MACROPHAGE PHOSPHOINOSITIDE 3-KINASE p110δ REGULATES INTESTINAL HOMEOSTASIS BY DIRECTING ADAPTIVE IMMUNITY AND ENHANCING MICROBIAL CLEARANCE

ERIN CATHLEEN STEINBACH

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Approved by:

Scott E. Plevy, MD.

R. Balfour Sartor, MD.

Virginia L. Miller, PhD.

Miriam Braunstein, PhD.

William E. Goldman, PhD.

ABSTRACT

ERIN CATHLEEN STEINBACH: Macrophage Phosphoinositide 3-kinase p110δ Regulates Intestinal Homeostasis by Directing Adaptive Immunity and Enhancing Microbial Clearance
(Under the direction of Scott E. Plevy, MD)

The human inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis, result from an inappropriately directed immune response to enteric microbiota in a genetically susceptible host. IBDs represent an increasing burden on the global health care system, as incidence is increasing and effective therapies remain elusive. Genome-wide association studies highlight the importance of host innate immune cell-microbial interactions in the pathogenesis of IBDs.

PI3K signaling regulates diverse functions, including cell growth, differentiation, proliferation and survival. The Class I_A PI3K catalytic subunit p110δ negatively regulates toll-like receptor signaling in innate immune cells. The importance of p110δ in intestinal homeostasis is shown in a mouse harboring a kinase-dead p110δ ($p110\delta^{KD}$) that develops spontaneous Th1/Th17-skewed colitis. We describe a requirement for the enteric microbiota to drive intestinal inflammation in $p110\delta^{KD}$ mice. Microbial-innate immune interactions maintain homeostasis through regulation of both protective (IL-10) and inflammatory (IL-12p40) cytokines, and p110δ is a central regulator of this balance. Additionally, p110δ positively regulates eradication of intracellular bacteria in macrophages. Persistence of intracellular bacteria and chronic stimulation in intestinal $p110\delta^{KD}$ macrophages propagates the cytokine imbalance. Furthermore, p110δ

orchestrates innate immune cell regulation of pathogenic adaptive immune responses. Importantly, in human CD, decreased intestinal *PIK3CD* gene expression and an inverse correlation with intestinal *IL12B*:*IL10* ratios are demonstrated. Thus, p110δ appears to be a central homeostatic switch in the intestine, governing the critical balance between IL-12/23 and IL-10 induced by the microbiota that determines the subsequent T cell response. Counter to prevailing paradigms where p110δ inhibition is a strategic approach in inflammatory diseases, strategies to induce p110δ gene expression could be a potential therapeutic approach in human IBDs.

To my parents, Jeanne and Chris

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LIST OF ABBREVIATIONS

APC, antigen presenting cell

BMDC, bone marrow-derived dendritic cell

BMDM, bone marrow-derived macrophage

CBL, cecal bacterial lysate

CD, Crohn's disease

cDC, conventional dendritic cell

CFU, colony forming unit

CGD, chronic granulomatous disease

CLR, C-type lectin receptor

CNV, conventionalized (murine housing)

DC, dendritic cell

DSS, dextran sulfate sodium

EEA1, early endosome antigen 1

ELISA, enzyme-linked immunosorbent assay

FITC, fluorescein isothiocyanate

Flt3, Fms-related tyrosine kinase 3

Flt3L, Fms-related tyrosine kinase 3 ligand

G-CSF, granulocyte colony-stimulating factor

GF, germ free

GFP, green fluorescent protein

GSK-3β, glycogen synthase kinase 3β

GWAS, genome-wide association studies

HKEC, heat-killed *Escherichia coli*

IBD, inflammatory bowel disease

IDO, indoleamine 2,3-dioxygenase

IEC, intestinal epithelial cell

IEL, intraepithelial lymphocyte

IFN, interferon

Ig, immunoglobulin

IL, interleukin

IRF, interferon regulatory factor

ITAM, immunoreceptor tyrosine-based activation motif

ITIM, immunoreceptor tyrosine-based inhibition motif

LP, lamina propria

LPDC, lamina propria dendritic cell

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MLN, mesenteric lymph node

MOI, multiplicity of infection

mTOR, mammalian target of rapamycin

MyD88, myeloid differentiation primary response gene 88

NFIL3, nuclear factor, interleukin-3 regulated

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NLR, nucleotide-binding oligomerization domain, leucine-rich repeat receptor

NO, nitric oxide

OVA, ovalbumin

p110δ^{KD}, mouse containing a knock-in of the *Pik3cd* locus with an inactivating point mutation in the kinase domain

