is the key inflammatory cytokine.

We found that IFN-1 was also involved in the expansion and maintenance of $Foxp3^+$ Treg cells. Like its effects on effector T cell expansion, IFN-1 did not signal directly onto Treg cells to mediate its effects as both the induction and function of Treg cells was unaffected by the absence of IFNAR on T cells (Fig 3-10, Fig 3-14). Moreover, IFNAR signaling on DCs was also not required for the induction of Treg cells from naïve T cells (Fig 3-11, Fig 3-12, Fig 3-13). Thus, although Th17 cells and Treg cells have reciprocally regulated

developmental programs (43), the mechanisms by which IFN-1 inhibits Th17 cell differentiation do not enhance Treg cell induction.

Despite being dispensable for the induction of Treg cells, we found that IFNAR signaling was necessary for the homeostasis of the Treg cell population in the intestine. When congenic $Foxp3^{+}$ Treg cells were co-transferred with naïve $CD4^{+}$ T cells into $RAG^{-/-}$ recipients, they retained Foxp3 expression and comprised approximately 5% of the $CD4^{+}$ T cells present in the colon (Fig 3-15A). Conversely, when the same co-transfer was performed into DKO hosts, there was a drastic reduction in the proportion of $Foxp3^{+}$ T cells present in the colon (Fig 3-15A). Furthermore, many of the congenic $Foxp3^{+}$ Treg cells lost Foxp3 expression and gained the ability to secrete pro-inflammatory cytokines in DKO recipients (Fig 3-15B, Fig 3-16). Treg cell expansion and homeostasis in lymphopenic hosts is dependent on IL-10 production by colon myeloid cells (327, 343). We therefore supplemented DKO recipients of naïve T cells plus $CD4^{+}Foxp3^{GFP+}$ Treg cells with recombinant IL-10, but were unable to observe any significant enhancement of Treg cell expansion or function (Fig 3-19A,B). Although there was a trend toward IL-10 maintaining Foxp3 expression on Treg cells (Fig 3-19C), this pilot experiment must be repeated with more mice to make any definitive conclusions. The poor results using recombinant IL-10 could also be due to lack of bioavailability of the protein in the colon or an insufficient dose. Previous studies successfully treating colitic mice with recombinant IL-10 used intestinal bacteria as a delivery mechanism for IL-10 (494) or a significantly higher dose of IL-10 (438).

We also found that $CD45.2^{+}$ cells isolated from the MLNs failed to expand or maintain expression of Foxp3 (Fig 3-15C,D). Because *de novo* induction of Treg cells was

equivalent in the MLNs (Fig 3-12, Fig 3-13A), reduced numbers of $Foxp3^+CD45.2^+CD4^+$ T cells in the MLNs are most likely secondary to the loss of $Foxp3^+$ Treg cells in the cLP. Treg cells are known to require the intestinal lamina propria for expansion, and the dissemination of mucosally expanded Treg cells is essential for tolerance to oral antigens (327). Thus, the role of IFN-1 in maintaining Treg cells in the colon during an inflammatory period may have reverberating effects throughout the body.

The plasticity of $Foxp3^+$ Treg cells is highly contested. Under a variety of inflammatory settings, Treg cells convert into pathogenic cells, regardless of whether they retain expression of the transcription factor, Foxp3 (343, 495, 496). $Foxp3^+$ T cells also convert to $Foxp3^-$ pathogenic T cells under lymphopenic conditions (497). However, fate mapping of $Foxp3^+$ T cells showed that they were remarkably stable in both inflammatory and lymphopenic conditions (498). The discrepancy between these studies may be explained by the presence of cells that transiently express Foxp3, but have not fully committed to the Treg cell lineage (499). Thus, studies claiming a "loss" of Foxp3 expression on Treg cells may instead reflect outgrowth of a subset of $Foxp3^+$ cells that were never developmentally regulatory T cells. The requirement of inflammation for the loss of Foxp3 (343, 495) suggests that inflammatory signals preferentially expand effector T cells and non-committed $Foxp3^+$ T cells over $Foxp3^+$ T cells committed to the Treg cell lineage. In our studies, we observed a loss of Foxp3 on Treg cells when transferred into DKO hosts compared to $RAG^{-/-}$ (Fig 3-15). Thus, it is possible that the same mechanisms responsible for enhanced effector T cell proliferation also lead to an apparent loss of Foxp3 in the Treg cell population.

Taken together, these results show that IFN-1 can control the expansion of both effector and regulatory T cells. Previous reports have found that exogenous IFN-1 was able to suppress the colitogenic potential of effector T cells and expand regulatory T cells (277, 485). We found, instead, that endogenous IFN-1 exerts its effects on T cell proliferation via an intermediate hematopoietic cell. The effects of IFN-1 on innate immune cells will be investigated in the following chapter.

Chapter 4: The effects of type I interferons on lamina propria mononuclear phagocytes

4.1 Introduction

The colon lamina propria (cLP) contains a heterogeneous population of mononuclear phagocytes (MPs), which includes both macrophages and dendritic cells. Studies on the colonic mucosa of patients with IBD revealed an infiltration of both macrophages and dendritic cells (405, 500). MPs recruited during the pathogenesis of IBD were functionally distinct from resident MPs in the healthy colon. MPs isolated from the healthy colon expressed low levels of the activation markers, CD80 and CD86 (402). They also expressed low levels of CD14, the co-receptor for LPS (400, 402, 403). Consequently, MPs from the steady-state colon did not produce IL-1b, IL-6 or TNF-α in response to stimulation with LPS (400). In contrast, infiltrating MPs present in the mucosa of IBD patients expressed CD14 and were responsive to LPS stimulation (405, 501, 502). Human MPs isolated from the inflamed colon released more pro-inflammatory cytokines including IL-1b, IL-6, IL-8, IL-12, IL-18, and IL-23 (404, 408, 409, 501, 503). Furthermore, MPs from the inflamed colon were found to induce greater T cell proliferation in *ex vivo* cultures (420). MPs that accumulated during intestinal inflammation were derived from blood monocytes (405). As such, peripheral blood mononuclear cells (PBMCs) isolated from IBD patients and differentiated into DCs by *in vitro* culture with GM-CSF were also more efficient at antigen presentation and T cell priming than equivalent DCs differentiated from healthy controls (421).

Antigen presentation and T cell priming are thought to occur in secondary lymphoid organs such as the Peyer's patches (PP) and MLNs (5), but interactions between

infiltrating MPs and lymphocytes could also be observed in the colon tissue from patients with IBD (504). These interactions may be important in facilitating the effector functions of both lymphocytes and myeloid cells in the tissue (505). Colonic lymphocytes from IBD patients expressed more CD40 ligand, which activates myeloid cells via their surface expression of CD40 (425, 444). Similarly, IFN-γ production by Th1 cells in the inflamed colon promoted IL-23 production from myeloid cells (404). As IL-23 signals directly onto $CD4^+$ T cells to drive their cytokine production (441), this generates a positive feedback loop that potentiates colitis.

Studies of myeloid cells have become complicated by the methodology used to distinguish macrophages from dendritic cells. Instead of exclusively relying on surface markers to define cells, work from our laboratory has characterized subsets of macrophages and dendritic cells in the mouse colon based on their functionality (11). Approximately 80% of MHC II^{hi} cells expressed high levels of F4/80 and were macrophages based on their morphological appearance, gene expression profiles and inability to prime T cell responses. The remaining MHC II^{hi} cells were $CD11c^+F4/80^{int/lo}$ and showed characteristics of DCs including a robust ability to induce T cell proliferation. Murine intestinal macrophages exhibited a tolerogenic phenotype, producing the anti-inflammatory cytokines, IL-10, IL-1RA and IL-27. Intestinal DCs, on the other hand, produced stimulatory cytokines such IL-6, IL-12, and Il-23 (11).

As in human IBD, induction of experimental colitis recruited a monocyte-derived population of cells with inflammatory DC-like characteristics to the cLP (11, 439, 506-508). Unlike resident mononuclear phagocytes, which are hyporesponsive to microbial stimuli (11, 341, 507), infiltrating cells responded to TLR ligands with robust production

of pro-inflammatory cytokines. These cells produced IL-6, IL-12, IL-23, and TNF-α (11, 439, 507, 508); migrated through the lymphatics to the MLNs (506); and primed T cell responses (11, 506). Recruitment of these cells to the intestinal mucosa was dependent on expression of the chemokine receptor, CCR2, and blockade of CCR2 has been shown to ameliorate DSS-induced acute colitis (506, 508).

The effects of IFN-1 on the regulation of mucosal myeloid cells are largely unexplored. IFNAR signaling on hematopoietic cells initiates classic antiviral programs to control intestinal reovirus infection (188), but their effects outside of a viral context are not as well defined. In the previous chapter, we identified IFNAR signaling on hematopoietic cells as a key mechanism of controlling T cell accumulation in the cLP and suppressing Th17 cell differentiation (Fig 3-4). IFN-1 was previously shown to induce protection against DSS colitis and IFNAR1$^{-/-}$ mice exhibited slightly worse disease (458). It was further shown that IFN-1 could act on adoptively transferred bone marrow derived macrophages (BMDMs) to suppress colitis. IFN-1 was also necessary to suppress the recruitment of neutrophils during colitis, although it was not determined on which cell type IFN-1 must act to exert this effect (285).

IFN-1 may suppress T cell mediated colitis by altering cytokine production from mucosal macrophages and DCs. IFN-1 has been shown to induce anti-inflammatory cytokines such as IL-10, IL-27 and IL-1RA from MPs outside the gut (241-243, 472, 509). Conversely, IFN-1 can inhibit production of the pro-inflammatory cytokines IL-12, IL-1α and IL-1β by MPs (238, 244, 245, 250, 251). The effects of IFN-1 on IL-1β secretion, however, depend crucially on the stimulus used to activate the inflammasome in myeloid cells. When ATP, alum, nigericin, or *Mycobacterium tuberculosis* triggered the

inflammasome, IFN-1 inhibited IL-1β secretion in part by inducing IL-10-mediated suppression of pro-IL-1β transcription (250, 251). However, when gram-negative bacteria, such as those that colonize the intestinal tract, were used to stimulate the inflammasome, IFN-1 enhanced IL-1β secretion by activating caspase-11, a signaling molecule that activates the NLRP3 inflammasome under certain conditions (252).

Another possibility is that IFN-1 affects not only the function of the MP populations in the intestine, but also their recruitment and lifespan. Upon inflammation, IFN-1 mobilized hematopoietic stem cells out of dormancy to initiate an immune response (233) and also accelerated their maturation upon differentiation into DCs (234, 235, 510, 511). However, after serving as a catalyst for the immune response, IFN-1 limited the immune response by inducing exhaustion in proliferating hematopoietic stem cells (512), suppressing migration of DCs from the tissue to the draining lymph node (489), and inducing apoptosis in mature DCs (513). It is unclear whether these processes were stimulated by a spike in IFN-1 during acute inflammatory responses or whether constitutively produced IFN-1 had similar effects during chronic inflammation.

In this chapter, we explored the role of endogenous IFN-1 in the homeostasis of intestinal macrophage and DC subsets. We also investigated how IFN-1 conditions the phenotype of intestinal MPs and their contributions to the development of colitis.

4.2 Results

4.2.1 LP MPs constitutively produce IFN-1

pDCs are the major producers of IFN-1 after viral infection (105-107). Upon enteric viral infection, IFN-1 was induced from both pDCs and other $CD11c^+$ cells at about equivalent levels (188). By contrast, pDCs isolated from steady state PPs were defective for IFN-1 production (3), whereas $CD11c^+$ cells from the steady state small intestinal lamina propria produced IFN-1 constitutively (7). The induction and source of IFN-1 in the colon lamina propria has not yet been investigated. Analysis of cell subsets from the cLP revealed that pDCs were completely absent from the colon tissue (A. Rivollier and K. Kitamura, unpublished data). We thus hypothesized that MPs, including macrophages and non-plasmacytoid DCs, could produce IFN-1 in the steady state colon.

We have previously defined colon MP subsets (11). Among live cells, cells were gated based on high expression of cell surface MHC II. Cells expressing CD19, TCRβ, or TCRγδ were excluded. Among MHC II^{hi} cells, cells were separated into five groups based on expression of the markers F4/80, CD11c, CD11b and CD103 (Fig 4-1A,B). Cells expressing the highest levels of F4/80 represented two macrophage populations (subsets 1 and 2), which could be separated based on CD11c expression. Cells expressing intermediate or low levels of F4/80 represented three DC populations: $CD11b^+CD103^-$ (subset 3), $CD11b^+CD103^+$ (subset 4) and $CD11b^-CD103^+$ (subset 5). In the steady state colon, WT and $IFNAR1^{-/-}$ mice had equal proportions of all subsets (Fig 4-1C). Because there was no difference in the distribution of cell subsets and isolating each individual subset yielded too few cells for functional experimentation, we pooled together all five

macrophage and DC subsets for all experiments in this thesis involving cLP myeloid cells unless otherwise specified.

We found that the pooled population of macrophages and DCs constitutively produced IFN-β, as determined by both quantitative PCR and ELISA (Fig 4-2A,B). IFN-1 can be induced upon recognition of pathogen-association molecular patterns by TLRs (514). TLRs signal using either TRIF or MyD88 as an adapter. We found that $TRIF^{-/-}/MyD88^{-/-}$ double knockout mice had a significantly reduced production of IFN-β from cLP MP populations (Fig 4-2A). When we analyzed $TRIF^{-/-}$ and $MyD88^{-/-}$ single knockout mice, we found that both transcription and secretion of constitutive IFN-β was dependent on TRIF (Fig 4-2A,B). TRIF is downstream of TLR3 and TLR4, sensors of endosomal dsRNA and LPS, respectively (98, 99). TRIF is also downstream of cytoplasmic helicases that detect cytosolic dsRNA (132). Thus, constant stimulation by viral and/or bacterial ligands in the mucosal microenvironment could drive constitutive IFN-1 production. However, mice raised in germ-free conditions, which are marketed as devoid of any bacteria or environmental viruses, had equivalent transcription and production of IFN-β (Fig 4-3).

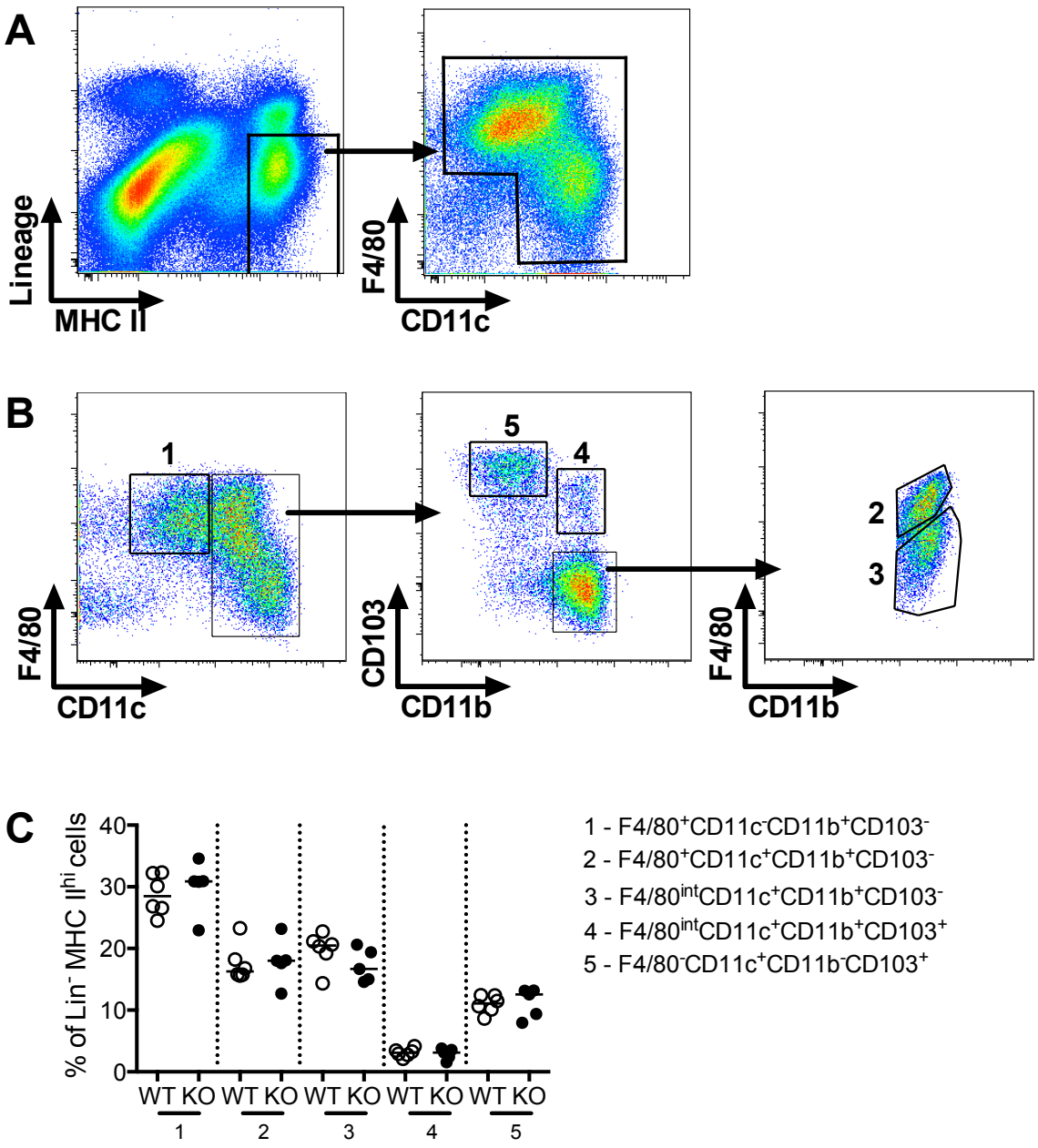


Figure 4-1. IFNAR signaling does not affect the distribution of steady state cLP MPs. (A, B) Cells were isolated from the colon LP and stained for surface markers. Representative FACS plots of the gating strategy for defining MP subsets in the colon are shown. Lineage markers used were CD19, TCRβ, and TCRγδ. (C) Subsets of different cell populations as defined in (B) from individual WT (open circles) or IFNAR1$^{-/-}$ (closed circles) mice are shown as a percentage of live lineage^{-} MHC II^{hi} cells. Horizontal bars represent the median compiled from two independent experiments, each with at least three mice per group.