PAMP, pathogen-associated molecular pattern

pDC, plasmacytoid dendritic cell

PI3K, phosphoinositide 3-kinase

PIP or PI(3)P, phosphoinositide 3-monophosphate

PIP_2, phosphoinositide 4,5-bisphosphate

PIP_3, phosphoinositide 3,4,5-triphosphate

PRR, pattern recognition receptor

PUFA, polyunsaturated fatty acid

RA, retinoic acid

RKO/δ^{KD}, *Rag1*$^{-/-}$ x p110δ^{KD} mouse

RLR, RIG-I-like receptor

ROS, reactive oxygen species

RT-PCR, reverse transcriptase polymerase chain reaction

sBLP, synthetic bacterial lipoprotein

SCFA, short chain fatty acid

SEM, standard error of the mean

SNP, single nucleotide polymorphism

TGF-β, transforming growth factor β

Th, $CD4^+$ T helper cell

TIR, toll-interleukin 1 receptor

TIRAP, TIR-domain-containing adaptor protein

TLR, toll-like receptor

TNBS, 2,4,6-trinitrobenzenesulfonic acid

TRAM, TRIF-related adaptor molecule

Treg, regulatory T cell

TREM-1, triggering receptor expressed on myeloid cells 1

T-RFLP, terminal restriction fragment length polymorphism

TRIF, TIR-domain-containing adapter-inducing interferon-β

TSLP, thymic stromal lymphopoietin

UC, ulcerative colitis

WT, wild type

CHAPTER 1

INTRODUCTION

1.1 Inflammatory Bowel Diseases

The human inflammatory bowel diseases (IBDs), Crohn's disease (CD) and ulcerative colitis (UC), result from an incompletely defined and complex interaction between host immune responses, genetic susceptibility, environmental factors and the enteric luminal contents (Xavier and Podolsky, 2007). In North America, CD prevalence is estimated to be 16.7 to 318.5 per 100,000 people, and UC prevalence is estimated to be 37.5 to 248.6 per 100,000 people (Molodecky et al., 2012). A recent systematic review revealed increases in the worldwide incidence and prevalence of both adult- and pediatric-onset IBDs (Burisch and Munkholm, 2013). Several studies have shown that having CD is associated with an incremental but statistically significant increase in all-cause mortality above the general population (Bewtra et al., 2013). IBDs account for emergency room visits, hospitalizations and surgical interventions that place a significant cost burden on health care (Park and Bass, 2011). Furthermore, IBDs impart significant emotional, psychological, and physiological stress on patients with these chronic diseases (Kemp et al., 2012). Thus, IBDs represent an increasing burden on global and United States health care systems.

The two most common types of IBDs are CD and UC. CD is characterized by chronic, transmural inflammation occurring anywhere along the digestive tract but most commonly affecting the terminal ileum. The disease course tends to be one of relapse and remission, and complications such as stricture and fistula formation often develop. This is in contrast to UC, where ulcerous lesions are typically confined to the superficial layer of the colonic mucosa, extending proximally from the rectum. Histopathologically, UC lesions demonstrate crypt abscesses, goblet cell depletion, and significant infiltration of neutrophils. CD lesions often contain non-caseating granulomas and infiltration of macrophages (Xavier and Podolsky, 2007).

Despite recent advances in identifying IBD susceptibility loci using population-based genome-wide association studies (GWAS), the etiology of IBDs remains elusive. There is a complex interaction of host susceptibility, enteric microbiota, immune system responses and unspecified environmental contributions to IBD pathogenesis. Single nucleotide polymorphisms (SNPs) associated with increased risk of developing IBDs were identified in genes involving microbial sensing (*NOD2*, *IRF5*, *NFKB1*, *RELA*, *REL*, *RIPK2*, *CARD9*, and *PTPN22*) and clearance (*ATG16L1*, *IRGM*, *NCF4*), and integrating antimicrobial adaptive immune responses (*IL23R*, *IL10*, *IL12*, *IL18RAP/IL1R1*, *IFNGR/IFNAR1*, *JAK2*, *STAT3*, and *TYK2*) (Jostins et al., 2012). Additionally, despite great advancements in the use of biologics as therapeutics, hospitalization rates have not decreased (Park and Bass, 2011). Furthermore, biologics are relatively expensive, and patients are not immune from failing this treatment option. Indeed, approximately 10% of patients with IBD lose their response to biologics every year (Gisbert and Panes, 2009).

Therefore, there is a real and pressing need to understand the pathways involved in IBD pathogenesis in order to develop safer and more effective therapies.

1.2 Macrophages and Dendritic Cells in Innate Immunity

1.2.1 Macrophages

Macrophages are a highly heterogeneous population of cells that demonstrate a continuum of activation states. The wide spectrum of macrophage phenotypes is often somewhat oversimplified into two functional groups: "inflammatory" M1 (high IL-12, low IL-10) and "wound healing" M2 (low IL-12, high IL-10) macrophages (Mosser and Edwards, 2008). Additionally, the recently appreciated subset of macrophages that produces high levels of IL-10 is referred to as "regulatory macrophages."

Specific combinations of cytokines within the tissue microenvironment polarize macrophages, and evidence suggests that macrophages maintain considerable plasticity between activation states. M1 macrophages are polarized by IFN-γ produced by natural killer and T helper (Th) 1 cells, TNF-α produced by granulocytes or other antigen presenting cells (APCs) and engagement of pattern recognition receptors (PRRs) by pathogen-associated molecular patterns (PAMPs), which activates suppressor of cytokine signaling 3 (SOCS3) to induce the M1 phenotype (Dale et al., 2008; Mackaness, 1977; Spence et al., 2013). M1 macrophages produce pro-inflammatory cytokines (TNF-α, IL-12, IL-6), and reactive oxygen and nitrogen species (ROS and RNS, respectively). Production of these mediators promotes the differentiation and activation of Th1 and Th17 cells (Bettelli et al., 2007; Edwards et al., 2006; Langrish et al., 2005; Mosser and Edwards, 2008). The Th1 response in turn helps macrophages by enhancing their ability

to clear intracellular pathogens. While M1 macrophages are essential for the eradication of intracellular infections, they also produce pro-inflammatory cytokines implicated in IBD pathogenesis. Furthermore, unregulated M1 macrophage activity can induce tissue damage, predispose the host to developing neoplastic lesions, and induce insulin resistance (Sica and Mantovani, 2012; Swann et al., 2008).