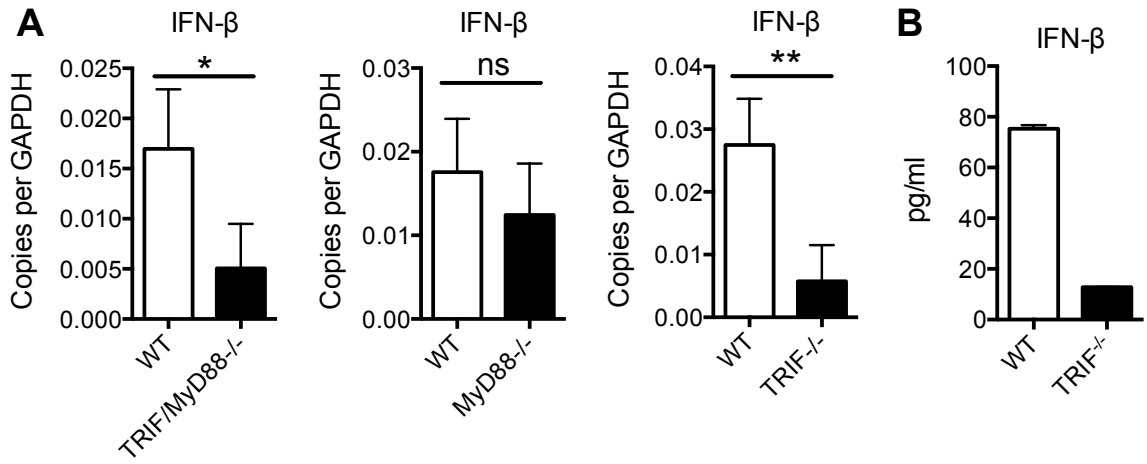


Figure 4-2. Constitutive production of IFN-1 by LP MPs is TRIF-dependent. cLP MPs were sorted as in Fig 4-1A from WT B6 and specified gene-knockout mice. (A) mRNA levels from sorted cells without additional stimulation determined by quantitative RT-PCR analysis are shown. Copies of the mRNA for *Ifnb1* were normalized to levels of the housekeeping gene, *GAPDH*. Data shown are the mean values ± SD of three or four independent experiments. (B) Sorted cLP MPs from WT B6 and $TRIF^{-/-}$ mice and placed in culture without stimulation for 24 hours. Protein levels in the culture supernatant from one of three independent experiments with similar results are shown. Values from each independent experiment in (A) or (B) were obtained by pooling cells from at least three mice per group. Statistics were calculated using a student's t-test. *$p<0.05$, **$p<0.01$.

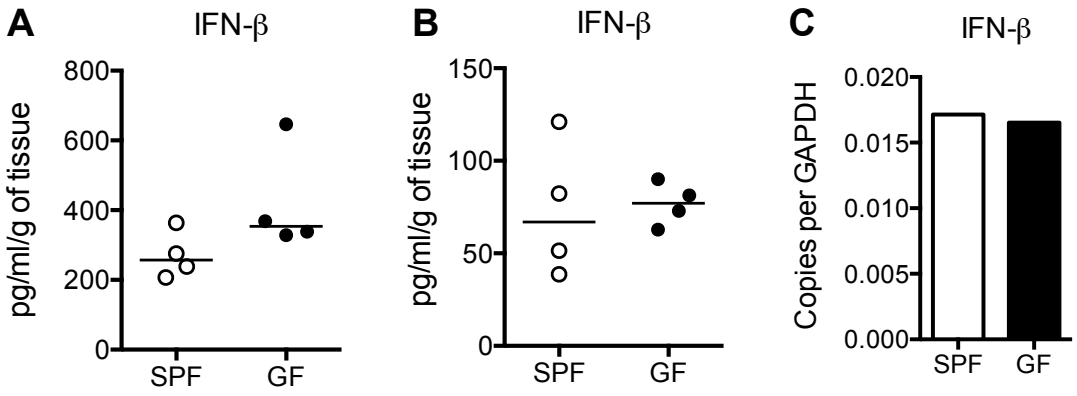


Figure 4-3. Constitutive production of IFN-1 is not dependent on the microbiota. Colons from specific pathogen-free (SPF) or germ-free (GF) mice were either (A) minced and placed in culture for 24 hours or (B) homogenized. IFN-β was measured from the culture supernatant (A) or homogenates (B) and normalized to the weight of tissue used. (C) cLP MPs were sorted as in Fig 4-1A from SPF or GF mice (n=4 for each) and transcription of IFN-β was measured by quantitative RT-PCR and normalized to levels of the housekeeping gene, GAPDH. Data shown in each plot is from one experiment. Symbols represent individual mice and horizontal lines represent the median.

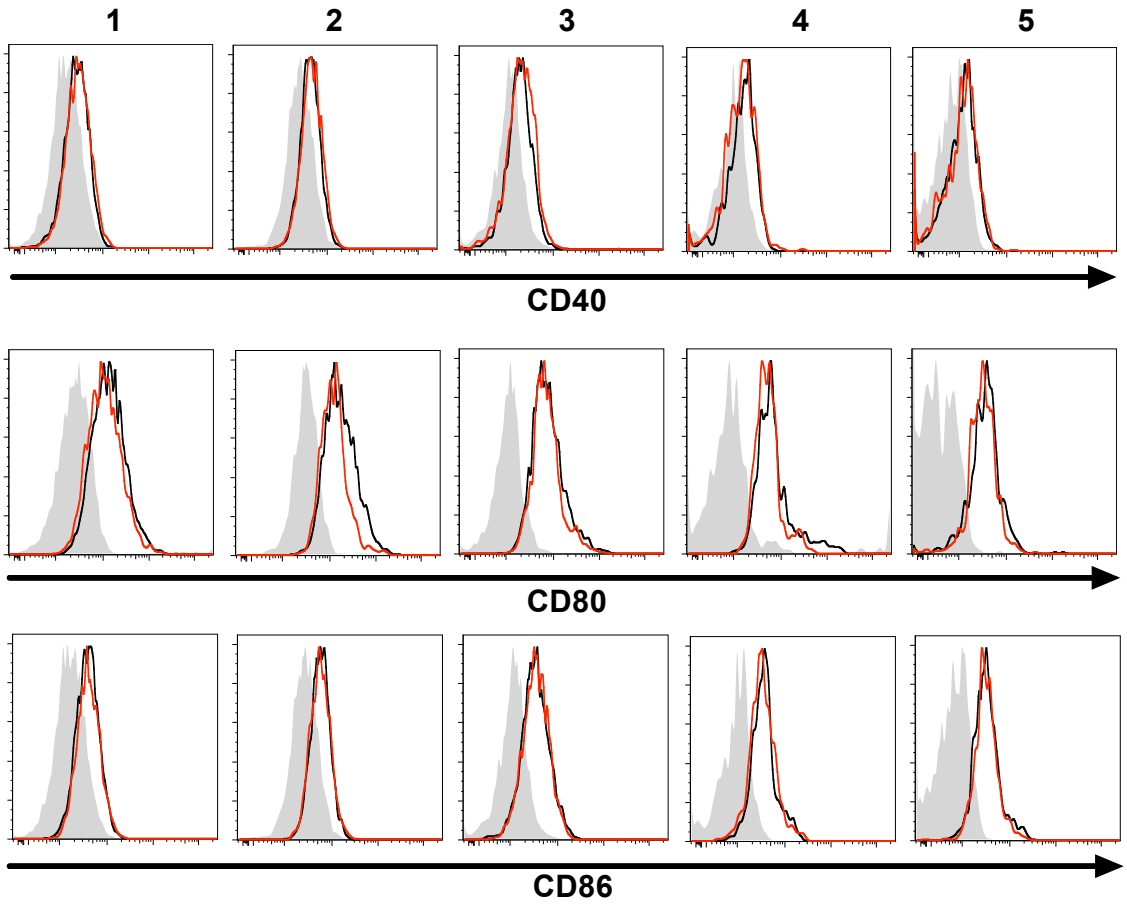
4.2.2 IFNAR signaling does not alter the steady state phenotype of LP MPs.

We next addressed whether IFNAR1$^{-/-}$ mice had a baseline difference in the phenotype of their myeloid cell subsets. We first looked at the activation state of MPs in the steady state colon. There were no significant or reproducible differences in the expression of the activation markers CD40, CD80 or CD86 between WT and IFNAR1$^{-/-}$ mice for any of the five subsets analyzed (Fig 4-4).

MP subsets in the colon were categorized as macrophages or DCs based on functional tests such as phagocytic capacity, ability to migrate and potential for T cell priming (11). Tissue resident macrophages have been found to have lifespans longer than other antigen-presenting cells, especially migratory DCs (515). We found that subsets 1 and 2, cells that exhibited characteristics of macrophages, had an incredibly slow turnover as only 20% of them were replaced over two weeks (Fig 4-5). By contrast, the three DC subsets showed significantly faster turnover as 50% of them were replaced after three days. A previous study had found that IFN-1 increased turnover of splenic conventional DCs (228). However, none of the macrophage or DC subsets from the steady state colon displayed different rates of turnover in the absence of IFNAR signaling (Fig 4-6). MLN DC turnover was also unaffected by IFNAR deficiency (Fig 4-7).

A previous study also suggested that IFNAR1$^{-/-}$ DCs were impaired in antigen presentation and unable to achieve optimal levels of T cell priming *in vivo* (516). However, we found that in mice lacking IFNAR signaling specifically on hematopoietic cells, T cell priming was actually enhanced (Fig 3-4). To address this discrepancy, we loaded DCs with whole ovalbumin protein and cultured them with naïve T cells. This *in vitro* system allowed us to investigate the ability of DCs to take up antigen, process it,

present it on MHC II molecules on the cell surface, and prime $CD4^+$ T cell responses. We found, however, that $CD4^+$ T cells with a TCR specific for ovalbumin proliferated robustly when cultured with either WT or $IFNAR1^{-/-}$ ovalbumin-loaded DCs (Fig 4-8). One caveat to this experiment, however, is that we did not include controls where the $CD4^+$ T cells were cultured without DCs or with DCs that had not been loaded with ovalbumin. Thus, it is possible, though unlikely, that the T cell proliferation observed could be independent of antigen-presentation entirely.



1 - F4/80^{+}CD11c^{-}CD11b^{+}CD103^{-}

2 - F4/80^{+}CD11c^{+}CD11b^{+}CD103^{-}

3 - F4/80^{int}CD11c^{+}CD11b^{+}CD103^{-}

4 - F4/80^{int}CD11c^{+}CD11b^{+}CD103^{+}

5 - F4/80^{-}CD11c^{+}CD11b^{-}CD103^{+}

Figure 4-4. IFNAR signaling does not affect the activation state of steady state cLP MPs. Cells isolated from the cLP and stained for extracellular markers were gated as in Fig 4-1A. Representative histograms plotting the surface expression level of indicated activation markers are shown. Data shown is from one experiment representative of two independent experiments. In each experiment, cells were pooled from three mice per group. Black line = WT; Red line = IFNAR1$^{-/-}$ KO; Gray fill = Isotype control.

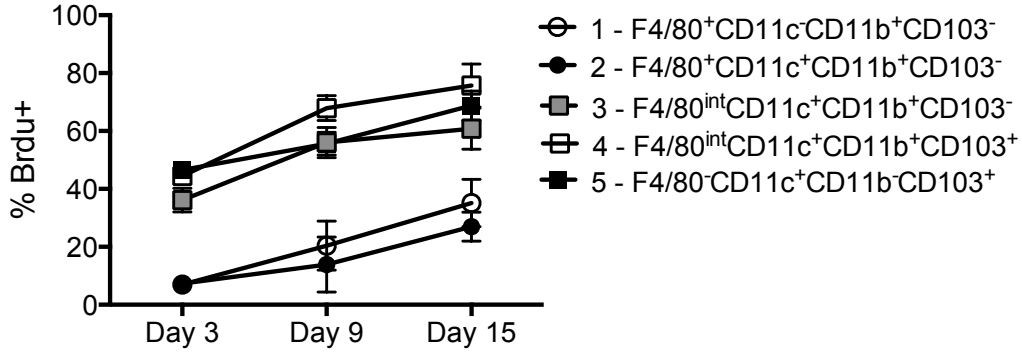


Figure 4-5. Macrophages and DCs in the cLP segregate by turnover rate. WT B6 mice were fed Brdu in their drinking water for 15 days. At indicated time points, cLP cells were isolated and stained for extracellular markers to define MP subsets and intracellular Brdu. Subsets were pre-gated on MHC $II^{hi}Lin^{-}$ live cells prior to analysis. Data show mean percent +/- SD of $Brdu^{+}$ cells in each subset from one experiment with three mice at each time point. The experiment is representative of three independent experiments with similar results.

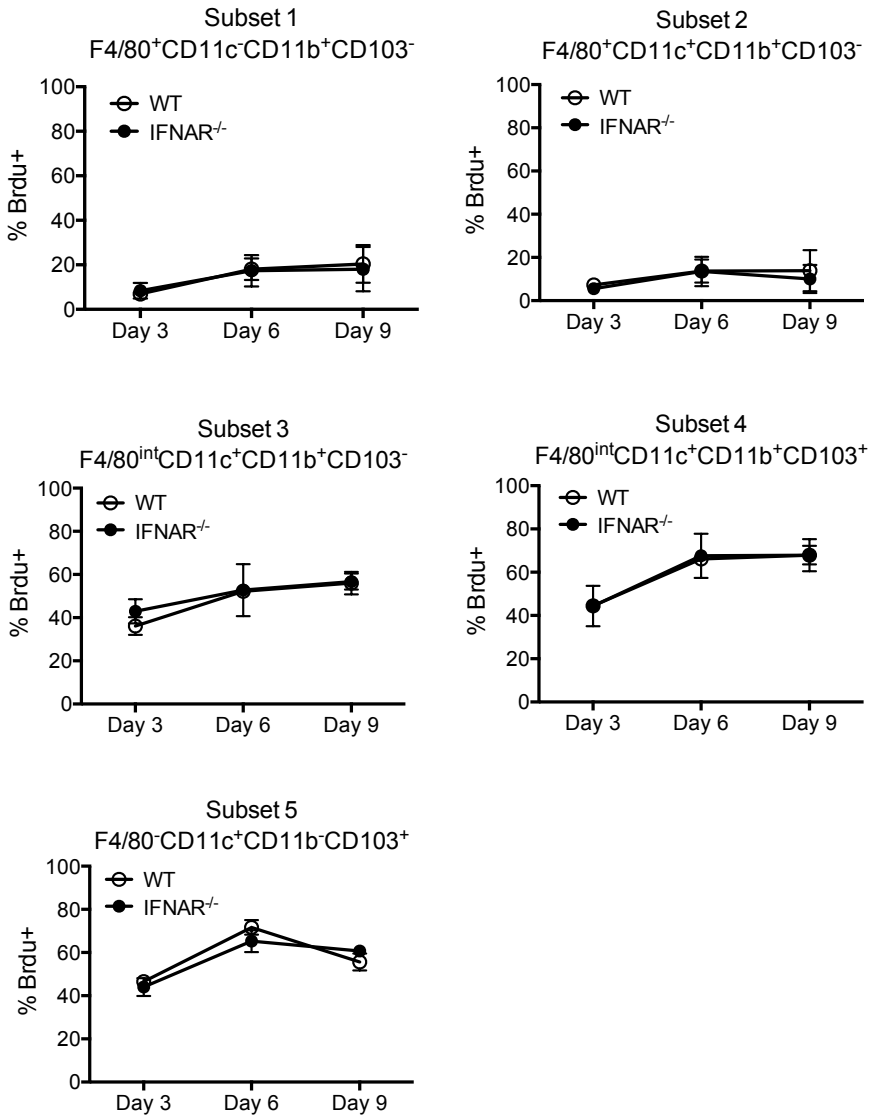


Figure 4-6. cLP MPs have identical turnover rates in steady state WT and IFNAR1$^{-/-}$ mice. WT and IFNAR1$^{-/-}$ mice were fed Brdu in their drinking water for 9 days. At indicated time points, cLP cells were isolated and stained for extracellular markers to define MP subsets and intracellular Brdu. Subsets were pre-gated on MHC II^{hi}Lin^{-} live cells prior to analysis. Data show mean percent +/- SD of Brdu^{+} cells in each subset from one experiment with three mice at each time point. The experiment is representative of two independent experiments with similar results.

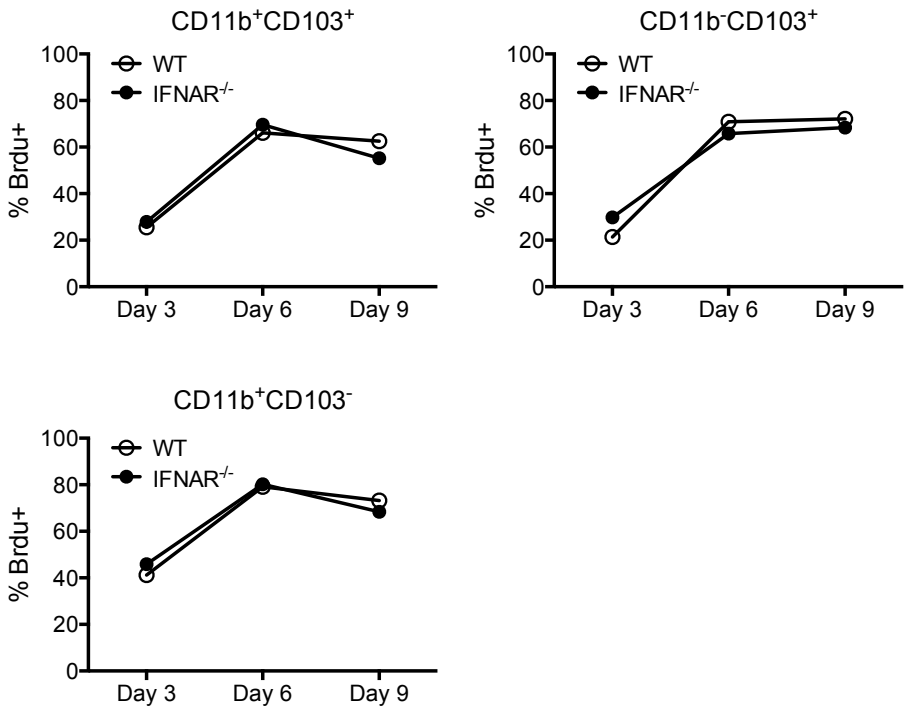


Figure 4-7. MLN DCs have identical turnover rates in steady state WT and IFNAR1$^{-/-}$ mice. WT and IFNAR1$^{-/-}$ mice were fed Brdu in their drinking water for 9 days. At indicated time points, MLN cells were isolated and stained for extracellular markers to define DC subsets and intracellular Brdu. Subsets were pre-gated on MHC II^{hi}Lin^{-}CD11c^{+} live cells prior to analysis. Data show the percent of Brdu^{+} cells in each subset from one experiment with pooled cells from three mice at each time point. The experiment is representative of two independent experiments with similar results.

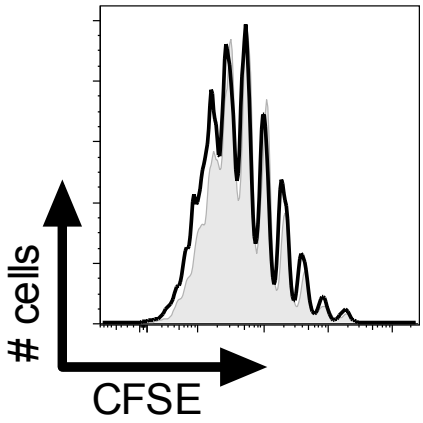


Figure 4-8. IFNAR1$^{-/-}$ DCs are not defective in antigen presentation. WT (gray fill) or IFNAR1$^{-/-}$ (black line) splenocytes MACS-enriched for CD11c^{+} DCs were irradiated and pre-loaded with 0.5 mg/mL ovalbumin protein before culture with CFSE-labeled naïve CD4^{+} T cells from OT-II mice at a 1:5 ratio. Five days later, cells were harvested and CD4^{+} T cells were analyzed by flow cytometry for CFSE dilution. Data shown is from one experiment.

4.2.3 IFNAR signaling is required for optimal anti-inflammatory cytokine production by colon MPs.

As there were no differences in the surface phenotype, turnover or antigen presentation capabilities between WT and IFNAR1$^{-/-}$ MPs in the steady state, we next looked at the ability of these cells to respond to external stimuli. We chose to stimulate MPs with FSL-1, a ligand for TLR2/6 heterodimers and flagellin, a ligand for TLR5. FSL-1 and flagellin were chosen due to the high expression of their sensing receptors on cLP MP subsets (11) and their previously demonstrated ability to elicit responses from gut myeloid cells (308). We found that, compared to WT MPs, IFNAR1$^{-/-}$ MPs produced less of the potent anti-inflammatory cytokine, IL-10, either with or without stimulation with TLR ligands (Fig 4-9A). IFNAR1$^{-/-}$ MPs also secreted significantly less IL-27 and IL-1RA, two other anti-inflammatory cytokines, in response to FSL-1. Adding a monoclonal antibody blocking IFNAR to cultures of FSL-1-stimulated WT MPs resulted in similar decreases in IL-10, IL-27, and IL-1RA secretion (Fig 4-9B). Because FSL-1 does not induce IFN-1 (87), this showed that constitutive production of IFN-1 and autocrine/paracrine IFNAR signaling is required for optimal production of anti-inflammatory cytokines by colon MPs. Furthermore, the effects of IFN-1 signaling were specific to anti-inflammatory cytokine production as the production of several pro-inflammatory cytokines including IL-1α, IL-23 and TNF-α was unaffected by the absence of IFNAR (Fig 4-10).

Many factors have been shown to be involved in the induction of IL-10 from myeloid cells. We have previously shown that colon macrophages constitutively produce IL-10 by both microbiota dependent and independent mechanisms (11). In BMDMs, IFN-1 and IL-27 were both crucial for the induction of IL-10 in response to LPS (241, 242). However, using primary cells from the cLP, we observed that addition of exogenous IFN-1 had no

effect on IL-10 production, either alone or in conjunction with FSL-1 (Fig 4-11A). IL-27 is thought to be downstream of IFN-1 in the LPS-induced production of IL-10 (242). However, addition of recombinant IL-27 was unable to rescue the poor production of IL-10 from TLR-stimulated IFNAR1$^{-/-}$ MPs (Fig 4-11B). Likewise, blocking IL-27 failed to reduce IL-10 production from TLR-stimulated WT MPs (Fig 4-11B). Thus, despite its requirement downstream of IFN-1 in BMDMs, IL-27 is dispensable for the induction of IL-10 from primary cLP MPs.

We next asked whether MLN DCs also showed alterations in cytokine production in the absence of IFNAR signaling. As cytokines can control the extent of T cell proliferation, especially during lymphopenic conditions (435, 479), an inability to produce anti-inflammatory cytokines could explain the enhanced ability of DCs to promote T cell proliferation in the MLNs of DKO mice (Fig 3-7). However, we found that IFNAR1$^{-/-}$ MLN DCs and WT MLN DCs secreted similar levels of pro-inflammatory and anti-inflammatory cytokines both in the presence or absence of TLR agonists (Fig 4-12).

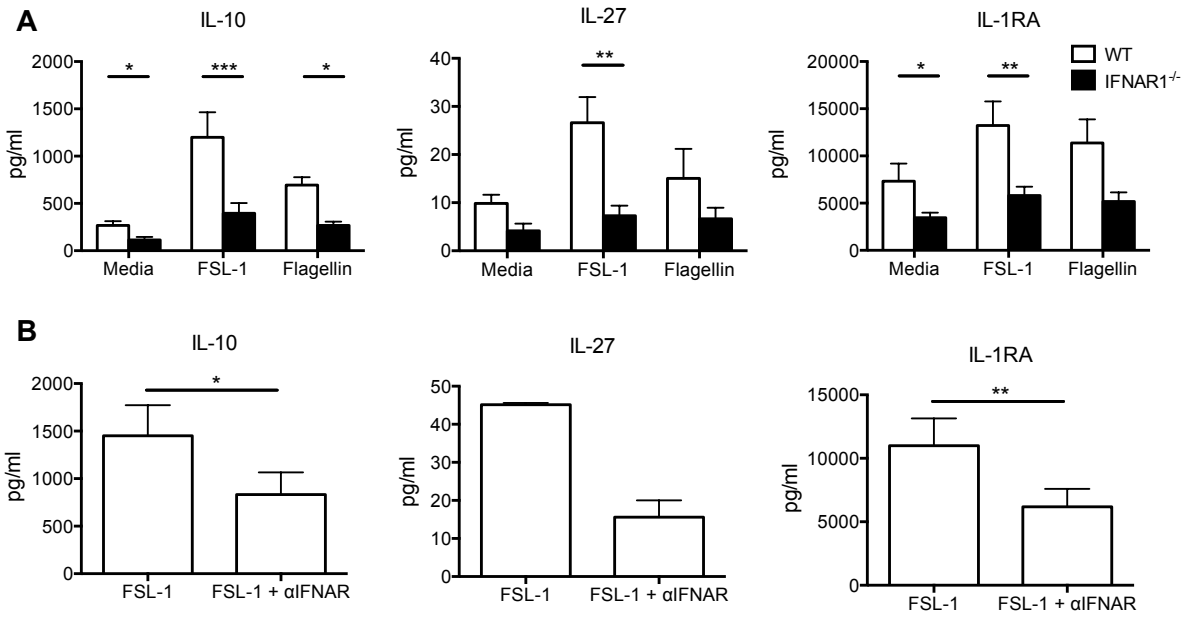


Figure 4-9. IFNAR signaling is required for optimal anti-inflammatory cytokine production. (A) FACS sorted MPs (as gated in Fig 4-1A) isolated from WT or IFNAR$^{-/-}$ colons were cultured (1x10^6 cells/mL) for 24 hours ± FSL-1 (500 ng/mL) or flagellin (1 μg/mL). Culture supernatants were analyzed for cytokine levels. (B) FACS sorted MPs (as gated in Fig 4-1A) from WT colons were cultured (1x10^6 cells/mL) for 24 hours with FSL-1 (500 ng/mL) ± αIFNAR (5 μg/mL). Data shown are means ± SD from two to four independent experiments, each conducted with cells pooled from ten mice per group. Statistics were calculated using a student's t-test. *p<0.05, **p<0.01, ***p<0.001.

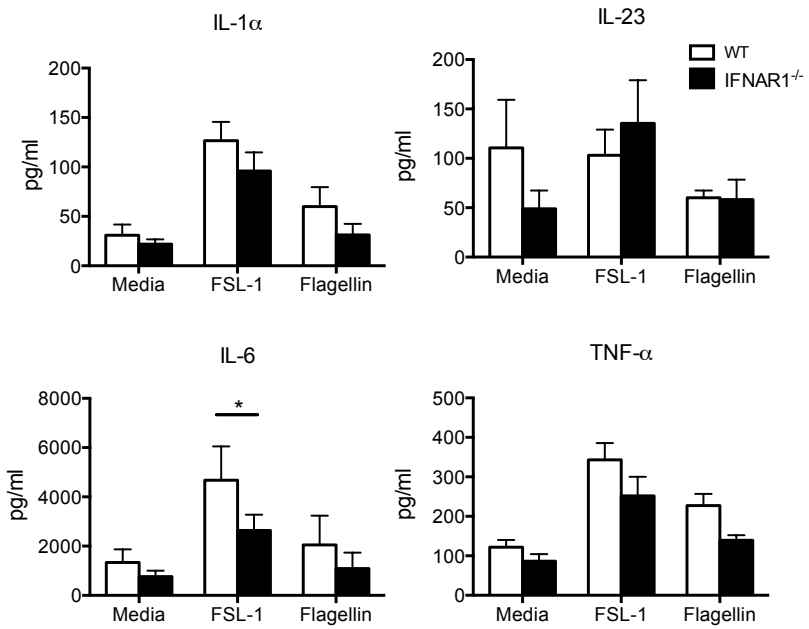


Figure 4-10. IFNAR signaling is not required for pro-inflammatory cytokine production. FACS sorted MPs (as gated in Fig 4-1A) isolated from WT or IFNAR$^{-/-}$ colons were cultured (1x10^6 cells/mL) for 24 hours ± FSL-1 (500 ng/mL) or flagellin (1 μg/mL). Culture supernatants were analyzed for cytokine levels. Data shown are means ± SD from three independent experiments, each conducted with cells pooled from ten mice per group. Statistics were calculated using a student's t-test. *$p<0.05$.

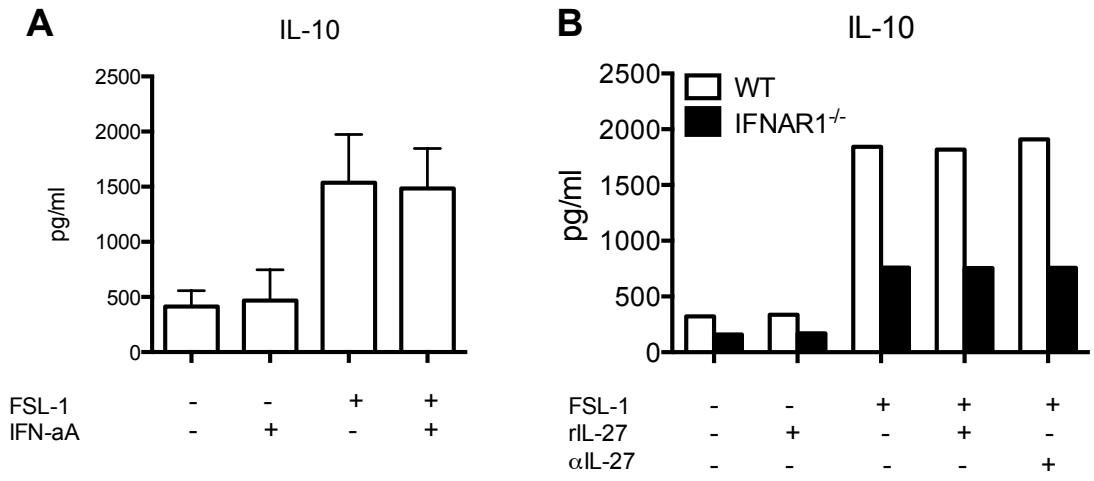


Figure 4-11. Exogenous IFN-1 or IL-27 does not augment IL-10 production by cLP MPs. FACS sorted MPs (as gated in Fig 4-1A) isolated from WT (A, B) or IFNAR$^{-/-}$ (B) colons were cultured (1x10^6 cells/mL) for 24 hours in the indicated conditions (FSL-1 500 ng/mL; IFN-aA 500 U/mL; rIL-27 20 ng/mL; αIL-27 10 μg/mL). Culture supernatants were analyzed for cytokine levels. (A) Data shown are means ± SD from three independent experiments, each using cells pooled from ten mice. (B) Data shown is from one experiment, using cells pooled from ten mice per group.

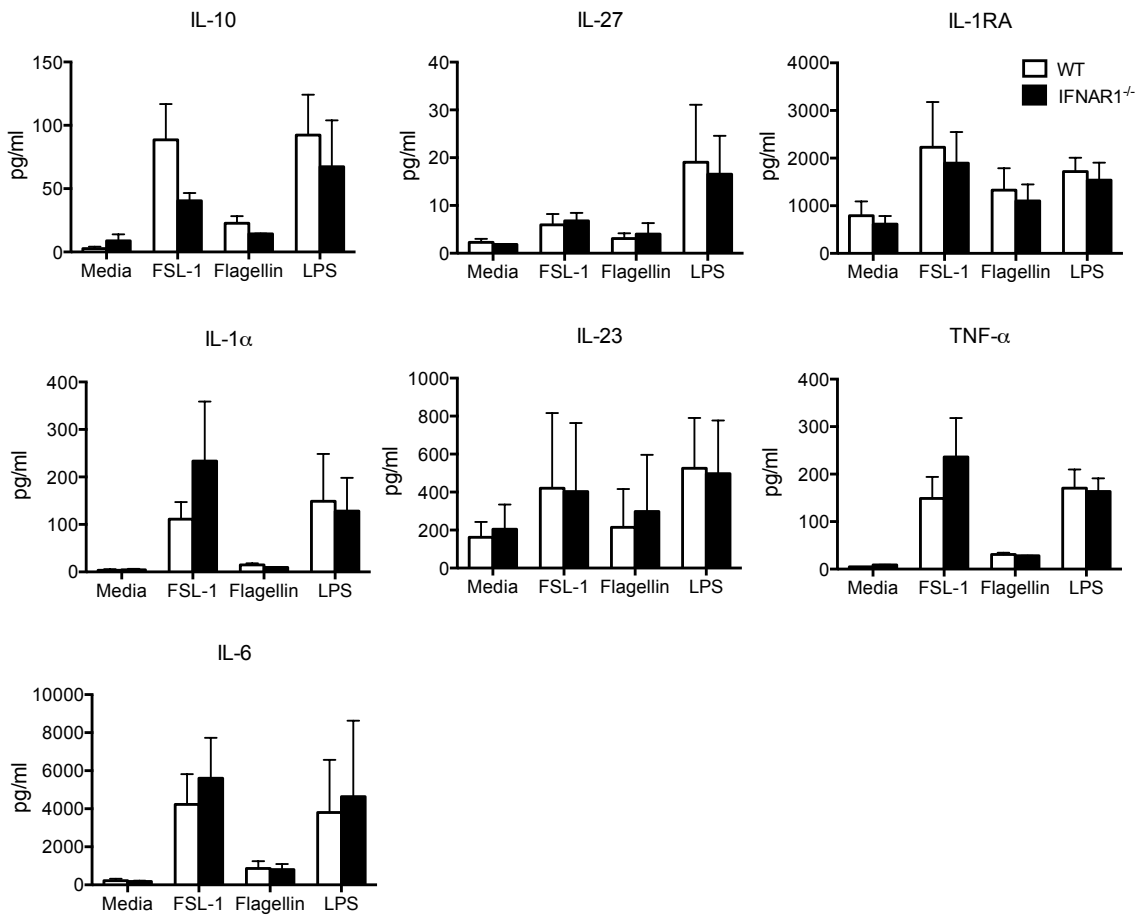


Figure 4-12. WT and IFNAR1$^{-/-}$ MLN DCs produce equivalent amounts of pro- and anti-inflammatory cytokines. FACS sorted CD11c^{+} DCs isolated from MLNs of WT or IFNAR1$^{-/-}$ mice were cultured (1x10^{6} cells/mL) for 24 hours in the presence or absence of FSL-1 (500 ng/mL), flagellin (1 μg/mL), or LPS (10 μg/mL). Culture supernatants were analyzed for cytokine levels. Data shown are means +/- SD from three independent experiments, each with cells pooled from ten mice per group.

4.2.4 Enhanced colitis is driven by IL-1-mediated accumulation of $CD11b^{+}CD103^{-}$ MPs in the MLNs of DKO mice.

Despite equivalent expression of co-stimulatory molecules by colon-resident MPs in the steady state, we hypothesized that migratory DCs in DKO mice were better able to stimulate T cell proliferation via enhanced expression of co-stimulatory markers during development of colitis. We isolated $CD11c^{+}$ cells from the MLNs ten days after transfer of naïve $CD4^{+}$ T cells into $RAG^{-/-}$ or DKO recipients. Neither group of mice had any signs of overt inflammation at this time point. We looked both at molecules that provide co-stimulation to T cells (CD80 and CD86) as well as receptors that receive stimulatory signals from T cells (CD40). MLN DCs isolated from DKO mice did not have any striking differences in any of the markers analyzed compared to MLN DCs isolated from $RAG^{-/-}$ recipients, although CD80 expression was slightly enhanced on the $CD11b^{+}CD103^{-}$ subset in DKO mice (Fig 4-13).

Further analysis of MLN DCs from pre-colitic mice ten days after naive $CD4^{+}$ T cell transfer showed that DKO mice had an increased accumulation of the $CD11b^{+}CD103^{-}$ subset (Fig 4-14A-B). This was not simply due to increased cellularity in the MLNs since the numbers of $CD103^{+}$ DCs were not increased in DKO mice (Fig 4-14B). We had previously shown that treatment with IL-1RA could suppress enhanced T cell proliferation in DKO mice (Fig 3-9) and in this chapter, we found that $IFNAR1^{-/-}$ MPs from the colon were defective in production of IL-1RA (Fig 4-9). However, it was previously shown that in this model of colitis, IL-1R signaling directly on T cells was dispensable for their initial proliferation in the MLNs (49). Thus, we hypothesized that IL-1RA indirectly exerts inhibitory effects on T cell proliferation by regulating the accumulation of stimulatory DCs in the MLNs. Indeed, when we treated DKO recipients

of $CD4^+$ T cells with anakinra, a recombinant form of IL-1RA, we found that the influx of $CD11b^+CD103^-$ DCs in the MLN was inhibited (Fig 4-14A-B). Moreover, the effects of anakinra treatment were specific to curtailing the accumulation of this subset of DCs, as anakinra had no effect on the accumulation of $CD103^+$ DCs in the MLNs of either $RAG^{-/-}$ or DKO mice (Fig 4-14B).