M2 macrophages are polarized by IL-4 produced by granulocytes and Th2 cells in response to tissue injury and activation by some fungi and parasites and initiation of SOCS2 signaling (Mosser and Edwards, 2008; Spence et al., 2013). M2 macrophages produce matrix metalloproteases, growth factors, and demonstrate efficient phagocytosis of debris without producing pro-inflammatory cytokines. Th2 responses are aimed at inducing wound healing and clearing parasites, although the exact mechanisms underlying parasite eradication are unknown. Indeed, the downregulation of microbicidal functions in M2 macrophages can render the host more susceptible to certain infections (Bishop et al., 2008; Harris et al., 2007; Kropf et al., 2005; Muller et al., 2007; Shirey et al., 2008; Tumitan et al., 2007). M2 macrophages are also efficient at recruiting $Foxp3^+$ T regulatory (Treg) cells, which would further downregulate local immune responses (Spence et al., 2013). Furthermore, unregulated M2 macrophage activity can promote the development of fibrotic lesions through elaboration of TGF-β and enhanced allergic responses (Fairweather and Cihakova, 2009; Murray et al., 2011).

Regulatory macrophages are polarized by a wide array of signals, including IgG immune complexes, IL-10, prostaglandins, and apoptotic cells, potentially by activation of the MAPK pathway extracellular signal-regulated kinase (ERK) (Mosser and Edwards, 2008). However, typically an additional signal is necessary to induce regulatory

macrophages, such as engagement of PRRs by PAMPs. Regulatory macrophages differ from M2 macrophages in that they do not produce extracellular matrix components but express high levels of co-stimulatory molecules (CD80, CD86) necessary for the activation of T cells. Like M2 macrophages, regulatory macrophages produce high amounts of the anti-inflammatory cytokine IL-10 and can render the host more susceptible to certain infections (Agrawal and Pulendran, 2004; Baetselier et al., 2001; Benoit et al., 2008; Kim et al., 2008; Mahalingam and Lidbury, 2002; Miles et al., 2005; Ruas et al., 2009). Furthermore, unregulated regulatory macrophage activity may also play a role in the induction of neoplastic lesions (Biswas et al., 2006; Lin et al., 2006).

1.2.2 Dendritic Cells

Broadly speaking, dendritic cells (DCs) are professional antigen presenting cells (APCs) with the ability to initiate adaptive immune responses against potential pathogens. Like macrophages, DCs comprise a heterogeneous population of cells with functional diversity. DCs originate from blood monocytes or a common DC progenitor (CDP) in the bone marrow at steady state. DCs repopulating tissues from monocyte precursors rely on granulocyte-macrophage colony stimulating factor (GM-CSF) for local proliferation (Rutella et al., 2004). Conventional DCs (cDCs) arising from the CDP express high levels of CD11c, varying levels of CD8α and CD11b, and reside in secondary lymphoid tissues. Plasmacytoid DCs (pDCs) also originate from the CDP and are specialized in the production of type I interferons. In addition to functional subsets of DCs, the maturation state of DCs has important implications in immunity. Mature DCs that have previously encountered microbial products and inflammatory stimuli are highly

specialized for antigen presentation. Thus, mature DCs express high levels of co-stimulatory molecules and tend to reside in secondary lymphoid organs where they are ideally positioned to prime antigen-specific T cells (Rescigno and Di Sabatino, 2009). On the other hand, immature DCs demonstrate low surface expression of co-stimulatory molecules and constitutively migrate in low numbers to lymph nodes, perhaps to maintain tolerizing signals there (Huang et al., 2000; Rescigno and Di Sabatino, 2009).

1.3 Recognition of Pathogen-associated Molecular Patterns

Macrophages and DCs sense conserved molecular patterns, or PAMPs, on microbes via germ-line encoded PRRs (Akira et al., 2001; Janeway and Medzhitov, 2002). PRRs are divided into four families based on shared functional domains: toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR) receptors (NLRs), C-type lectin receptors (CLRs), and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) (Kawai and Akira, 2011). Signaling downstream of each family of PRRs culminates in activation of central immune response pathways: nuclear factor kappa-light-chain-enhancer of B cells (NF-κB), the mitogen-activated protein kinases (MAPKs), and interferon regulatory factors (IRFs) (Akira et al., 2006). Upon engagement of PRRs, immune and non-immune cells produce inflammatory cytokines, type I interferons, chemokines, and antimicrobial peptides. As a result, neutrophils are recruited and macrophages are activated, leading to the direct killing and clearance of microbes. Additionally, these inflammatory products induce the maturation of DCs, promoting the induction of adaptive immune responses. A carefully orchestrated process, microbial sensing and subsequent immune responses are highly regulated.

Dysregulation of these pathways can lead to both enhanced susceptibility to infections and development of chronic inflammatory diseases (Kawai and Akira, 2011).