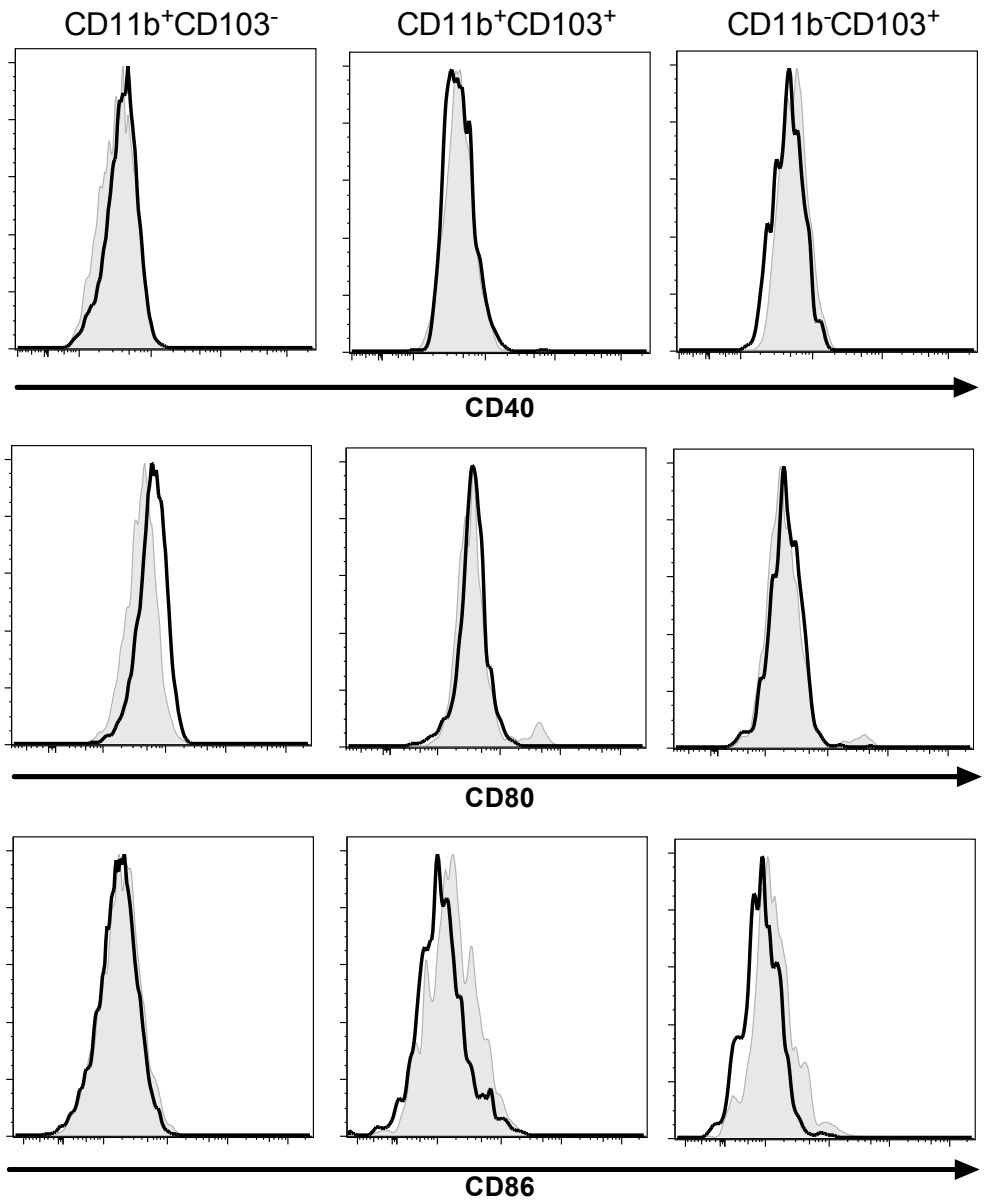


Figure 4-13. RAG$^{-/-}$ and DKO mice have equivalent expression of activation markers on CD11c^{+} cells isolated from MLNs during colitis. 3x10^{5} WT naïve CD4^{+} T cells were transferred into either RAG$^{-/-}$ (gray fill) or DKO (black line) hosts. Ten days later, cells were isolated from the MLNs and stained for surface markers. Histograms are pre-gated on live CD11c^{+} cells. Data shown is from one experiment representative of two independent experiments with similar results, each with cells pooled from five mice per group.

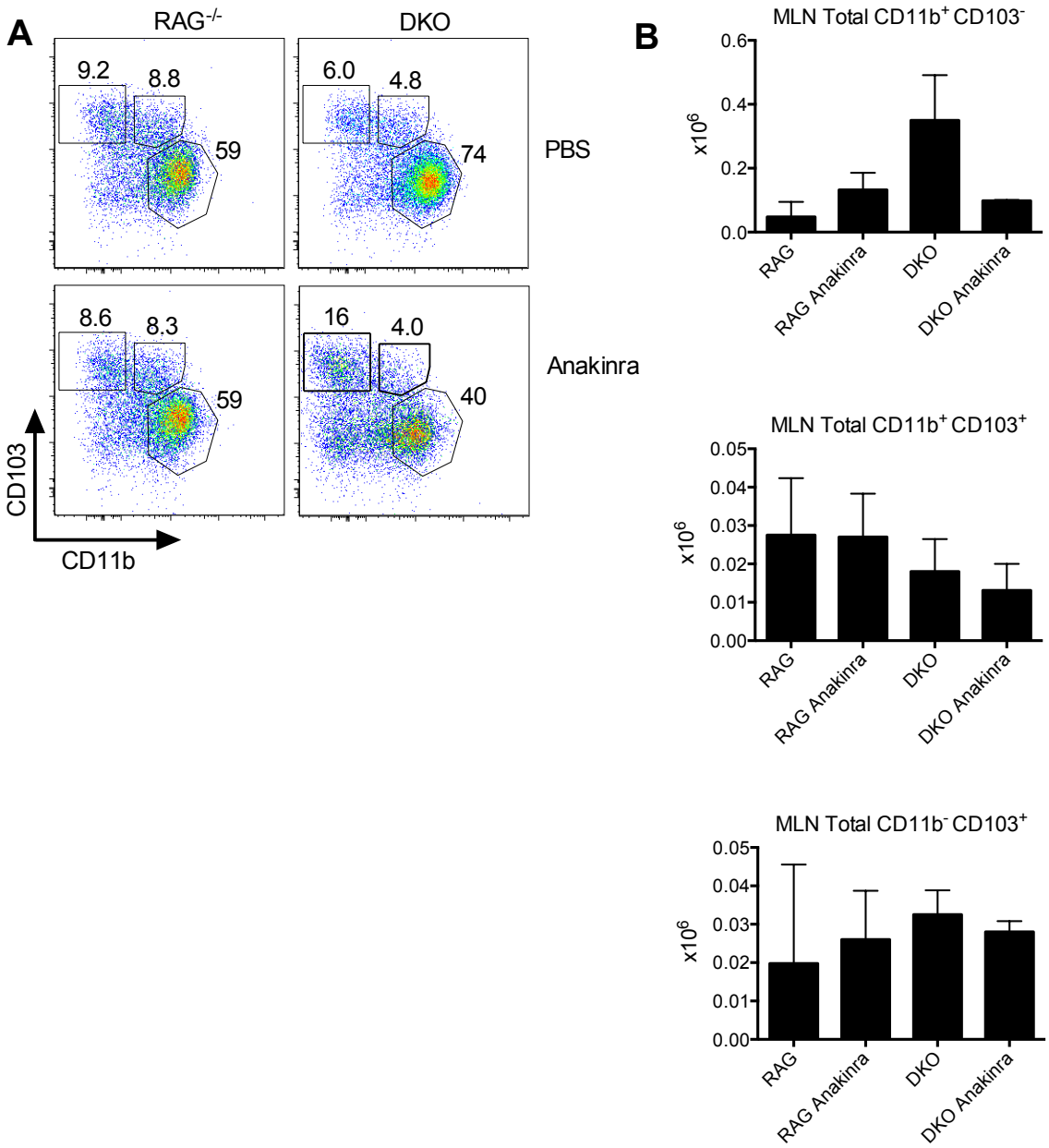


Figure 4-14. IL-1RA suppresses the accumulation of CD11b$^+$CD103$^-$ MPs in the MLNs of DKO mice during colitis. 3x10^5 WT naïve CD4$^+$ T cells were transferred into either RAG$^{-/-}$ or DKO hosts. Mice were given daily i.p. injections of either PBS or anakinra (1 mg daily) throughout the study. Ten days later, cells were isolated from the MLNs and stained for surface markers. Cells were analyzed by flow cytometry and data shown is pre-gated on live CD11c$^+$ cells. (A) FACS plots are shown from one experiment representative of two independent experiments with similar results, each with cells pooled from at least five mice per group. (B) Total numbers of DCs per mouse in each experiment were counted. Data shown is the mean +/- SD of two independent experiments with similar results, each with cells pooled from at least five mice per group.

4.2.5 DKO mice do not have worse anti-CD40-mediated innate colitis

To determine whether the effects of IFN-1 on myeloid cells were restricted to their ability to amplify T cell-dependent colitis, we moved to an acute model of innate colitis. A single injection of an agonistic CD40 antibody into $RAG^{-/-}$ mice activates myeloid cells to drive both systemic and mucosal pathology, characterized by rapid weight loss in the first four days after CD40 ligation and development of inflammation in the proximal colon (444). However, we found almost identical kinetics of weight loss and recovery, and similar levels of proximal colon inflammation in $RAG^{-/-}$ and DKO mice after anti-CD40 treatment (Fig 4-15A-B).

Anti-CD40-mediated innate colitis is driven by IL-23-responsive innate lymphoid cells (16). IL-23 induced both IL-17 and IFN-γ from ILCs. Given that IFNAR signaling could regulate IL-17 production from $CD4^{+}$ adaptive lymphoid cells (Fig 3-3, Fig 3-4), we asked whether it had the same effect on ILCs. However, both IL-23p19 and IL-17A gene expression were equivalently increased in $RAG^{-/-}$ and DKO mice (Fig 4-15C). Finally, although anti-CD40 did induce IL-10 production, levels were again equivalent between $RAG^{-/-}$ and DKO mice (Fig 4-15C).

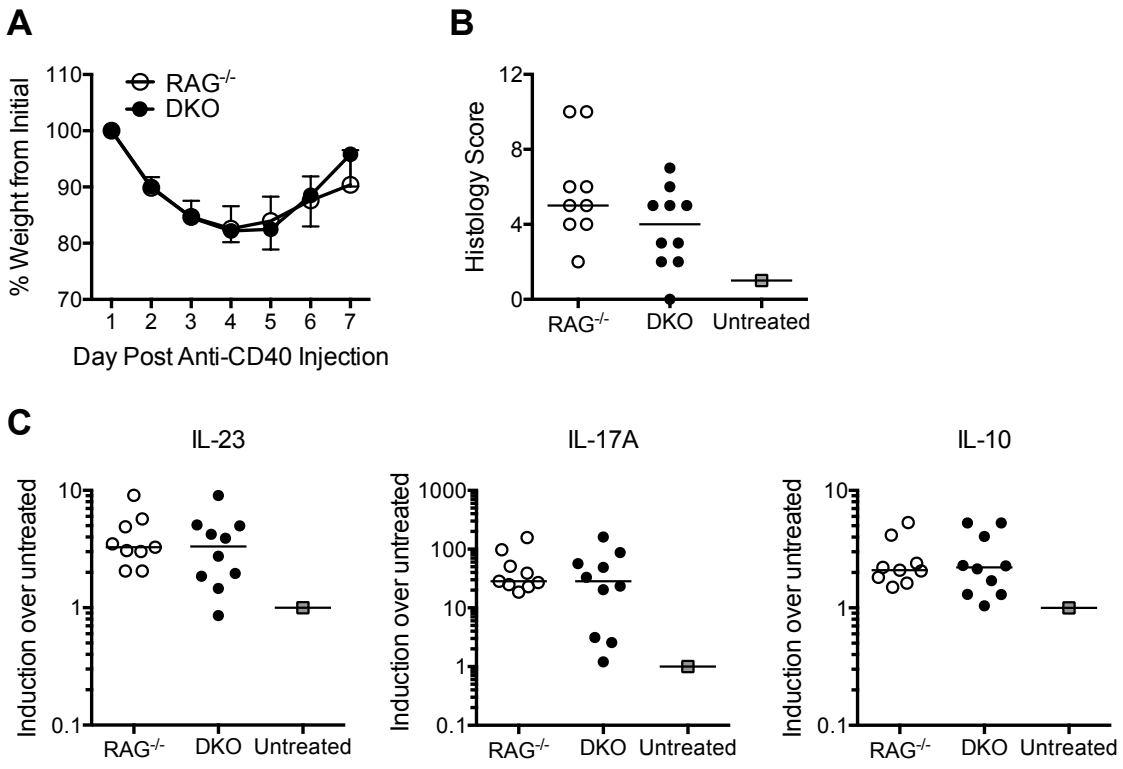


Figure 4-15. DKO mice do not have worse anti-CD40-mediated innate colitis. RAG$^{-/-}$ and DKO mice were treated with a single i.p. injection of 40 μg anti-CD40 agonistic antibody. (A) Mice were weighed daily. Mean deviation from initial body weight +/- SD is shown from one experiment representative of two independent experiments with similar results, each with five mice per experimental group. (B) On day 7 after anti-CD40 injection, the proximal colon was sectioned and scored for histopathological features. (C) Gene expression from proximal colon tissue sections isolated at day 7 is shown. Gene transcripts for *Il23p19*, *Il17a*, and *Il10* were normalized to levels of the housekeeping gene, *GAPDH*, and then plotted as induction over baseline levels of gene transcription in untreated mice (set to 1). Data shown in (B) and (C) are compiled from two independent experiments with similar results. Circles represent individual mice and horizontal bars represent the median.

4.3 Discussion

The colon lamina propria consists of several populations of macrophages and dendritic cells. At homeostasis, macrophages serve largely anti-inflammatory functions while a minor population of dendritic cells retains stimulatory activity (11). The mechanisms by which cLP cells are conditioned to adopt an anti-inflammatory phenotype have not been fully elucidated. We found that colon MPs constitutively produced IFN-1 when assayed *ex vivo* (Fig 4-2). We hypothesized that IFN-1 was produced at low levels continuously in part to promote the anti-inflammatory functions of these cells by autocrine and paracrine signaling.

Constitutive production of IFN-1 was dependent on signaling via the adapter molecule, TRIF (Fig 4-2). TRIF is essential for the IFN-1 response against gastrointestinal pathogens (14, 252), most likely due to pathways recognizing LPS on the bacterial cell surface. Also, the presence of commensal bacteria enhances IFN-1 production in response to viruses (517, 518). However, we found cLP MPs isolated from germ-free mice produced equivalent amounts of IFN-1, suggesting that despite being necessary for optimal induction of IFN-1 during a viral infection, commensals were dispensable for spontaneous production of IFN-1 (Fig 4-3). Germ-free mice, though devoid of living commensals, may still receive microbial signals in the form of endogenous retroviruses (519) or dead bacteria in autoclaved sterile feed (520). Alternatively, constitutive IFN-1 could be induced physiologically by endogenous ligands such as self nucleic acid or ceramide on apoptotic cells (120, 163, 521) or CSF-1 (158). It is also possible that a ligand is not necessary for IFN-1 production. Rather, IFN-1 could be constitutively produced by default unless repressed by antagonistic transcription factors such as IRF-2 (155).

IFN-1 signaling on myeloid cells was important for suppressing T cell-dependent colitis, but not T cell-independent colitis (Fig 3-4, Fig 4-15). Thus, we explored the mechanisms by which IFN-1 promoted the ability of myeloid cells to stimulate T cell accumulation. Despite enhanced T cell proliferation in the MLNs of DKO recipients of naïve $CD4^+$ T cells as compared to $RAG^{-/-}$ recipients (Fig 3-7), $IFNAR1^{-/-}$ DCs did not exhibit an increased ability to stimulate T cell proliferation *in vitro* (Fig 3-5, Fig 4-8). In concordance, expression of co-stimulatory molecules including CD40, CD80 and CD86 by MPs was not significantly altered in mice lacking IFNAR signaling either in the steady state cLP (Fig 4-4) or in the MLNs of mice undergoing colitis (Fig 4-13).

Increased migration of DCs to the lymph node could also increase T cell proliferation by providing greater antigen presentation. Increased migration could be reflected by increased Brdu uptake by precursor cells that must proliferate to replace the emigrated cells. We found that in the steady state, WT and $IFNAR1^{-/-}$ mice had equivalent rates of turnover in all myeloid cell subsets examined. Our results conflict somewhat with previously published results, showing decreased rates of DC turnover in $IFNAR1^{-/-}$ mice (228). However, Brdu uptake is influenced by several variables, including proliferation of hematopoietic stem cell progenitors, egress of cells from the bone marrow, migration in and out of tissues, and apoptosis. IFN-1 has been shown to either promote or inhibit DC migration depending on the cell subset and the context (228, 489). Likewise, IFN-1 can either promote or inhibit DC apoptosis depending on the cell's maturation status or antigen load (229, 513). Thus, opposing effects of IFN-1 on DCs may complicate interpretation of the turnover rate as measured by Brdu uptake.

Despite lack of evidence for altered migration in the steady state, we found that during colitis, DKO mice showed an early accumulation of $CD11c^{+}CD11b^{+}CD103^{-}$ cells in the MLNs to a greater extent than in $RAG^{-/-}$ mice. Although we did not determine the source of these cells, this population has been shown by other groups to traffic from the colon tissue to the MLNs and prime T cell responses (344, 506). These DCs expressed equivalent levels of co-stimulatory molecules during colitis (Fig 4-13) and $IFNAR1^{-/-}$ DCs were equally adept at processing proteins for antigen-specific T cell proliferation *in vitro* (Fig 4-8), suggesting that IFNAR signaling on hematopoietic cells controlled T cell proliferation primarily by regulating the number of inflammatory DCs that accumulate and are able to interact with T cells in the MLNs. Furthermore, this accumulation could be inhibited by treatment of DKO mice with recombinant IL-1RA (Fig 4-14), a protein that blocks IL-1 binding to cells. IL-1 was previously shown to promote migration of DCs out of tissue into the draining lymph node (490, 491). Treatment of $RAG^{-/-}$ mice with IL-1RA had no effect on accumulation of $CD11c^{+}CD11b^{+}CD103^{-}$ cells in the MLNs. Taken together, these results suggest that IFN-1 blocked IL-1-dependent migration of inflammatory DCs from the colon to the MLNs.

Indeed, we found that IL-1RA is produced by cLP myeloid cells both constitutively and upon stimulation in a IFN-1-dependent manner (Fig 4-9A,B). In addition to driving DC migration, IL-1 has also been proposed to promote the differentiation and survival of Th17 cells in the colon (49, 50). Accordingly, we found that DKO mice did indeed have increased IL-17-producing T cells in the cLP during colitis (Fig 3-3, Fig 3-4).

In addition to IL-1RA, secretion of IL-10 and IL-27 was found to be defective in $IFNAR1^{-/-}$ MPs from the cLP. Impaired anti-inflammatory cytokine production was

evident under baseline conditions, but was even more pronounced following stimulation with TLR agonists (Fig 4-9A). Additionally, WT cLP MPs treated with an anti-IFNAR monoclonal neutralizing antibody displayed an impaired ability to produce IL-10, IL-27 and IL-1RA (Fig 4-9B). In contrast, despite being essential for anti-inflammatory cytokine production, IFN-1 was dispensable for pro-inflammatory cytokine production in the colon (Fig 4-10).

IL-10 is a potent anti-inflammatory cytokine vital for intestinal homeostasis. Commensal bacteria are partially responsible for IL-10 production by colon MPs (11). We show here that constitutive IFN-1 signaling is also vital for secretion of IL-10 by cLP MPs. Exogenous IFN-1, however, did not enhance IL-10 production from primary gut cells (Fig 4-11), although IFN-1 has been shown to induce IL-10 from BMDMs (241). Similarly, while BMDMs required IL-27 downstream of IFN-1 to induce IL-10 (242), cLP MPs did not show an analogous requirement (Fig 4-11). It is possible that cLP MPs receive environmental signals that prime IL-10 production. Not having received these signals, *in vitro* generated BMDMs require IL-27 for IL-10 production and are more sensitive to exogenous IFN-1.

IL-10 maintains intestinal homeostasis not only by suppressing effector T cells, but also by maintaining the Treg population. Il10rb$^{-/-}$ Tregs placed into a lymphopenic host lost expression of Foxp3 to a greater extent than WT Tregs that were co-transferred into the same hosts (343). Also, IL-10 is necessary for the expansion of Tregs that maintain tolerance to orally ingested antigens (327). IL-27 signaling on Tregs promotes expression of T-bet and CXCR3, which are necessary for Tregs to suppress mucosal Th1 inflammatory responses (522). Thus, IFN-1 induces anti-inflammatory cytokines that

enhance the Treg population by multiple mechanisms and this could explain why Tregs transferred into DKO hosts show poor stability (Fig 3-15, Fig 3-16).