1.3.1 Toll-like Receptors (TLRs)

The best-characterized PRRs are the TLRs (Kawai and Akira, 2010). TLRs are transmembrane proteins with an extracellular LRR domain and an intracellular Toll-interleukin 1 receptor (TIR) domain. Upon binding its ligand to the LRR domain, homo- and heterodimerized TLRs recruit TIR domain-containing adaptor proteins. TLRs signal through one or both of two adapters: myeloid differentiation primary response gene 88 (MyD88) and/or TIR-domain-containing adapter-inducing interferon-β (TRIF) (Akira et al., 2006). TLR3 signaling is TRIF-dependent, whereas TLR4 can signal through both the MyD88- and TRIF-dependent pathways, and the remaining TLRs are MyD88-dependent (Akira et al., 2006). MyD88-dependent signaling activates transforming growth factor-β-activated kinase 1 (TAK1), a kinase of inhibitor of NF-κB (IκB) kinase (IKK). IKK phosphorylates IκB, leading to its degradation and subsequently releasing the transcription factor NF-κB from sequestration in the cytoplasm (Bhoj and Chen, 2009). NF-κB then translocates to the nucleus and initiates transcription of a large number of inflammatory response genes. TAK1 also activates MAPKs, leading to the activation of other inflammatory transcription factors, including activator protein 1 (AP-1) (Yamamoto et al., 2006). The TRIF-dependent pathway initiates the production of type I interferons via the activation of IRF3 by TANK-binding kinase 1 (TBK1) (Kawai and Akira, 2008).

1.3.2 Nucleotide-binding Oligomerization Domain (NOD), Leucine-rich Repeat (LRR) Receptors (NLRs)

NLRs are cytosolic PRRs that can respond to a diverse array of stimuli, from bacteria to viruses and particulates involved in pathogenic states (e.g., monosodium urate crystals in gout and asbestos in mesothelioma and pneumoconiosis) (Kersse et al., 2011). NLRs contain a NACHT (present in NAIP, CIITA, HET-E, and TP-1) oligomerization domain, an LRR domain (except NLRP10 and NAIP), an N-terminal effector domain (except NLRX1), and an N-terminal homotypic protein-protein binding domain. Based on their N-terminal effector domain, the NLRs are divided into four subfamilies: NLRA, NRLB, NLRC, and NLRP. The sole member of NLRA is CIITA, which contains an acidic transactivation domain. NRLB proteins contain a baculovirus inhibitor repeat domain, NRLCs contain a caspase recruitment domain (CARD), and NRLPs contain a pyrin domain. Activation of NLRs can lead to several consequences, from inflammasome assembly and pro-IL-1β and pro-IL-18 processing (Bergsbaken et al., 2009), to caspase-1-dependent pyroptotic cell death (Schroder and Tschopp, 2010), and activation of NF-κB and MAPKs (Ting et al., 2010). Because the array of ligands that activate NLRs is so diverse, a controversial theory is that NLRs actually respond to a more universal physiologic change within the cell, such as a decrease in intracellular potassium concentration or an increase in extracellular calcium (Rajamaki et al., 2013; Rossol et al., 2012). Nevertheless, NLRs are important for the clearance of certain intracellular pathogens, maintenance of intestinal epithelial cell (IEC) homeostasis (Marques and Boneca, 2011), and promotion of local and systemic immune responses to perceived dangers (Kersse et al., 2011).

1.3.3 C-type Lectin Receptors (CLRs)

CLRs are indispensable for immunity against fungal pathogens (Hardison and Brown, 2012). Activation of these transmembrane proteins containing a C-type lectin domain induces binding of the pathogen and phagocytosis, activation of antifungal programs, and production of cytokines and chemokines important in antifungal immunity. Dectin-1, Dectin-2, and Mincle signal through Syk to activate NF-κB, MAPKs, and nuclear factor of activated T cells (NFAT), inducing ROS generation and the production of cytokines and chemokines (Goodridge et al., 2007; Gross et al., 2006; LeibundGut-Landmann et al., 2007; Strasser et al., 2012). In contrast, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) signals through Raf-1 to modulate NF-κB activity (Gringhuis et al., 2007). CLRs demonstrate extensive crosstalk with other PRRs, enhancing antifungal defenses. CLRs are especially important at mucosal surfaces where they induce Th17 responses (Iliev et al., 2012; Vautier et al., 2010).

1.3.4 Retinoic Acid-inducible Gene-1 (RIG-I)-like Receptors (RLRs)

Finally, RLRs are cytoplasmic RNA helicases that recognize viral RNA and are expressed in many cell types in addition to immune cells (Loo and Gale, 2011). RLRs contain N-terminal tandem CARDs, a DExD/H box RNA helicase domain, and a C-terminal repressor domain (Saito et al., 2007; Yoneyama et al., 2005; Yoneyama et al., 2004). Without viral RNA present, the C-terminal repressor domain interacts with the CARDs to block the central RNA helicase domain. Binding of viral RNA, especially RNA with 5’ triphosphorylated ends, to the repressor domain frees the CARDs to

multimerize and interact with the adaptor protein interferon-β promoter stimulator 1 (IPS-1) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). Recruitment of other proteins into the IPS-1 signalosome ultimately leads to type I interferon production through activation of IRF3, IRF7, and NF-κB (Paz et al., 2006). Like CLRs, RLRs demonstrate extensive crosstalk with other PRR signaling pathways.

1.3.5 Functional Integration of Pathogen Recognition Receptor Signaling

PRR recognition of its cognate PAMPs culminates in the initiation of pathogen-specific programs designed to eradicate the prevailing insult. But how does the host recognize an intact microbe and decide which program to initiate? In reality, one microbe has many different PAMPs, and many PRRs may recognize one PAMP. Additionally, different cell types express unique sets of PRRs, and each PRR may play fundamentally different roles in the temporally distinct phases of an infection (i.e., initial infection versus memory response). The complex crosstalk between PRR families also confers specificity to and regulates each immune response. Thus, the assembly of a successful immune response to microbes depends on the context of the infection, the cell types responding to it, and the array of PRRs that are engaged during the infection (Kawai and Akira, 2011). Furthermore, the local microenvironment provides contextual cues to immune cells via cytokines and growth factors produced by host cells and metabolic products from microbes (Danese, 2008). It is likely through this complex context of recognition that innate immune cells distinguish between commensal and pathogenic microbes and initiate an appropriate response program. However the precise mechanism of discernment of helpful from harmful microbes and regulation of subsequent immune

responses and how this relates to intestinal homeostasis and IBDs remains incompletely understood.

1.4 Macrophage Intracellular Bactericidal Functions

A central function of macrophages, literally translated as "big eater", is to engulf and eradicate pathogens and other debris/stimuli. These functions are critical to maintaining intestinal homeostasis, as demonstrated by IBD-associated SNPs identified in genes regulating bactericidal activity (*ATG16L1*, *IRGM*, *NCF4*) (Jostins et al., 2012). Macrophages can kill or limit the replication of microorganisms through many possible mechanisms. Macrophages can limit the availability of essential nutrients, as well as produce antimicrobial peptides, lysosomal enzymes, and ROS/RNS (Ismail et al., 2002). Oxygen-dependent metabolites are perhaps the most efficient anti-microbial effectors produced by macrophages. NADPH oxidases and associated accessory proteins are therefore essential components of cellular responses to microbial invasion. Hemoprotein complexes of the NADPH phagocyte membrane-bound gp91phox and p22phox subunits, along with the cytosolic p40phox, p47phox, p57phox and Rac proteins, can consume molecular oxygen to produce ROS/RNS (Rada et al., 2008; Robinson, 2008). The cytosolic components stabilize and activate phagocyte NADPH oxidase (Nox2). Compartmentalization of membrane-bound and cytosolic components of Nox2 ensures that the production of cytotoxic oxygen radicals is prevented in resting cells, thus avoiding "collateral damage".

Another important bactericidal pathway in macrophages following the phagocytosis of a microbe is phagosome maturation to the actively bactericidal vesicle,

the phagolysosome. The nascent phagosome goes through three stages of maturation: early, late, and lysosome-interacting (Fairn and Grinstein, 2012). Rab-family GTPases mediate the maturation process. Early phagosomes are marked by Rab5 decoration, which allows fusion with early endosomes via interactions with endosomal early antigen 1 (EEA1) (Christoforidis et al., 1999; Kinchen et al., 2008). Recruitment of maturation factors such as EEA1 is mediated by the generation of membrane-bound phosphoinositide 3-phosphate (PIP) molecules by the Class III phosphoinositide 3-kinase (PI3K) vacuolar protein sorting 34 (Vps34) (Kinchen et al., 2008; Scott et al., 2002). GTPase activity on late phagosomes transitions from Rab5 to Rab7, which mediates intracellular trafficking and fusion with lysosomes (Harrison et al., 2003; Johansson et al., 2007). The resulting phagolysosome drives a critical drop in intravesicular pH by pumping H^+ into the lumen via V-ATPase (Flannagan et al., 2009). The low pH of phagolysosomes directly impairs microbe function, activates host hydrolytic enzymes and assists in the generation of superoxide by NADPH oxidase. Furthermore, the H^+ gradient is used to pump essential microbial nutrients out of the phagolysosome.

Another indirect pathway that macrophages use to eradicate intracellular bacteria is autophagy. Autophagy is a normal cellular process use for recycling intracellular organelles and is typically activated during cell stress (Levine and Deretic, 2007). However, the autophagy machinery can be hijacked to eliminate phagocytosed microbes (Andrade et al., 2006; Birmingham et al., 2006; Checroun et al., 2006; Gutierrez et al., 2004; Ling et al., 2006; Nakagawa et al., 2004; Ogawa et al., 2005; Py et al., 2007; Rich et al., 2003; Singh et al., 2006). Autophagy is initiated by the formation of a phagophore membrane that envelopes damaged organelles or microbes into a double-membranous

vesicle termed the “autophagosome” (Levine and Deretic, 2007). The autophagosome then fuses with lysosomes to form autolysosomes where degradation of intravesicular contents occurs. Indeed, autophagy is central to intestinal homeostasis, as several IBD-associated SNPs in genes involved in autophagy have been identified (Jostins et al., 2012).

1.5 Lamina Propria Mononuclear Cells in the Healthy Gastrointestinal Tract

1.5.1 Lamina Propria Mononuclear Cells

The gut-associated lymphoid tissue (GALT) represents the largest aggregate of lymphoid tissue in the body. GALT includes various organized collections of immune cells within the gastrointestinal tract, such as Peyer’s patches in the small intestine and cryptopatches in the large intestine; and the diffuse arrangement of intestinal mononuclear cells within the lamina propria (LP). The close proximity of lamina propria mononuclear cells (LPMCs) to the enteric luminal compartment, separated by an epithelial cell monolayer, is important for several reasons: LPMCs (1) sample luminal antigens that gain access to the LP under normal physiologic conditions to maintain local and systemic tolerance, and (2) efficiently clear microbes and stimuli that cross the IEC barrier. Resident LP macrophages demonstrate distinct attributes from peripheral monocyte populations. While LP macrophages maintain robust microbicidal effector functions, they do not produce inflammatory mediators upon encountering microbial stimuli (Mowat and Bain, 2011). Additionally, LP macrophages promote the transition from protective inflammatory responses to resolving anti-inflammatory responses upon

encountering a danger signal. Thus, LPMCs are integral to directing appropriate immune responses and maintaining intestinal homeostasis in the gut.