In conclusion, constitutively produced IFN-1 acts on cLP MPs to induce three key anti-inflammatory cytokines: IL-1RA, IL-10, and IL-27. These cytokines suppress T cell mediated colitis by restricting the traffic of inflammatory DCs to the MLNs where they can prime T cell responses, promoting regulatory T cell responses, and suppressing effector T cell function in the cLP.

Chapter 5: The role of IFN-1 in regulating intestinal bacteria

5.1 Introduction

Colitis, including the T cell adoptive transfer model of colitis used in this study, is heavily dependent on the presence of bacteria. Reduction of bacterial load by antibiotic treatment ameliorated the severity of colitis in lymphopenic mice given naïve T cells (523). Furthermore, transfer of $CD45RB^{hi}$ naïve $CD4^+$ T cells into SCID mice raised under germ-free conditions could not induce colitis (524, 525). T cell adoptive transfer colitis is dependent on both antigen-non-specific spontaneous proliferation as well as antigen-specific clonal expansion (329). However, both stages of proliferation require the presence of bacteria, since ovalbumin-specific $CD4^+$ T cells were able to proliferate in conventionally reared ovalbumin-free mice, but this spontaneous proliferation was completely abrogated in germ-free mice (329). Commensal bacteria were similarly found to enhance T cell responses in an antigen-non-specific manner in a different model of gastrointestinal infection as well (330). In humans, antibodies against bacterial flagellin can be found in patients with Crohn's disease (374) and studies have reported beneficial effects of antibiotic treatment on colon inflammation in CD patients (378, 379).

Despite a generalized requirement for microbiota in inducing T cell-mediated colitis, there is some degree of specificity to the bacteria needed. Germ-free mice monoassociated with several individual strains of bacteria failed to induce colitis, although monoassociation with *Helicobacter muridarum* was sufficient to induce colitis after transfer with $CD45RB^{hi}$ naïve $CD4^+$ T cells (524, 526). Furthermore, colonization of mice by a related bacterium, *Helicobacter hepaticus*, in the context of the normal flora significantly worsened the severity of colitis in this model (527). Studies in other models

of colitis show that transfer of gut flora from a susceptible mouse strain to a WT mouse can transmit colitis to the previously unsusceptible mouse (528, 529). These studies clearly show that while microbiota are required for the development of colitis, certain species of bacteria have greater colitogenic potential than others.

Alterations in the innate immune system have been found to have profound effects on the composition of the intestinal flora (296, 528). The effects of IFN-1 on shaping the commensal microbial landscape have not yet been defined. However, the effects of IFN-1 on specific gastrointestinal pathogens has been studied. IFN-1 contributes to a protective response against a variety of enteric bacterial and parasitic infections including *Helicobacter pylori* (284), *Cryptosporidium parvum* (530), *Salmonella typhimurium* (531), *Yersinia enterocolitica* (14), and *Toxoplasma gondii* (532). *In vitro* studies have found that IFN-1 greatly enhances the pro-inflammatory response against gram-negative bacteria, which are common in the intestinal tract (252). Conversely, IFN-1 has also been found to enhance susceptibility to certain mucosal pathogens, including the bacterium, *Listeria monocytogenes* (488, 533, 534), and the parasitic nematode, *Nippostrongylus brasiliensis* (535). Thus, IFN-1 has the potential to significantly influence the response to intestinal pathogens.

In this chapter, we explored the contributions of IFN-1 to regulating intestinal commensals, opportunistic pathogens, and classical pathogens in the context of colitis.

5.2 Results

5.2.1 The microbiota contributes to colitis in DKO mice

There is considerable variability in the length of time required for T cell adoptive transfer colitis to develop, depending on the facility (unpublished observations). It is thought that microbiota differences in various facilities contribute to this variability. $RAG^{-/-}$ mice used in these studies were purchased from Taconic Farms, while DKO mice, although originally generated by crossing together strains obtained from Taconic, were subsequently bred in-house. After purchase, $RAG^{-/-}$ mice were housed in the same room and given the same feed as DKO mice before and during colitis. However, $RAG^{-/-}$ mice may still have maintained the different flora they acquired while housed at Taconic. Alternatively, a deficiency in IFNAR signaling in the DKO mice may have allowed for the bloom of specific bacteria that have greater colitogenic potential.

To determine whether bacterial microbiota differences might have contributed to the accelerated colitis observed in DKO recipients of $CD4^{+}$ naïve T cells (Fig 3-2), we performed a series of co-housing experiments in which $RAG^{-/-}$ mice were placed in the same cage as DKO mice for at least two weeks prior to induction of colitis. Because of coprophagy, bacterial populations passed in the feces of one mouse can be transmitted to other mice in the same cage. Despite considerable experimental variation, data compiled from three independent experiments showed that DKO mice co-housed with $RAG^{-/-}$ mice still displayed significantly worse colitis, although the difference was diminished when compared to colitis induced in $RAG^{-/-}$ and DKO recipients that were housed separately (Fig 5-1).

To further clarify the role of intestinal microbiota in accelerated colitis in DKO mice, we generated $RAG^{-/-}$ and DKO littermates. At birth, both genotypes of mice were weaned on

the same mother and thus acquired the same microbiota (536). We then separated $RAG^{-/-}$ and DKO mice from each other and housed them in cages specific to their genotype for four weeks. This allowed the mice to develop any genotype-specific alterations in the microbiota without the confounding factor of coprophagy. We found that DKO recipients of $CD4^{+}$ naïve T cells exhibited more severe colitis when compared to either their $RAG^{-/-}$ littermates or $RAG^{-/-}$ mice purchased from Taconic (Fig 5-2). Thus, the enhanced colitis in DKO recipients cannot be attributed solely to random differences in the microbiota due to housing conditions. A role for IFN-1-specific alterations in the microbiota, however, cannot be completely excluded.

To directly assess potential differences in intestinal microbiota, we collected stool samples from $RAG^{-/-}$ mice purchased from Taconic and DKO mice bred in-house. We isolated DNA from fecal pellets and used deep sequencing to analyze the bacterial populations contained in the intestinal tract (537). Although sequencing data for each individual species of bacteria is not yet available, we were able to analyze the diversity of species within the entire microbiome. Diversity, or the number of different species of bacteria in the microbiome, has been associated with intestinal health. Accordingly, patients with Crohn's disease and ulcerative colitis have reduced diversity within their fecal microbiota (538-540). We found that even prior to induction of colitis, DKO mice had reduced diversity as determined by the Shannon diversity index (Fig 5-3A), a measure that quantifies the inability to guess the identity of a bacterial species randomly selected from the pool of all species in the microbiome (541). As the number and proportional abundance of different species in the pool increases, the probability of incorrectly guessing a randomly selected species also increases. DKO mice also showed lower diversity as measured by rarefaction analysis (Fig 5-3B), a method where the

number of different bacterial species is quantified as a proportion of the total number of bacteria present (542). Bacteria were speciated based on whether their DNA sequence constituted a unique operational taxonomic unit (OTU). The reduced diversity in DKO mice became even more apparent upon induction of colitis (Fig 5-3A,B).

One specific bacterium, segmented filamentous bacteria (SFB), has been linked recently to enhanced intestinal Th17 responses (449), and the presence of this bacterium was associated with more severe pathology during T cell adoptive transfer colitis (526). Because enhanced colitis in DKO mice was associated with increased numbers of IL-17-producing $CD4^+$ T cells (Fig 3-3, Fig 3-4), we examined whether DKO mice harbored a higher load of SFB than their $RAG^{-/-}$ counterparts, using specific primers for quantitative PCR (461). However, we found that both $RAG^{-/-}$ and DKO mice were colonized with SFB to a similar extent (Fig 5-4). Stool samples isolated from WT mice purchased from Jackson Labs were used as negative controls (449). Thus, the enhanced Th17 responses observed in DKO mice were not due to increased colonization with SFB.

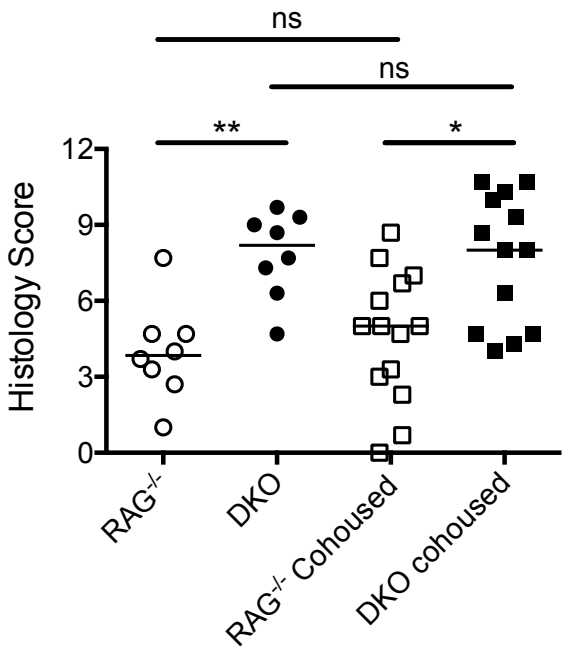


Figure 5-1. Co-housing diminishes, but does not eliminate the differences in severity of colitis between RAG$^{-/-}$ and DKO recipients. 3x10^5 naïve CD4$^+$ T cells were injected intraperitoneally into RAG$^{-/-}$ mice purchased from Taconic or DKO mice bred in-house. Mice were housed separately or together as indicated. Co-housed mice were caged together for two weeks prior to transfer of T cells and throughout the course of disease. Mice were killed at four weeks. Composite histology scores shown are compiled from three independent experiments. Each symbol represents an individual mouse, horizontal bars represent group medians. Statistics were calculated using a Mann-Whitney U-test. *$p<0.05$, **$p<0.01$.

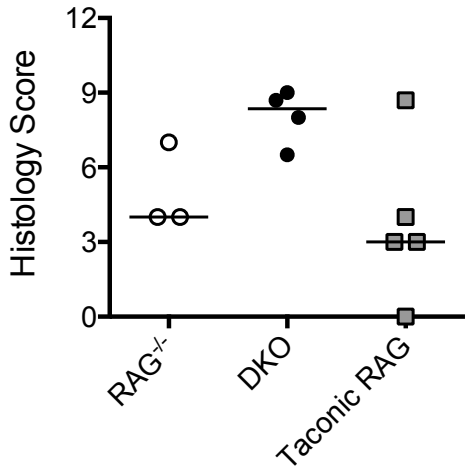


Figure 5-2. DKO mice have worse colitis than RAG$^{-/-}$ littermates. RAG$^{-/-}$ and DKO littermates were separated into individual cages for four weeks prior to injection with $3x10^5$ naïve T cells. Colon histology scores four weeks after T cell transfer are shown. Data shown is from one experiment. Each symbol represents an individual mouse, horizontal bars represent group medians.

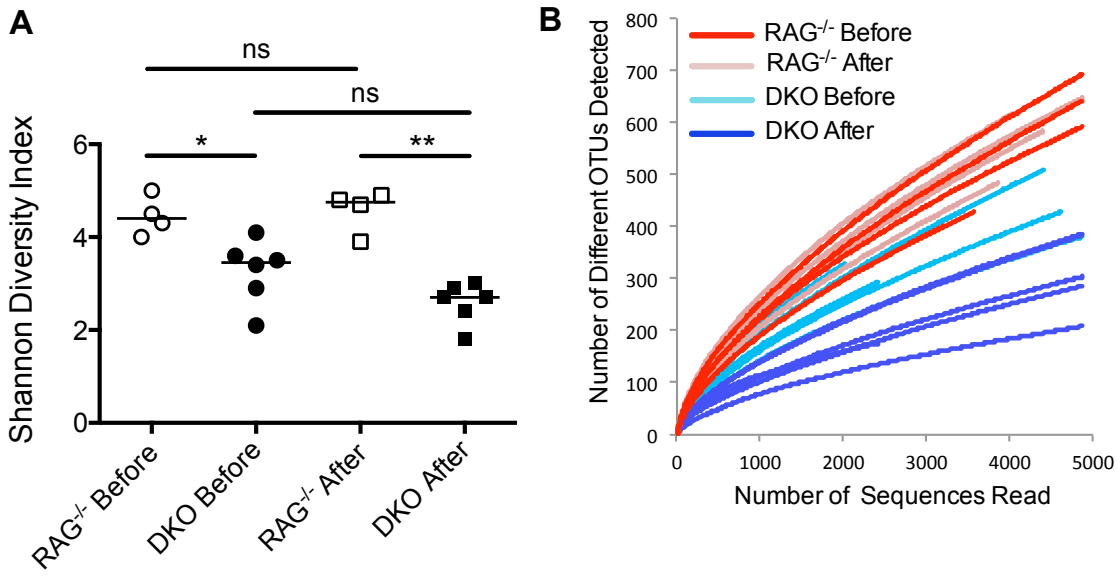


Figure 5-3. DKO mice have a different microflora with reduced diversity prior to induction of colitis. Stool samples were collected from RAG$^{-/-}$ mice purchased from Taconic and DKO mice bred in-house before and four weeks after induction of T cell transfer colitis ($3x10^5$ naïve T cells injected intraperitoneally). DNA was isolated from the stools and sequenced. Sequences were analyzed using MOTHUR software. Median Shannon Index diversity scores (A) and rarefaction analysis (B) are shown from one experiment. Each symbol (A) or line (B) represents an individual mouse in the indicated group. Statistics were calculated using a Mann-Whitney U-test. *$p<0.05$, **$p<0.01$.

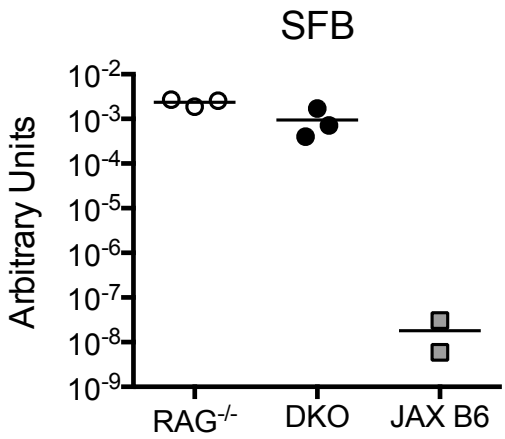


Figure 5-4. Fig 5-4 DKO mice do not have a difference in levels of segmented filamentous bacteria. DNA was isolated from stool samples collected from indicated mice without induction of colitis. Quantitative PCR using primers specific for SFB 16S ribosomal RNA (461) was performed and normalized to the total level of eubacterial 16S ribosomal RNA. Units represent SFB-specific sequence expression relative to universal eubacterial sequence expression. Data shown is from one experiment. Each symbol represents an individual mouse, horizontal bars represent medians.

5.2.2 IFNAR deficiency does not predispose mice to colitis induced by an opportunistic pathogen

We next asked whether IFN-1 influenced colonization of the mouse intestine with a specific commensal bacterium with colitogenic potential, *Helicobacter hepaticus*. *H. hepaticus* can be found in mouse colonies worldwide (445), but does not usually cause spontaneous colitis on its own. Rather, *H. hepaticus* requires defects in immunoregulatory mechanisms and the presence of other bacteria to trigger colitis (543). IL-10 production is one regulatory mechanism that suppresses *H. hepaticus*-induced colitis, and blockade of IL-10R signaling with a neutralizing antibody leads to typhlocolitis in *H. hepaticus*-infected mice (446, 447). Therefore, as IFNAR1$^{-/-}$ mice showed a defect in innate IL-10 production, we investigated whether IFN-1 was necessary for regulating *H. hepaticus*-induced colitis.

We observed that, similar to WT mice, infection of IFNAR1$^{-/-}$ mice with *H. hepaticus* did not induce spontaneous inflammation in the colon or cecum (Fig 5-5A,B). Furthermore, we found equal amounts of inflammation in both the colon and cecum when IL-10 signaling was blocked in WT or IFNAR1$^{-/-}$ mice infected with *H. hepaticus* (Fig 5-5A-B). Using quantitative PCR to detect *H. hepaticus*-specific DNA (462), we found that both WT and IFNAR1$^{-/-}$ hosts had equivalent levels of colonization with *H. hepaticus* after oral infection (Fig 5-5C). *H. hepaticus* infection induces IL-10 production from antigen-specific T cells (334). *In vivo*, *H. hepaticus* colonization induced equivalent amounts of IL-10 production by colon T cells in either WT or IFNAR1$^{-/-}$ mice (Fig 5-5D).

We next asked whether IFN-1 regulated *H. hepaticus*-induced IL-10 production from innate immune cells. We cultured BMDMs with live *H. hepaticus* and measured IL-10 in

the culture supernatant 12 hours later. We found that both WT BMDMs and IFNAR1$^{-/-}$ BMDMs produce IL-10 in response to *H. hepaticus* (Fig 5-6). IFNAR1$^{-/-}$ BMDMs showed a trend towards lower IL-10 secretion, although this difference did not reach statistical significance (Fig 5-6).

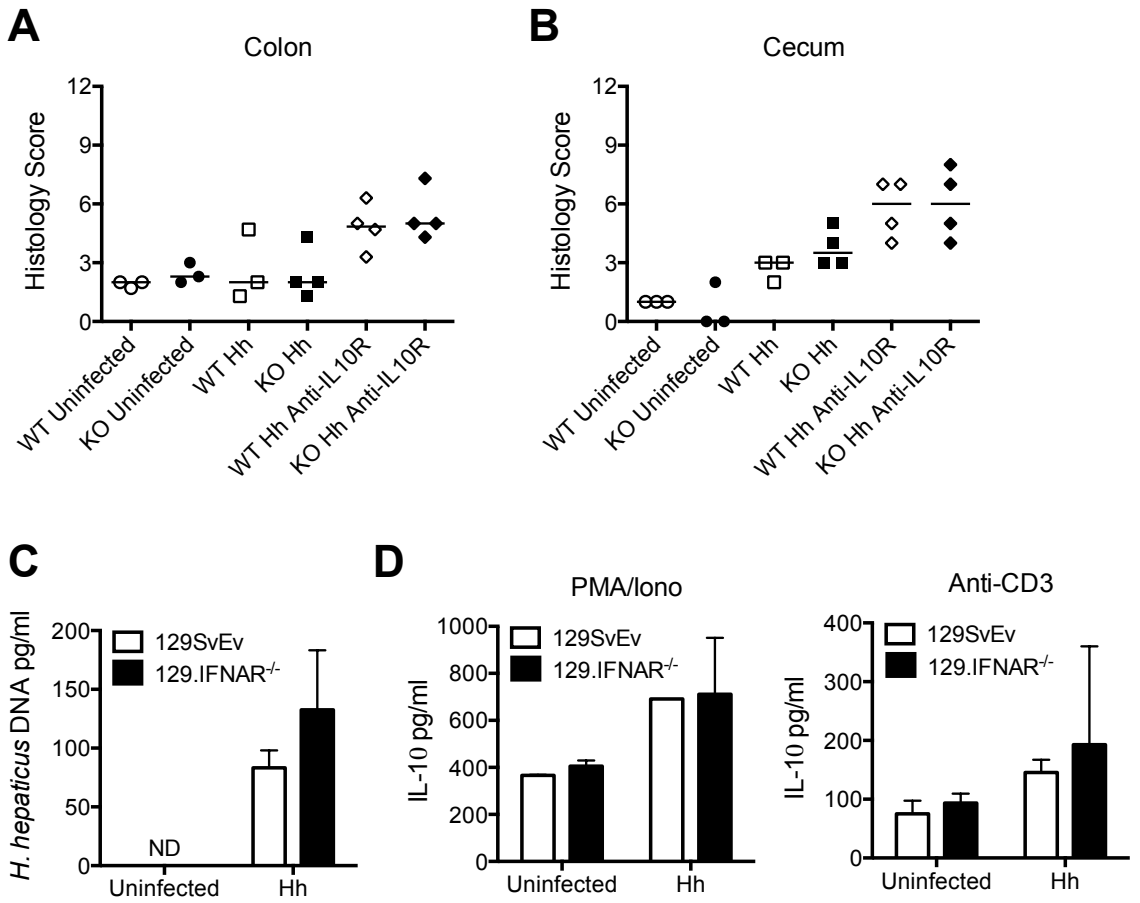


Figure 5-5. IFNAR1$^{-/-}$ mice do not develop exacerbated *Helicobacter hepaticus*-driven typhlocolitis. WT or IFNAR1$^{-/-}$ mice on the 129 background were either infected by oral gavage with 10^8 CFU *H. hepaticus* +/- treatment with anti-IL-10R antibody (1 mg per mouse weekly i.p.), or left uninfected. Mice given *H. hepaticus* only (n=2-4 per group) were followed for 8 weeks while mice given *H. hepaticus* plus anti-IL-10R (n=4 per group) were followed for 4 weeks. Colon (A) and cecal (B) histology scores are shown. (C) DNA was isolated from cecal contents of mice infected with *H. hepaticus* for 8 weeks. Mean levels of *H. hepaticus* DNA +/- SD are shown. (D) Cells isolated from the cLP of 8 week *H. hepaticus*-infected mice were stimulated *in vitro* (10^6 cells/mL) with PMA (50 ng/mL) and ionomycin (500 ng/mL) or anti-CD3 (1 μg/mL) overnight. Supernatants were harvested and IL-10 content assayed by ELISA. Mean amounts of IL-10 in the culture supernatant +/- SD are shown (n=2-4). All data shown is from one experiment. Each symbol in (A) and (B) represents an individual mouse and horizontal bars represent the median.

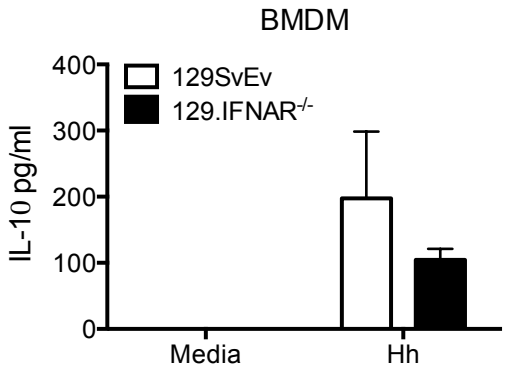


Figure 5-6. IL-10 induction by *H. hepaticus* in BMDMs is partially dependent on IFN-1. BMDMs grown *in vitro* were stimulated (10^6 cells/mL) with live *H. hepaticus* (MOI: 15) for 12 hours. IL-10 was measured in the culture supernatant by ELISA. Mean +/- SD from two independent experiments is shown.

5.2.3 IFNAR deficiency does not affect acute colitis induced by a gastrointestinal pathogen

Although IFN-1 did not alter colonization or inflammation during *H. hepaticus* infection, we asked whether a IFN-1 response would be necessary for protection against an acute gastrointestinal pathogen. We infected mice with *Citrobacter rodentium*, a murine pathogen that closely resembles enterohemorrhagic and enteropathogenic *Escherichia coli* in humans. Disease induced by these gram negative bacterial pathogens is characterized by attachment and effacement of the epithelial cell border, leading to malabsorption and diarrhea (544).

We infected WT and IFNAR1$^{-/-}$ mice on the 129 background with *C. rodentium*. Although IFNAR1$^{-/-}$ mice showed slightly greater, but statistically insignificant, weight loss (Fig 5-7A), both strains of mice showed equivalent levels of intestinal inflammation in the colon and cecum at days 8 and 14 post-infection (Fig 5-7B,C). Furthermore, both WT and IFNAR1$^{-/-}$ also showed equivalent levels of *C. rodentium* colonization throughout the infection as measured in fecal pellets, and in cecum, colon, and spleen tissue samples isolated at necropsy (Fig 5-8A-C).

Strikingly, when compared to WT mice on the B6 background, both WT and IFNAR1$^{-/-}$ mice on the 129 background shed about 100 times more *C. rodentium* in their feces at early time points (Fig 5-8A). Additionally, both genotypes of 129 mice showed systemic spread of the bacteria to the spleen (Fig 5-8D). In our animal facility, WT B6 mice infected with the same strain of *C. rodentium* do not normally exhibit bacterial translocation to the spleen (G. Song-Zhao and N. Srinivasan, personal communication). The comparatively poor ability of 129 mice to control *C. rodentium* infection may be due

to the fact that mice on the 129 background lack caspase-11, a critical component of the inflammasome response against gram-negative bacteria (252, 253). Accordingly, BMDMs grown from WT and IFNAR1$^{-/-}$ mice on the 129 background failed to secrete IL-1β when stimulated with *C. rodentium in vitro*, whereas WT B6 BMDMs responded robustly (Fig 5-9). Treatment of BMDMs with IFN-1 prior to stimulation with *C. rodentium* marginally increased inflammasome activation in B6 BMDMs as shown before (252), but had little effect on the low levels of IL-1β secreted by 129 BMDMs (Fig 5-9).

C. rodentium has previously been shown to induce Th17 cell differentiation (47), and T cell-derived IL-17 was associated with clearance of *C. rodentium* (321). Because we found increased Th17 cell differentiation in the absence of IFNAR signaling in another model of intestinal inflammation (Fig 3-3, Fig 3-4), we examined whether IFNAR1$^{-/-}$ mice had increased levels of IL-17 in response to *C. rodentium*. Analysis of whole colon tissue revealed that IL-17A, but not IL-17F, was markedly induced after *C. rodentium* infection in both WT and IFNAR1$^{-/-}$ mice (Fig 5-10). As predicted, *C. rodentium* infected IFNAR1$^{-/-}$ mice did show significantly greater IL-17A expression than *C. rodentium* infected WT mice (Fig 5-10); however, this did not translate to greater protection against growth of *C. rodentium* (Fig 5-8). IFN-γ was also induced upon infection with *C. rodentium*, but there were no differences in the levels of this cytokine between WT and IFNAR1$^{-/-}$ mice either (Fig 5-10).

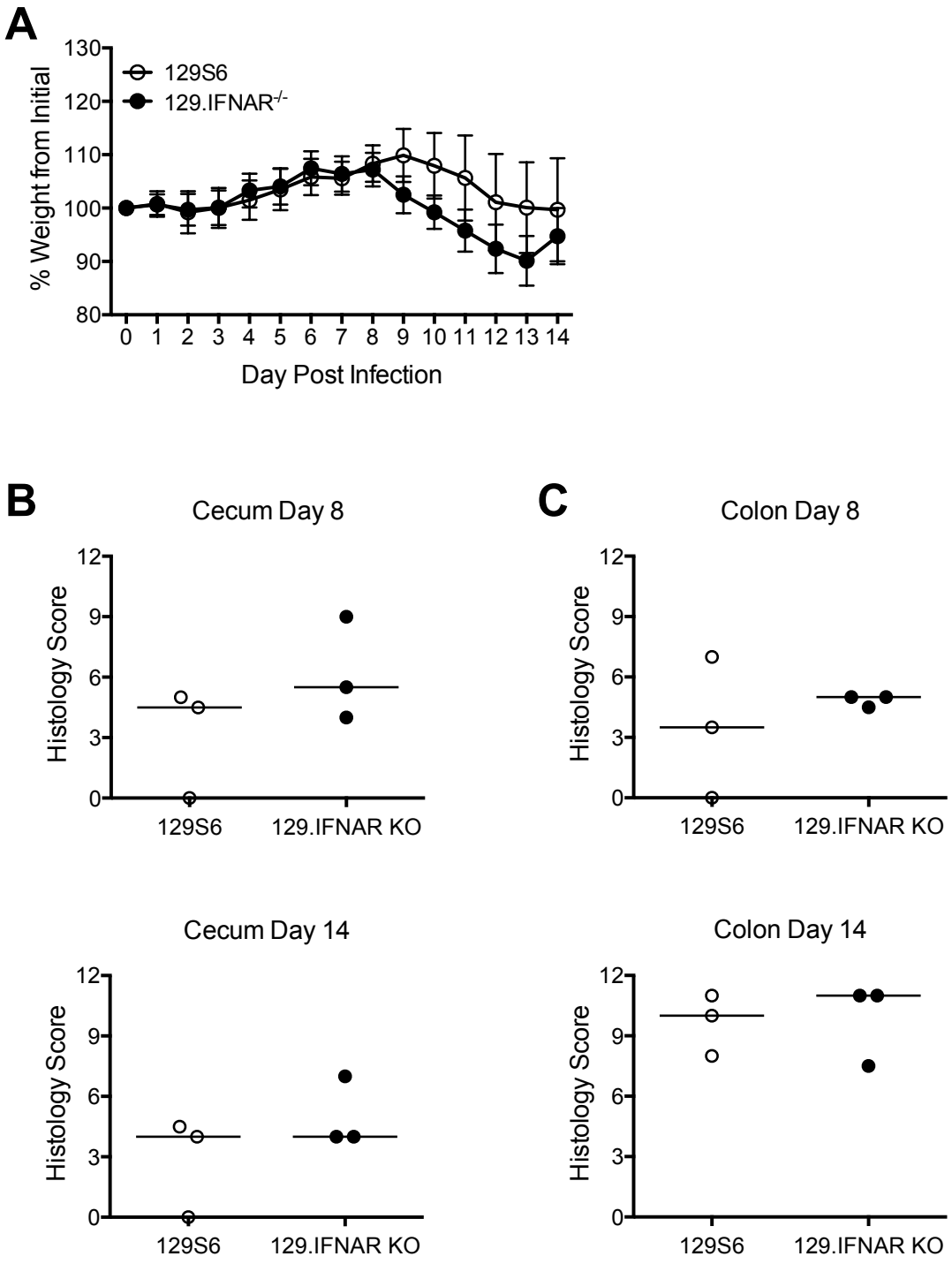


Figure 5-7. 129 IFNAR1$^{-/-}$ mice do not develop worse *Citrobacter rodentium*-induced colitis. WT and IFNAR1$^{-/-}$ mice were fed 10^8 CFU *C. rodentium* by oral gavage. Mice were weighed daily and groups of mice were killed at day 8 and day 14 after infection. (A) Mean body weight +/- SD is shown (n=6 for days 1-8, n=3 from day 9-14). (B-C) Histology scores from the cecum or colon as indicated at days 8 and 14 post-infection are shown. All data shown is from one experiment. Each symbol in (B-C) represents an individual mouse, horizontal lines represent group medians.

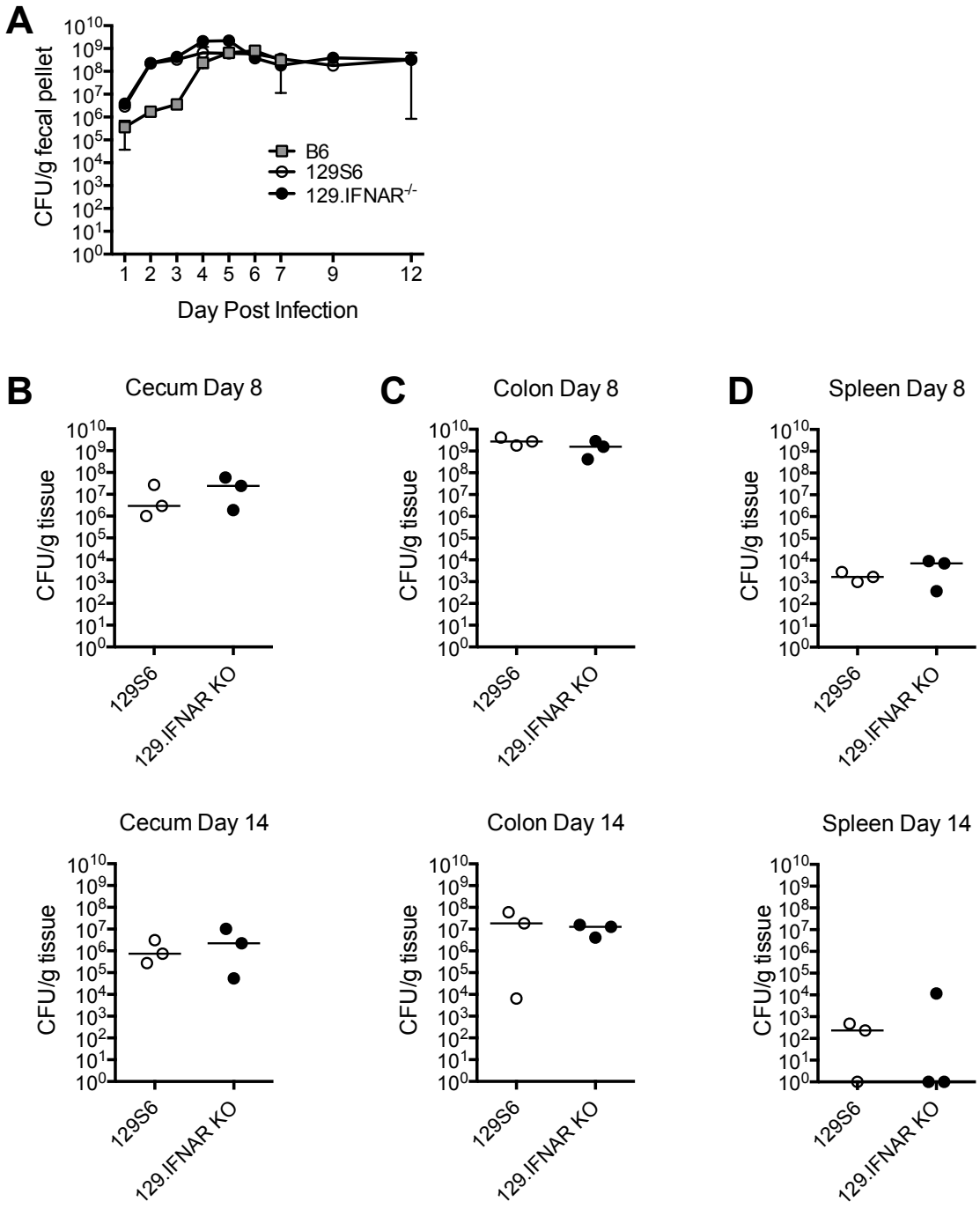


Figure 5-8. IFNAR1$^{-/-}$ mice have equivalent bacterial loads of *C. rodentium* after infection. WT and IFNAR1$^{-/-}$ mice were fed 10^8 CFU *C. rodentium* by oral gavage (n=4-6 for days 1-8, n=3 for days 9-14). Bacteria were grown and CFUs were counted from fecal pellets (A) or cecum, colon, or spleen homogenates (B-D) collected at indicated time points. All data shown is from one experiment. Data in (A) represent mean CFU +/- SD. Each symbol in (B-D) represents an individual mouse, horizontal lines represent group medians.

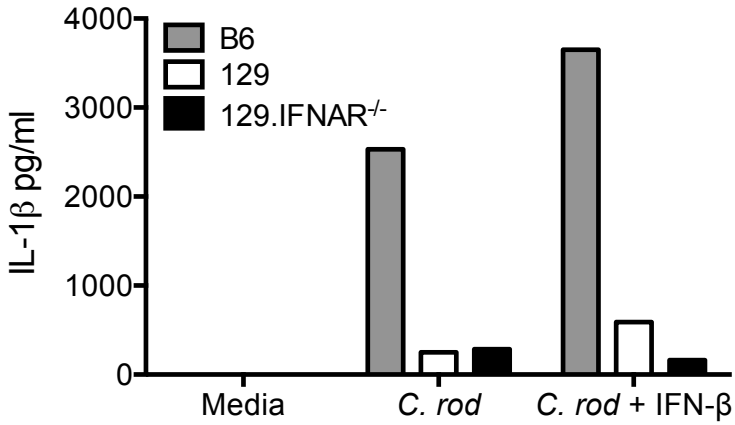


Figure 5-9. BMDM from 129 strain mice do not produce IL-1β in response to *C. rodentium*. BMDMs were grown from indicated mouse strains and incubated overnight with either media or 500 U/mL IFN-β at a concentration of 10^6 cells/mL. Cells were then incubated with LPS (100 ng/mL) for four hours prior to stimulation with *C. rodentium* (MOI: 20) for seven hours. IL-1β in the culture supernatants was measured by ELISA. Data shown is from one experiment.

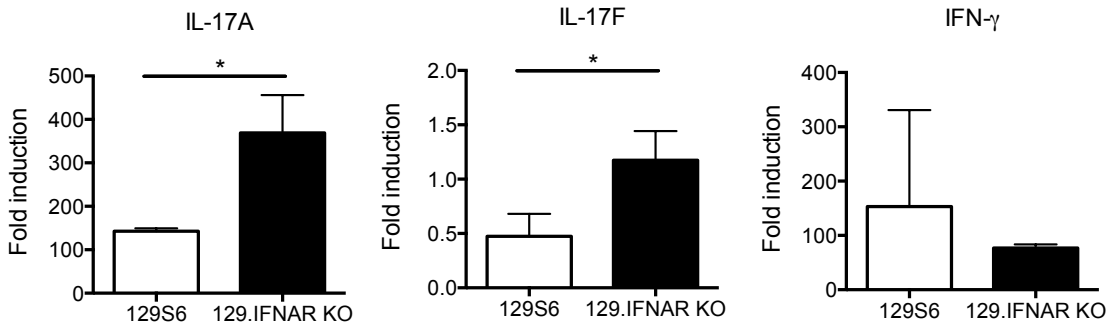


Figure 5-10. IFNAR1$^{-/-}$ mice have increased IL-17 production after *C. rodentium* infection. WT or IFNAR1$^{-/-}$ mice were fed 10^8 CFU *C. rodentium* by oral gavage. 14 days later, RNA was isolated from sections of distal colon. Expression of the indicated cytokine genes was measured by RT-PCR and normalized to transcript levels of HPRT. Mean levels of induction over WT uninfected from mice in one experiment are shown (n=3 per group). Error bars represent the standard deviation. *$p<0.05$.