There remains active debate about the classification and ontogeny of LP macrophages and LP dendritic cells (LPDCs). The surface integrins CD11b and CD11c are routinely used to distinguish between macrophages and DCs in peripheral lymphoid tissues ($CD11b^{+}CD11c^{-}$ and $CD11b^{+/-}CD11c^{high}$ are characterized as macrophages and DCs, respectively). However, the distinction between LP macrophages and LPDCs is less clear, as LP macrophages express both CD11b and CD11c (Mowat and Bain, 2011). It has been proposed that differential expression of CX_3CR1 (the receptor for the chemokine fractalkine, CX_3CL1) and CD103 ($\alpha_E\beta_7$ integrin) reliably distinguish between LP macrophages and LPDCs (Mowat and Bain, 2011; Schulz et al., 2009). $CD103^{-}$ CX_3CR1^{hi} LP macrophages express the classical macrophage marker F4/80, demonstrate ultrastructural characteristics of macrophages, and under physiologic conditions do not traffic to draining mesenteric lymph nodes (MLNs) where priming of adaptive immune responses is initiated. However, there is evidence that CX_3CR1^{hi} LP macrophages do travel to MLNs during enteric microbial dysbiosis (Diehl et al., 2013). Conversely, $CD103^{+}CX_3CR1^{lo}$ LPDCs are $F4/80^{-}$ and perform functions typically associated with DCs, including constitutive trafficking to MLNs, antigen presentation to T cells, and inducing gut homing receptors on T cells. Both LP macrophages and LPDCs express high levels of MHC class II, demonstrating their ability to interact with and shape adaptive immune responses. While controversy remains over the exact nature and origin of these LP subsets, for our purposes, we will classify LP macrophages as $CD103^{-}CX_3CR1^{hi}$ and LPDCs as $CD103^{+}CX_3CR1^{lo}$ cells.

1.5.2 Lamina Propria Macrophages

LP macrophages are unique tissue resident macrophages characterized by the inability to produce inflammatory cytokines in response to microbial stimuli. However, these cells maintain robust phagocytic and microbicidal effector capabilities. The tolerant phenotype of LP macrophages is likely conditioned by locally produced IL-10 and TGF-β (Denning et al., 2007; Smythies et al., 2010). However, the ontogeny of these cells is unknown. LP macrophage maintenance may depend on local proliferation rather than repopulation from migrating blood monocytes, but this is experimentally difficult to determine due to the extremely low turnover rate of these cells. Additionally, the context during which blood monocytes are recruited to the intestines may determine the final phenotype of the LP macrophages. During non-inflammatory homeostatic conditions, $Ly6C^{hi}$ monocytes almost exclusively repopulate the LP with $CD11c^{+}$ ($F4/80^{hi}CX_3CR1^{hi}CD11b^{+}CD103^{-}$) LP macrophages (Rivollier et al., 2012). In contrast, under inflammatory conditions, $Ly6C^{hi}$ monocytes recruited to the LP differentiate into $CD103^{+}CX_3CR1^{int}CD11b^{+}$ DCs that produce high levels of the inflammatory cytokines IL-12, IL23, iNOS, and TNF-α (Rivollier et al., 2012).

CX_3CR1^{hi} LP macrophages extend dendrites between IECs to sample luminal antigens and promote local tolerance through constitutive production of the anti-inflammatory cytokine IL-10 (Hadis et al., 2011), the absence of an inflammatory response to activating stimuli, very low expression of co-stimulatory molecules CD80, CD86 and of the macrophage activating receptor CD40 (Smythies et al., 2010). Although these cells that sample the luminal environment were originally defined as DCs (Niess et

al., 2005), recent work supports that they may represent a macrophage population (Medina-Contreras et al., 2011). IL-10 produced by LP macrophages promotes the persistence of Foxp3 expression in Treg cells in the intestine (Murai et al., 2009). Additionally, CX_3CR1^{hi} LP macrophages participate in the induction of systemic oral tolerance (Hadis et al., 2011). It has been suggested that CX_3CR1^{hi} LP macrophages sample luminal antigens and deliver them to $CD103^+$ LPDCs, which are then able to traffic to MLN to prime adaptive immune responses (Ruane and Lavelle, 2011). However, there is recent compelling evidence that CX_3CR1^{hi} LP macrophages traffic to MLNs in a CCR7-dependent manner during dysbiosis of the enteric microbiota (Diehl et al., 2013).

Unique intracellular signaling pathways contribute to the inflammation anergic characteristic of LP macrophages; however, it remains unclear exactly what makes LP macrophages distinct from circulating monocytes and other tissue resident macrophages. Additionally, inflammation anergic LP macrophages are distinct from the more widely studied endotoxin-resistant macrophages. For one, LP macrophages do express PRRs, contrary to conventional thought. Recent studies suggest that the enteric microbiota are not necessary to program LP macrophages to express high amounts of the anti-inflammatory cytokines IL-10 and TGF-β (Kobayashi et al., 2012) (Maheshwari et al., 2011). One enticing candidate for inducing LP macrophage non-responsiveness to PAMPs is IL-10. Importantly, IL-10-deficient mice (Kuhn et al., 1993) and mice with myeloid-specific ablation of the IL-10 signaling molecule STAT3 (Takeda et al., 1999) develop spontaneous colitis reminiscent of human IBDs. Additionally, blocking IL-10 restores PAMP responsiveness in LP macrophages. Our lab described a mechanism for

IL-10-mediated suppression of IL-12p40 via altering histone acetylation and RNA polymerase II accessibility to the *Il12b* promoter (Kobayashi et al., 2012), suggesting that IL-10 directly inhibits the production of pro-inflammatory cytokines in response to PAMP stimulation. IL-10 additionally exerts its anti-inflammatory effects on the innate immune system by regulating transcriptional elongation (Smallie et al., 2010), microRNA induction (McCoy et al., 2010), mRNA stability (Schaljo et al., 2009), and transcriptional repressors and co-repressors (El Kasmi et al., 2007).