5.3 Discussion

In this chapter, we provide evidence that IFN-1 has a minor role in shaping the composition of the intestinal microbiota. We found that DKO mice did have a different microbiota than non-littermate control $RAG^{-/-}$ mice (Fig 5-3). However, this difference in microbiota had variable effects on the severity of T cell adoptive transfer colitis. When we housed mice of different genotypes in the same cage to equalize intestinal flora, we found that in some cases, DKO mice co-housed with $RAG^{-/-}$ mice developed less severe colitis, while in other experiments, co-housing led to $RAG^{-/-}$ mice getting more severe colitis (data not shown). However, overall, DKO mice co-housed with $RAG^{-/-}$ mice still showed worse T cell-mediated colitis than their $RAG^{-/-}$ cagemates (Fig 5-1). The reason for the minor experimental variation was unclear, but may be explained by stochastic transfer of protective bacterial species from $RAG^{-/-}$ to DKO mice or transfer of colitogenic bacteria from DKO to $RAG^{-/-}$ mice. Further work involving microbiota sequencing will need to be conducted to define which bacterial species modify the kinetics and severity of colitis in DKO mice.

Although there were differences in the microbiota between $RAG^{-/-}$ and DKO mice, it is not clear whether these differences arose specifically due to a lack of IFNAR signaling or due to maternal inheritance of bacteria, as has been previously suggested (536). We generated $RAG^{-/-}$ and DKO littermates that were nursed on the same mother and thus, acquired the same flora during weaning. However, when DKO mice were separated from their $RAG^{-/-}$ littermates after weaning, they still developed worse colitis (Fig 5-2). Furthermore, we have previously shown that irradiated $RAG^{-/-}$ mice reconstituted with bone marrow from DKO mice developed worse T cell transfer colitis than $RAG^{-/-}$ mice reconstituted with bone marrow from $RAG^{-/-}$ mice (Fig 3-4). In these experiments, the

mice receiving naïve T cells were all RAG$^{-/-}$, eliminating the confounding factor of host microbiota. In addition, we re-derived DKO mice into two animal facilities at the NIH, each with different levels of cleanliness and different microbiota. DKO mice from either animal facility got more severe T cell transfer colitis compared to RAG$^{-/-}$ mice (Fig 3-2 and data not shown). Taken together with the co-housing data (Fig 5-1), these data indicate that random differences in host microbiota cannot fully explain the increased susceptibility of DKO mice to T cell adoptive transfer colitis.

However, altered microbiota in DKO mice may play a partial role in influencing the severity of colitis. Moreover, DKO littermates and RAG$^{-/-}$ mice receiving DKO bone marrow may still develop a microbiota that is functionally different from RAG$^{-/-}$ littermates or RAG$^{-/-}$ mice receiving RAG$^{-/-}$ bone marrow, if given enough time. We are currently planning experiments to sequence the microbiota of RAG$^{-/-}$ and DKO littermates over time to determine whether a lack of IFNAR signaling can directly alter the microbial composition.

We also looked at the role of IFN-1 in controlling pathology induced by *H. hepaticus*. *H. hepaticus* is a commensal of the murine intestinal tract in most immune competent strains of mice, but has the potential to cause colitis in mice with immune dysregulation, particularly when IL-10-dependent anti-inflammatory pathways are disrupted (446). Because IFNAR1$^{-/-}$ mice showed defects in IL-10 production (Fig 4-9), we hypothesized that they may not be able to suppress *H. hepaticus*-induced colitis. However, IFNAR1$^{-/-}$ mice did not develop colitis in response to *H. hepaticus* or show any differences in bacterial loads (Fig 5-5A-C). When we blocked IL-10 signaling with a neutralizing antibody against the IL-10 receptor, *H. hepaticus*-infected IFNAR1$^{-/-}$ mice developed

inflammation in the cecum and colon that was of similar severity to that seen in WT mice (Fig 5-5A,B). These results suggest that IFNAR1$^{-/-}$ mice do possess IL-10-dependent mechanisms of suppressing *H. hepaticus*-induced disease.

While *H. hepaticus* is capable of inducing either T cell-dependent or independent inflammation depending on the genotype of the host mice, CD4^{+} regulatory T cells can inhibit both types of inflammation in an IL-10 dependent manner (61, 334). Furthermore, when RAG$^{-/-}$ mice with intact IL-10 production in the innate immune system are reconstituted with bulk CD4^{+} T cells from IL-10$^{-/-}$ mice, they still develop *H. hepaticus*-induced immunopathology (334). Thus, adaptive immune cells appear to be a more critical source of IL-10 than innate immune cells for the control of disease instigated by this opportunistic pathogen. Although IFNAR signaling was essential for the optimal secretion of IL-10 from innate immune cells (Fig 4-9, Fig 5-6), T cells from IFNAR1$^{-/-}$ mice were able to produce IL-10 in response to *H. hepaticus* infection to the same extent as T cells from WT mice (Fig 5-5D). Thus, this may explain how IFNAR1$^{-/-}$ mice are able to control *H. hepaticus*-induced immunopathology despite a defect in innate immune cell-derived IL-10.

Finally, we analyzed the ability of IFNAR1$^{-/-}$ mice to handle an acute enteric pathogen, *Citrobacter rodentium*. Due to restrictions on availability, these experiments were conducted in mice on the 129 background. These mice are deficient in caspase-11, a protein that is essential for non-canonical activation of the NLRP3 inflammasome when stimulated with gram-negative bacteria or bacterial toxins (252, 253). Consistent with these reports, we found that both WT and IFNAR1$^{-/-}$ BMDMs from mice on the 129 background were unable to secrete IL-1β in response to *C. rodentium*, while WT B6

BMDMs displayed a robust response (Fig 5-9 and (252)). In WT mice, *C. rodentium* is a self-limiting, subclinical infection. NLRP3-deficient mice, however, are unable to control *C. rodentium* bacterial loads and experience more severe intestinal inflammation (G. Song-Zhao, unpublished observations and (451)). Thus, the inflammasome is crucial for control of *C. rodentium*, especially at early time points after infection (G. Song-Zhao and N. Srinivasan, unpublished observations). Therefore, it was not surprising that both WT and IFNAR1$^{-/-}$ mice on the 129 background showed increased bacterial loads for the first four days after infection, when compared to WT B6 mice (Fig 5-8A).

The use of mice on the 129 background did allow us to examine the inflammasome-independent effects of IFN-1 on control of *C. rodentium* infection. IL-17 and other Th17-related cytokines, including IL-23 and IL-22, have been associated with a protective response against *C. rodentium*. Mice deficient in IL-22 are unable to control bacterial loads at early points after infection (545) and recent studies have shown that ILCs in the lamina propria are key sources of this protective cytokine during the very early stages of *C. rodentium* infection (365). However, a very recent study found that six days post-infection, T cells became the predominant source of IL-22 crucial for controlling *C. rodentium* infection (450). IL-22 limits growth of *C. rodentium* via induction of the antimicrobial peptide, RegIIIγ (545). Mice deficient in IL-23 also show defects in control of *C. rodentium* (47). Although IL-23 is necessary for the maintenance of Th17 cells (47), it also acts upon ILCs to induce IL-22 and other "Th17" signature cytokines (16). IL-17A itself was found to be important for controlling *C. rodentium* bacterial loads two weeks after infection, and the predominant source of IL-17A was adaptive lymphoid cells, suggesting a role for Th17 cells after the initial wave of innate immunity subsided (321). Because IFNAR1$^{-/-}$ mice have increased Th17 cell differentiation (Fig 3-3, Fig 3-4 and

(247, 472)), we hypothesized that they would be better able to control bacterial loads at later time points. However, despite increased production of IL-17A in IFNAR1$^{-/-}$ mice, they did not show any greater decrease in *C. rodentium* bacterial loads compared to WT mice two weeks post-infection (Fig 5-8, Fig 5-10). These data provide further evidence that an early innate response to *C. rodentium* is more effective at reducing bacterial loads and controlling pathology than an adaptive Th17 cell response. We are currently investigating the role of IFN-1 in the early immune response against *C. rodentium* by analyzing the kinetics of infection in WT and IFNAR1$^{-/-}$ mice on the B6 background, which are sufficient for the inflammasome component, caspase-11.

Bacteria are necessary for many models of colitis and play a key role in human IBD. IFN-1 is induced by various bacterial species (64), and thus may contribute to the immune response that shapes the microbiota. While mice deficient in IFNAR signaling did display an altered microbiota, this had only a minor effect on the severity of colitis. Furthermore, IFNAR1$^{-/-}$ mice did not have worse bacteria-induced colitis in two separate models. These results suggest that IFN-1 controls the development of colitis independent of effects on the microbiota.

Chapter 6: General Discussion

6.1 Summary

The data presented here suggests that IFN-1 signaling licenses the innate immune system to tune the magnitude of the adaptive immune response. While direct IFNAR signaling on $CD4^+$ T cells did not enhance or limit their proliferation, IFNAR signaling on innate hematopoietic cells did limit the proliferation and accumulation of $CD4^+$ T cells in the MLNs and cLP, respectively. IFN-1 did not alter the ability of DCs to process antigen, present antigen, express co-stimulatory molecules, or seed the cLP. Rather, IFN-1 appeared to control the production of anti-inflammatory cytokines in the colon. IFN-1 also suppressed IL-1-mediated accumulation of a subset of inflammatory $CD11b^+CD103^-$ dendritic cells in the MLNs during colitis. In the steady state, $CD11c^+$ cells from the MLNs did not have any alterations in cytokine production, further supporting the hypothesis that the infiltration of this inflammatory cell population was the cause of enhanced T cell proliferation during colitis.

IFN-1 signaling on DCs also suppressed Th17 cell differentiation both *in vitro* and *in vivo*. IFN-1 was previously reported to have this effect via induction of IL-27, a known suppressor of Th17 cell differentiation (247, 472). However, we found that in the presence of additional Th17 cell polarizing reagents, the ability of IFN-1 to inhibit Th17 cell differentiation was diminished. *In vivo*, mice lacking IFNAR signaling had a greater accumulation of IFN-γ^+IL-17^+ $CD4^+$ T cells, a population that has been correlated with severe intestinal inflammation (441) and has been shown to convert into pathogenic Th1 cells that drive colitis (492). Concordantly, IFN-1 also suppressed IL-17 production in a *Citrobacter rodentium* infectious model of colitis.

IFNAR signaling on non-T cells was also shown to promote the suppressor phenotype of Treg cells. In the absence of IFNAR signaling on non-T cells, Treg cells lost expression of Foxp3 and some began to produce the pro-inflammatory cytokines IFN-γ and IL-17. Despite playing a prominent role in suppressing expansion of the effector $CD4^+$ T cell niche, IFN-1 seemed to be dispensable for regulating the size of the Treg niche. Thus, in the absence of IFNAR signaling, effector $CD4^+$ T cells accumulated in significantly greater numbers than regulatory $CD4^+$ T cells, leading to an imbalanced intestinal T cell response.

Finally, IFNAR signaling was shown to play a minimal role in immunity against specific pathogens we tested, although they may have a more general role in shaping commensal bacterial populations in the intestine. These data are preliminary and will be explored further in future studies.

In this section, I put into perspective the role that IFN-1 has on homeostasis and immunity, as well as its application to human disease. I also describe future directions that can be pursued from these data.

6.2 Type I interferons as a bridge between innate and adaptive immunity

IFN-1 can greatly enhance the T cell priming capacity of the DCs. IFN-1 activity on monocytes leads to differentiation of dendritic cells with potent T cell proliferative and Th1 cell polarization capacities *in vivo* (235). Likewise, culture of human peripheral blood mononuclear cells (PBMCs) in the presence of IFN-α led to the differentiation of

DCs with potent antigen-presenting capability. HLA and co-stimulatory molecule expression increased in response to IFN-1 in a dose-dependent manner (234, 510). Furthermore, bone marrow-derived DCs required constitutive autocrine IFNAR signaling for optimal expression of co-stimulatory molecules and T cell proliferative capacity (511).

IFN-1 augments not only the T cell effector response, but the B cell response as well. Administration of the IFN-1-inducing agent, poly (I:C), or treatment with IFN-1 itself greatly enhanced the humoral response (248). The ability of IFN-1 to serve as an adjuvant for antibody responses relied on IFNAR signaling on dendritic cells (248), at least partially due to its ability to elicit DC production of IL-6 (249). IL-6, in turn, signals on T cells to support their differentiation into T follicular helper cells (546), a $CD4^+$ T cell subset specialized in providing help to B cells (547). Direct IL-6 signaling on B cells also enhanced their differentiation and antibody secretion (548, 549). The adjuvanticity of CpG DNA motifs is also dependent on IFN-1 (550), although it has not been determined whether IFNAR signaling is required on DCs in this case. In addition to IFN-1 induced by adjuvants, endogenous IFN-1 was also necessary for the development of optimal antibody responses (248).

Despite these data and further evidence to the contrary (516), we found that $IFNAR1^{-/-}$ DCs were efficient at processing antigen, presenting antigen, and stimulating T cell proliferation. $IFNAR1^{-/-}$ DCs also seemed to accumulate to a greater extent in the draining lymph nodes and consequently drove increased T cell proliferation. The anti-inflammatory effects of IFN-1 on myeloid cells seemed to specifically regulate their ability to promote T cell responses since IFNAR signaling did not alter disease severity in an innate anti-CD40-mediated model of colitis. At this point, it is still unclear whether

IFN-1 suppression of T cell proliferation was predominantly due to IL-1RA-mediated inhibition of DC migration or whether the activity of other anti-inflammatory cytokines induced by IFN-1 also contributed to control of colitis.

To further explore this hypothesis, it must be determined whether migration of antigen-bearing inflammatory DCs from the colon tissue to the lymph nodes is dependent on IL-1 signaling during colitis. This could be accomplished by collecting the fluid from the lymphatics between the cLP and the MLNs from colitic mice that have been treated with either PBS or with the soluble IL-1RA, anakinra (551), the prediction being that fewer $CD11c^{+}CD11b^{+}CD103^{-}$ cells with a DC morphology would be recovered from anakinra-treated mice. Alternatively, $RAG^{-/-}$ mice could be crossed to $IL\text{-}1R^{-/-}$ mice to generate lymphopenic hosts that are unable to respond to IL-1 signaling. If IL-1 signaling is vital for the migration of inflammatory DCs, naïve $CD4^{+}$ T cells transferred into these recipients should be unable to induce colitis. However, in the absence of IL-1, it is possible that TNF-α or other DC-derived pro-inflammatory cytokines may substitute for the purposes of inducing DC migration out of tissue (490).

IL-1RA induced from myeloid cells may also block IL-1 signaling directly on $CD4^{+}$ T cells in the cLP. Indeed, previous work in our lab demonstrated that IL-1 is necessary for the survival of Th17 cells in the cLP, but it also controls the accumulation of other $CD4^{+}$ T cell populations (49). Although these other $CD4^{+}$ T cells do not make IFN-γ or IL-17, they may produce other effector cytokines such as GM-CSF, TNF-α, or IL-21. Consistent with this hypothesis, we found that a lack of IFNAR signaling not only led to an increase in the percentage of $IL\text{-}17^{+}$ $CD4^{+}$ T cells, but to an increase in total number of all $CD4^{+}$ T cell subsets as well.

IFN-1 also induced IL-10 from myeloid cells in the cLP. IL-10 may also work to block migration of DCs out of the colon, as treatment of DCs with IL-10 reduces the expression of CCR7 (552), a chemokine receptor necessary for the egress of DCs from the tissue to the lymph nodes (553). Accordingly, DCs exposed to IL-10 either exogenously or endogenously migrated to the draining lymph node to a lesser extent than untreated WT DCs or DCs from IL-10$^{-/-}$ mice (554, 555). Furthermore, other myeloid cells may be the source of IL-10 *in vivo*, suggesting that IL-10 works locally in an autocrine or paracrine manner to suppress DC migration (556, 557). Finally, IL-10 activity on myeloid cells can also suppress their T cell-polarizing cytokine production. Blockade of IL-10 signaling allowed normally unresponsive myeloid cells to produce IL-12p70 in response to LPS (341) and myeloid cells isolated from non-inflamed IL-10$^{-/-}$ mice had greater expression of IL-12p35 and IL-23p19 (11), two cytokines that heavily influence T cell-mediated colitis. *In vivo*, IL-10 regulated colitis by suppressing MyD88 signaling on myeloid cells (558). Strikingly, mice selectively deficient in STAT3 in their myeloid cells, rendering their myeloid cells unresponsive to IL-10, developed a spontaneous colitis dependent on IL-12p40, the shared subunit of IL-12 and IL-23 (559).

IL-10 produced by myeloid cells can signal directly onto T cells as well. However, IL-10 signaling on CD45RB^{hi} CD4^{+} T cells did not affect their accumulation in the cLP (343). Furthermore, bulk CD4^{+} T cells isolated from IL-10$^{-/-}$ mice were able to cause colitis when transferred into RAG$^{-/-}$ mice, suggesting that IL-10 derived from host myeloid cells was unable to control the pathogenicity of the Th1-type effector T cells (427). In contrast, IL-10 signaling on Treg cells was necessary for their ability to suppress colitis induced by CD45RB^{hi} cells (343). This effect was due to the role of myeloid cell-derived IL-10 in

both expanding Treg cells over effector T cells and maintaining Foxp3 expression on Treg cells (327, 343).

IL-27 is yet another myeloid cell-derived cytokine elicited by IFN-1 that can tune adaptive immune responses. IL-27 may control the adaptive immune response indirectly by acting on macrophages and inducing their secretion of IL-10 (242) and/or inhibiting their secretion of IL-12 (560). Consistent with these effects, $RAG^{-/-}$ x $IL\text{-}27R^{-/-}$ mice developed significantly worse dextran sodium sulfate-induced colitis (561). However, the role of IL-27 signaling in T cells is significant and has been very well characterized. IL-27R signaling was originally described to be crucial for the development of sustained Th1 immunity (562). Accordingly, $IL\text{-}27R^{-/-}$ $CD45RB^{hi}$ $CD4^{+}$ T cells failed to differentiate into Th1 cells or induce colitis (563) and were more prone to activation-induced cell death (564). In contrast, IL-27 inhibited Th17 cell differentiation and Th17 cell-mediated pathology (565). In addition, IL-27 signaling was crucial for the generation of $IL\text{-}10^{+}$ regulatory and effector T cells (566, 567) and the function of mucosal $Foxp3^{+}$ Treg cells (522). Paradoxically, although IL-27 enhanced the function of mucosal Treg cells, IL-27R signaling on naïve T cells inhibited their initial differentiation into $Foxp3^{+}$ Treg cells (563). Thus, IL-27 is a cytokine critical for both the development of $CD4^{+}$ T cell effector immunity as well as self-regulation of the immune response.

To determine whether IFN-1 control of T cell proliferation is dependent on the activity on any of these cytokines, DKO mice reconstituted with $CD45RB^{hi}$ $CD4^{+}$ T cells could be treated with IL-1RA, IL-10, or IL-27. As mentioned earlier, we have already treated mice with IL-1RA and observed a decrease in early T cell accumulation (Fig. 3-9). Whether this inhibition of early T cell accumulation is enough to suppress long-term colitis

remains to be determined. In any case, caution must be employed when considering systemic administration of these cytokines as a long-term treatment option in humans, since they are potent immunosuppressive cytokines that may increase the incidence of infections in patients.

Although we found that IFNAR1$^{-/-}$ DCs had a greater T cell proliferative capacity, it remains to be determined whether they increase T cell proliferation in an antigen-dependent manner. To determine this, we could transfer CFSE-labeled CD4^{+} T cells with a TCR specific for ovalbumin peptide into RAG$^{-/-}$ or DKO recipients and analyze T cell proliferation in the presence or absence of ovalbumin in the drinking water. Finally, although IFNAR signaling on DCs controlled T cell proliferation, it does not preclude a concomitant role in inhibiting CD4^{+} T cell survival. IFN-1 induced both IL-1RA and IL-27 secretion from cLP myeloid cells, which could either inhibit or promote the survival of activated CD4^{+} T cell subsets (49, 564).

6.3. Opposing effects of constitutive and acute IFNAR signaling in homeostasis and immunity

The fact that IFN-1 serves potent immunoregulatory roles in this model of chronic inflammation while amplifying the immune response during acute viral infections raises the question of how IFN-1 can have seemingly opposing effects. During acute infections, a swift and potent response is needed. A burst of IFN-1 is produced by epithelial cells (123), conventional DCs (109), and plasmacytoid DCs (105) upon viral recognition. IFN-1 acts locally on surrounding cells to promote apoptosis and inhibit transcription and translation. At the same time, pDCs and cDCs migrate from the tissue to secondary lymphoid organs (106) to create a systemic IFN-1 state that greatly enhances the ability

of NK and T cells to produce IFN-γ (71). Because of constitutive expression of IRF7, pDCs produce IFN-1 rapidly after detection of nucleic acids and in very high quantities (104). Indeed, depletion of pDCs led to defects in the early response against viruses (108). Finally, the IFN-1 response in this setting is self-limiting as IFN-1 controls a feedback loop inducing the apoptosis of pDCs (568).

In contrast, constitutive IFN-1 is produced in low, barely detectable quantities (149, 155) and has very different effects on the immune system. Constitutive production of IFN-1 is essential for the expression of IRF-7 (154), the master regulator of IFN-1 production during immune responses (569). Thus, low levels of IFN-1 production prime immune cells for a rapid response generating high levels of IFN-1 upon induction by pathogens (570).

However, constitutive IFN-1 must also prevent immune activation in the absence of infection. One mechanism by which IFN-1 accomplishes this is to decrease macrophage responsiveness to type I and type II interferons. When colonized by a commensal strain of bacteria engineered to constitutively produce IFN-β, myeloid cells in the colon adapted by downregulating IFNAR on their cell surface (571). IFN-1 also blocks IFN-γ signaling. While micromolar levels of IFN-1 had agonistic effects on macrophages, femtomolar levels (9 orders of magnitude lower) had the opposite effect and rendered macrophages unresponsive to activation by low levels of IFN-γ (572). It was found that IFN-1 can bind to the IFN-γR with a higher affinity than IFN-γ itself (572). IFN-1 was also found to directly downregulate the IFN-γR on macrophages (256). Accordingly, IFN-1 blocks many of the immune activating effects of IFN-γ on macrophages (256, 572-574). IFN-1 also induces STAT1 in cells (153), a key transcription factor that blocks IFN-γ production

from NK and T cells (174). Thus, steady state IFN-1 blocks innate and adaptive immune activation by several mechanisms. However, high levels of IFN-γ, as would be present during infections, can overcome the inhibitory effects of IFN-1 (572).

Several pathogens that establish latency or chronic infection exploit the inhibitory effects of constitutive IFN-1. *Listeria monocytogenes*, a gram-positive bacterium, which has adapted the ability to escape macrophage phagocytic vacuoles and establish intracellular residence, elicits a stronger immune response in the absence of IFNAR signaling (488, 533, 534). This was partially due to IFN-1-mediated sensitization of lymphocytes to *L. monocytogenes*-induced apoptosis (488). Curiously, *L. monocytogenes* was able to induce IL-12 production and a strong Th1 response (39), but *L. monocytogenes*-infected macrophages were unable to respond to IFN-γ (256). It was later found that this was due to IFN-1-mediated downregulation of the IFN-γR (256).

Chronic mycobacterial infections are also more persistent in the presence of IFNAR signaling. IFNAR1$^{-/-}$ mice showed lower bacterial loads when challenged with *Mycobacterium tuberculosis* (288), a chronic granuloma-forming infection of humans and mice. Furthermore, pharmaceutical induction of IFN-1 greatly increased the burden of infection in WT mice, but not IFNAR1$^{-/-}$ mice (288). IFN-1 recruited a population of myeloid cells that were permissive to *M. tuberculosis* infection (288), but simultaneously inhibited pulmonary myeloid cell production of IL-1α and IL-1β (251), two cytokines critical for host defense against the pathogen (575). IFN-1 also inhibited the host response against *Mycobacterium leprae*, the causative agent of human leprosy (576). IFN-1 induced IL-10 and inhibited IFN-γ-mediated macrophage microbicidal activity, leading to lesions that permitted the growth of the bacterium (576). Accordingly, self-healing

lesions displayed a IFN-γ gene signature, while lepromatous lesions displayed a IFN-1 gene signature and greater bacterial growth (576).

Finally, chronic viral infections are also negatively impacted by IFN-1 signaling despite the necessity of IFN-1 in clearance of acute infections. During chronic lymphocytic choriomeningitis virus (LCMV) infection in mice, IFN-1 levels were found to be systemically elevated (577, 578). Blockade of IFNAR resulted in decreased expression of IL-10 in the serum of LCMV-infected mice and downregulation of the immune inhibitory molecule, PD-L1, on DCs (577). In addition, treatment with an IFNAR neutralizing antibody enhanced IFN-γ production by $CD4^+$ T cells, which was required for expulsion of the virus (577, 578). Thus, prolonged IFN-1 signaling induces IL-10 and suppresses Th1 cells in a chronic infection model, mirroring our observations in a non-infectious model of chronic inflammation. While treatment with IFN-1 may suppress disease in chronic inflammatory disorders, blockade of IFN-1 may be beneficial in reactivating the immune system against persistent viral infections, such as HIV or chronic hepatitis.

In the colon environment, cells are conditioned by a constant supply of low-level IFN-1. Macrophages and cDCs are the predominant source of IFN-1 in the colon as pDCs are absent from this location (K. Kitamura, unpublished observations; and data not shown). It is possible that this low-level constitutive signaling not only promotes anti-inflammatory responses in the colon, but also renders cells refractory to immune activating signals. We found that $IFNAR1^{-/-}$ myeloid cells expressed similar levels of the CD40 receptor as WT cells, but we did not examine their ability to receive other immune activating signals. Specifically, it would be interesting to examine whether constitutive IFNAR signaling regulates the expression level of the IFN-γR, either in the steady state or during colitis.

IFN-γ signaling via the IFN-γR is essential for the classical activation of macrophages (316). Thus, IFNAR1$^{-/-}$ macrophages may be more sensitive to IFN-γ produced by CD4^{+} T cells during colitis and secrete greater amounts of pro-inflammatory cytokines such as IL-1, TNF-α and IL-6. Activated macrophages also release chemokines that attract other immune cells and therefore, IFN-1 may function to dampen this pro-inflammatory positive feedback loop.

Colon macrophages in the steady state display many properties of alternatively-activated macrophages, which can be divided into two groups: wound-healing macrophages and regulatory macrophages (579). Wound-healing macrophages are activated by IL-4 and promote Th2 responses and repair of the extracellular matrix (579). In response to IL-27, wound-healing macrophages are inhibited in their pro-inflammatory cytokine production (580). The stimuli for differentiation of regulatory macrophages is not as well defined, although they appear to be a population induced by adaptive immune responses as a compensatory mechanism to limit ongoing inflammation (579). We found IFN-1 to be essential for myeloid cell production of IL-10, IL-27 and IL-1RA, three cytokines typical of regulatory macrophages. It is possible, thus, that constant microbial stimulation in the colon induces regulatory macrophages in a IFN-1 dependent manner. As IFN-1 also induces IL-27 in the colon, it may be necessary for the differentiation of wound-healing macrophages as well. Further biochemical analysis and surface marker phenotyping will be necessary to determine to what extent IFN-1 controls the differentiation of each of these macrophage subsets.

It also remains to be determined how IFN-1 specifically promotes anti-inflammatory cytokine production with only a minimal effect on pro-inflammatory cytokine production.

IL-10 and IL-27 induction by IFN-1 requires STAT1 phosphorylation (242, 246, 250). IL-1β, IL-12p40, IL-6 and TNF-α are all also produced in a STAT1 dependent manner (581, 582), but unlike requirements for IL-10 and IL-27 production, STAT1 phosphorylation for pro-inflammatory cytokine production is IFN-1 independent and driven by TLR activation (79, 581). In fact, some reports suggest that IFN-1 can even inhibit TLR-dependent STAT1 phosphorylation (581). Because pro- and anti-inflammatory cytokine production both rely on STAT1, IFN-1 may induce additional STAT proteins that interact with STAT1 to specifically promote anti-inflammatory cytokine production. IFN-1 may also activate JAK/STAT-independent pathways as has been shown for IL-1RA and early IL-10 induction (243, 583). Thus, future work will need to focus on the signaling pathways induced by IFN-1 in colon myeloid cells.

The inhibition of pro-inflammatory signaling and the promotion of alternatively-activated macrophage subsets may be a mechanism by which constitutive IFN-1 conditions an anti-inflammatory phenotype on colon myeloid cells. In turn, these cells are essential for establishing intestinal homeostasis, but may also leave organisms susceptible to persistent infections.

6.4 The use of IFN-1 for human therapeutics

The pleiotropic effects of IFN-1 have important implications in designing human therapeutics. As mentioned earlier, IFN-1 enhances both T and B cell responses via signaling on DCs (248, 258, 584). Hence, IFN-1 is already being used as an adjuvant in several vaccine formulations, with promising efficacy in healthy volunteers (585). However, in clinical trials with patients that were previously unresponsive to vaccination,

the elderly, or in patients receiving a mucosal vaccine, IFN-1 did not enhance adaptive immune responses (586-588). These results highlight important considerations in patient selection and route of administration when designing an appropriate vaccine formulation. For example, patients with lower immune responses, such as non-responders to vaccines and the elderly, may already be refractory to the effects of exogenous IFN-1. Patients with chronic IFN-1-mediated immune activation may also be refractory to exogenous IFN-1. Finally, vaccines administered via mucosal routes where IFN-1 signaling is constitutive may require a higher dose of acute IFN-1 to overcome the immune inhibitory effects of constitutive IFN-1.

IFN-1 has also been used in clinical trials for the treatment of IBD, albeit with limited success (452-455). For patients with Crohn's disease, immunosuppressive steroids are the first-line treatment option, but come with significant side effects such as increased predisposition to infectious diseases. However, neither IFN-α nor IFN-β was able to substitute for steroids to achieve clinical remission (454, 455). IFN-1 showed greater promise for the treatment of ulcerative colitis (UC). In two independent clinical trials, a majority of patients with active UC responded to IFN-β (452, 453). In addition to a reduction in clinical disease scores, IFN-β treatment reduced T cell production of IL-13, the signature effector cytokine of UC (453, 465).

These results from clinical trials suggest that IFN-1 is capable of inhibiting ongoing Th2-driven disease in UC patients, but not ongoing Th1/Th17-driven disease in CD patients. Data presented in this thesis suggests that IFN-1 has no effect on Th1 cell polarization, but rather has a global effect on the accumulation of all $CD4^+$ T cells. T cell expansion in this model of colitis is driven by lymphopenia, which is unlike common etiologies of

human IBD. Furthermore, we did not attempt to treat ongoing colitis with recombinant IFN-1 in this project. Our results do not differentiate whether IFNAR signaling is more important during the induction of T cell-mediated colitis or for the suppression of ongoing disease. There are several ways to address this issue. The simplest would be to treat $RAG^{-/-}$ recipients of naïve $CD4^{+}$ T cells with recombinant IFN-1 at various doses and for varying durations during and after the induction of colitis. An alternative approach would be to transfer WT myeloid cells, sufficient for IFNAR signaling, into colitic DKO mice to determine whether the introduction of cells capable of receiving endogenous IFN-1 signals can suppress ongoing colitis. Finally, generation of transgenic mice with a floxed IFNAR1 gene and a Cre recombinase under the control of a tamoxifen-inducible promoter would allow us to control the expression of IFNAR at various times before and after the induction of colitis. If IFNAR expression prior to the induction of colitis protected mice from accelerated colitis, then IFNAR signaling would be important for the prevention of colitis. On the other hand, if IFNAR expression induced after the development of colitis lessened the severity of disease, then that would suggest IFN-1 actively suppresses ongoing inflammation.

The data presented in this thesis is more consistent with the hypothesis that IFNAR signaling is more important for preventing colitis than curing it. Although constitutive IFN-1 was necessary for optimal induction of anti-inflammatory cytokines by colon myeloid cells, addition of exogenous IFN-1 did not further augment anti-inflammatory cytokine production. This result suggests that while endogenous IFN-1 are important for the proper functioning of colon myeloid cells, treatment with IFN-1 is unlikely to provide further therapeutic benefit.

DKO mice more prone to develop accelerated and severe colitis may also represent IBD patients that are hyporesponsive to IFN-1. Mutations in the locus containing IFNAR1 and IFNAR2 have been found to be weakly associated with Crohn's disease (457). It is unknown whether these mutations reflect a loss-of-function or gain-of-function in IFNAR signaling. However, intestinal mononuclear cells from IBD patients were found to respond poorly to IFN-1. IFN-1 treatment of intestinal mononuclear cells from healthy controls enhanced cell-mediated cytotoxicity in vitro, while treatment of cells from IBD patients did not (456). Thus, another reason why IFN-1 shows poor efficacy for the treatment of IBD may be that patients with IBD are inherently hyporesponsive to IFN-1. Our results also suggested that inhibition of the IL-1 axis could inhibit the development of accelerated and severe colitis observed in DKO recipients of naïve $CD4^+$ T cells. Perhaps hyporesponsiveness to IFN-1 marks a subset of IBD patients that would respond well to anakinra, a human antagonist of IL-1 signaling.

6.5 Concluding remarks

Intestinal $CD11b^+CD103^-$ cells, which are functionally characteristic of dendritic cells, carry antigen from the tissue to lymph nodes where they drive T cell proliferation (344). This population of migratory cells expands greatly during conditions of inflammation (11, 506). Inflammation may be crucial for the T cell priming capacity of these cells as TNF-α, IL-1, and stimulated DCs can all drive the migration of these cells out of tissue and into lymphatics draining the lymph node (490). The data presented in this thesis provides evidence for the notion that constitutive IFN-1 signaling inhibits the migration of these cells and subsequent T cell priming by promoting the secretion of anti-inflammatory cytokines such as IL-1RA that counteract the pro-inflammatory cytokines that drive DC

migration. IFN-1 induced anti-inflammatory cytokines may concurrently play roles in expanding and maintaining regulatory T cells, which balance the effects of effector T cells. A schematic depicting these actions of IFN-1 is shown in Fig 6-1. In conclusion, these results suggest that IFN-1 plays a non-redundant role in intestinal homeostasis by promoting multiple anti-inflammatory pathways. Enhancing IFN-1 signaling or its downstream effectors may provide therapeutic benefit in some IBD patients.

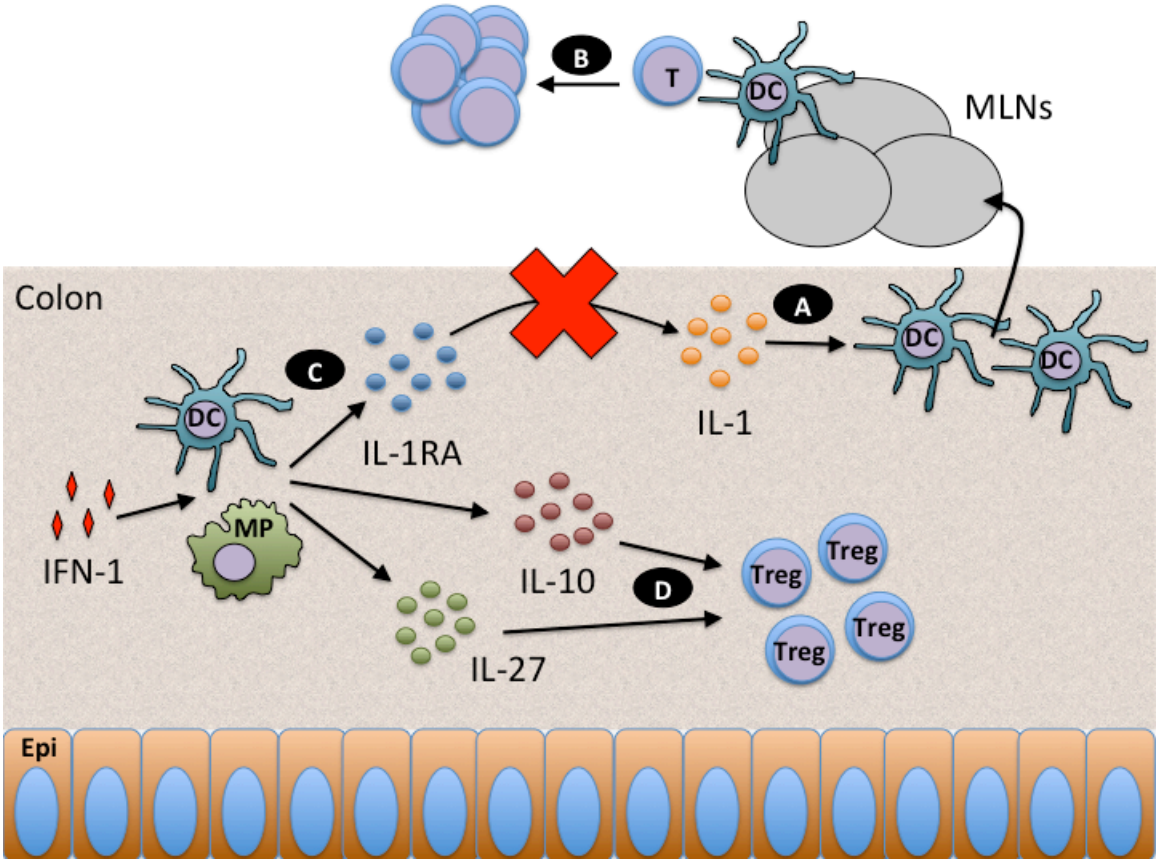


Figure 6-1. Schematic of mechanisms by which IFN-1 promotes anti-inflammatory responses in the colon. (A) IL-1 stimulates the migration of $CD11b^{+}CD103^{-}$ DCs to the MLNs. (B) In the MLNs, DCs interact with $CD4^{+}$ T cells and stimulate T cell proliferation. (C) IFN-1 activity on intestinal myeloid cells induces the release of IL-1RA, which counteracts the IL-1-mediated migration of inflammatory DCs. (D) Concurrently, IFN-1 induces myeloid cell secretion of IL-10 and IL-27, which act on Treg cells to promote the stability and function of the population.

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