Additionally, the PI3K pathway negatively regulates signaling through TLRs in macrophages (Fukao and Koyasu, 2003). In particular, the catalytic subunit of PI3K, p110δ, is enriched in leukocytes and regulates IL-12p40 production in LP macrophages in response to microbial stimulation. PI3K p110δ is indispensable for intestinal homeostasis as mice harboring an inactivating point mutation in p110δ (p110δ kinase-dead, or $p110\delta^{KD}$ mice) develop spontaneous colonic inflammation (Uno et al., 2010). LP macrophages from $p110\delta^{KD}$ mice produce significantly more IL-12p40 and less IL-10 upon stimulation with heat-killed *Escherichia coli* (Uno et al., 2010). Thus, a loss in the critical negative regulation of TLR signaling results in the disruption of intestinal homeostasis.

1.5.3 Lamina Propria Dendritic Cells

LPDCs also comprise a heterogeneous group of cells in the intestines. Only recently has it also been appreciated that LPDCs play an active and direct role in maintaining peripheral tolerance to self and intestinal luminal antigens. Like LP macrophages, LPDCs represent a spectrum of functionally distinct phenotypes. $CD8\alpha^{+}$

pDCs in the LP are capable of inducing Treg cells and supporting their function (Bilsborough et al., 2003). While the majority of LPDCs are $CD11b^{+}CD8\alpha^{-}$, $CD11b^{-}CD8\alpha^{+}$ and $CD11b^{-}CD8\alpha^{-}$ subsets are also present. These DCs weakly stimulate antigen-specific T cell proliferation and constitutively express IL-10 and type I interferons (Chirdo et al., 2005). Furthermore, LPDCs are divided into $CD103^{+}$ and $CD103^{-}$ (E-cadherin receptor) populations, each demonstrating distinct functions. $CD103^{+}$ LPDCs are able to induce Foxp3-expressing Treg cells (Coombes et al., 2007; Mucida et al., 2007; Sun et al., 2007), whereas $CD103^{-}$ LPDCs are efficient at inducing Th17 cells when stimulated with flagellin or microbial ATP (Atarashi et al., 2008; Kinnebrew et al., 2012; Uematsu et al., 2008). While the Th17 response is important for antimicrobial immunity, dysregulation of Th17 lymphocytes and cytokines is implicated in a number of autoimmune disorders (Bettelli et al., 2007).

$CD103^{+}$ LPDCs represent a population of tolerizing innate immune cells that express the enzyme retinaldehyde dehydrogenase (RALDH), which produces retinoic acid (RA) from retinaldehyde, and the important regulatory cytokine TGF-β. Both $CD103^{+}$ LPDC-produced RA and TGF-β are necessary for the induction of Treg cells in the intestine (Coombes et al., 2007; Mucida et al., 2007; Sun et al., 2007). Additionally, $CD103^{+}$ LPDCs produce indoleamine 2,3-dioxygenase (IDO), which participates in the induction of Treg cells and suppression of Th cell proliferation (Matteoli et al., 2010).

The induction of CD103 expression in LPDCs is dependent on the vitamin A metabolite retinoic acid (RA) and the local production of factors from IECs and stromal cells. IECs induce CD103 expression in LPDCs in an RA-, TGF-β-, and contact-dependent manner (Iliev et al., 2009). In addition to TGF-β, stromal cells in the LP

constitutively produce prostaglandin E2, which inhibits the production of pro-inflammatory cytokines in DCs (Newberry et al., 2001). Importantly, thymic stromal lymphopoietin (TSLP) produced by IECs conditions LPDCs to induce Th2 cell differentiation (Iliev et al., 2009), although its necessity in inducing and maintaining Treg cells is controversial. Nonetheless, TSLP produced by IECs confers a homeostatic phenotype on LPDCs to protect mice from colitis (Iliev et al., 2009; Liu et al., 2007; Rimoldi et al., 2005; Taylor et al., 2009). $CD103^+$ LPDC differentiation is dependent on Notch2 signaling, as *Notch2*$^{-/-}$ mice demonstrate a selective loss of $CD11b^+CD103^+$ LPDCs (Lewis et al., 2011). Furthermore, the preferential expansion of $CD103^+$ LPDCs depends on the DC differentiating molecule Fms-related tyrosine kinase-3 ligand (Flt3L) (Collins et al., 2012). The function of $CD103^+$ LPDCs depends on several factors. Dietary vitamin A induces RALDH expression in $CD103^+$ LPDCs (Molenaar et al., 2011) and is necessary for these cells to imprint T cells with gut-homing receptors (Jaensson-Gyllenback et al., 2011; Wang et al., 2011b).

Aside from inducing Th17 differentiation, $CD103^-$ LPDCs are involved in the induction of immunoglobulin A (IgA) class switching of B cells, both in the Peyer's patches and intestinal LP. IgA is abundantly produced in the intestine and prevents bacterial overgrowth and adhesion to IECs in the intestinal lumen (Mantis et al., 2011). In the isolated lymphoid follicles of the LP, $CD70^+$ LPDCs expressing TLR5 and any of various ATP receptors induce IgA class switching in RA-dependent and T cell-independent manners (Uematsu et al., 2008). LPDCs that produce iNOS and TNF-α also support IgA class switching (Tezuka et al., 2007). Cytokines produced by IECs, stromal cells and LPDCs, including B cell activating factor (BAFF), a proliferation-inducing

ligand (APRIL), IL-4, TGF-β, and IL-10, support the induction, maintenance, and expansion of IgA^+ plasma cells (Bemark et al., 2012).

LPDCs have a higher turnover rate than LP macrophages due to frequent trafficking to MLN to present antigen to naïve T cells (Rescigno and Di Sabatino, 2009). Evidence suggests that $CD103^+CD11b^-$ LPDCs are replenished by DC-committed precursors (pre-cDC) in a Flt3L-dependent manner (Liu et al., 2009b), whereas $CD103^-$ $CD11b^+$ LPDCs are derived from circulating $Ly6C^{hi}$ monocytes in a GM-CSF-dependent manner (Varol et al., 2009). Additionally, the preferential expansion of regulatory $CD103^+$ LPDCs is also Flt3L-dependent (Collins et al., 2012). The conditions under which precursors are recruited to and the existing microenvironment of the LP likely determine the final phenotype of LPDCs. For instance, under steady-state conditions $F4/80^{lo}CD103^+CD11c^+$ LPDCs are repopulated from circulating $Ly6C^{hi}$ monocytes (Rivollier et al., 2012). Mice with experimental colitis and reconstituted with $Ly6C^{hi}$ monocytes demonstrated intestinal accumulation of inflammatory $CD103^-$ $CX3CR1^{int}CD11b^+$ LPDCs and exacerbated colitis (Rivollier et al., 2012; Varol et al., 2009).

1.6 Lamina Propria Mononuclear Cells in IBD

1.6.1 Murine Experimental IBD

There are a number of phenotypic and functional alterations described in LP macrophages and LPDCs during IBDs. Recent research highlights a central role for macrophages and DCs in the pathogenesis of colitis, as numerous IBD susceptibility SNPs affecting innate immune cell functions have been identified (Cho and Brant, 2011;

Jostins et al., 2012). Additionally, the selective depletion of macrophage and DC subsets in mouse models of colitis has been particularly informative about the protective and pathogenic roles innate immune cells play during discrete stages of disease pathogenesis. Lymphocyte deficient mice (severe combined immunodeficiency, SCID) develop colitis upon treatment with the intestinal irritant dextran sodium sulfate (DSS), suggesting that macrophages and DCs are pathogenic in this model in the absence of mature lymphocytes (Dieleman et al., 1994). Depletion of phagocytes in *Il10*$^{-/-}$ mice (Watanabe et al., 2003), and blocking myeloid cell recruitment in both 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced (Palmen et al., 1995) and T cell adoptive transfer (Kanai et al., 2006) colitis ameliorate disease, as does selective depletion of LPDCs during DSS colitis (Abe et al., 2007; Berndt et al., 2007). Contrary to these findings, depletion of LP macrophages and LPDCs *prior* to the induction of DSS colitis results in exacerbated disease (Qualls et al., 2006; Qualls et al., 2009). Furthermore, different subsets of macrophages and DCs have distinct effects on the severity of colitis in animal models. M2 polarized macrophages protect mice from DSS colitis, whereas M1 polarized macrophages contribute to disease pathogenesis (Arranz et al., 2012; Hunter et al., 2010; Weisser et al., 2011). Selective expansion of CD103^{+} LPDCs by Flt3L protects TNFΔARE mice from ileitis (Collins et al., 2012), but E-cadherin-expressing DCs increase colonic pathology in DSS colitis (Siddiqui et al., 2010). Thus, the protective/pathogenic role of distinct macrophage and DC populations in the LP remains an active area of investigation.

In general, there are three ways in which defects in innate immune cell functions can initiate IBD development: (1) by responding inappropriately to normally benign stimuli such as commensal microbes, (2) by inefficiently clearing microbes, leading to

chronic immune stimulation, and (3) by failing to switch from an appropriate pro-inflammatory response to an inflammation-resolving anti-inflammatory response. Here we will discuss each of these defects and how each leads to chronic inflammation and IBDs.

The enteric microbiota is essential for the development of colonic inflammation in most murine models of colitis (Guarner, 2008; Sartor, 2008). Perturbations in the negative regulation of innate immune responses to stimuli enhance susceptibility to colitis development. The well-characterized *Il10*$^{-/-}$ murine model of spontaneously developing colitis demonstrates the necessity of the potent anti-inflammatory cytokine IL-10 in the maintenance of intestinal homeostasis (Kuhn et al., 1993). Indeed, LP macrophages derived from germ free (GF) *Il10*$^{-/-}$ mice produce increased IL-12p40 compared to GF WT LP macrophages at baseline, suggesting that IL-10 is the critical driver of the LP macrophage phenotype (Kobayashi et al., 2012). Furthermore, IL-10 produced by CD11b^{+} LP macrophages is necessary for the maintenance of Foxp3 expression in Treg cells and protection from colitis (Murai et al., 2009). The important IL-10- and microbiota-inducible nuclear transcription factor, interleukin-3 regulated (NFIL3) negatively regulates IL-12p40 production in LP macrophages and has been recently implicated in intestinal homeostasis (Kobayashi et al., 2011). Thus, studying the regulation of IL-10 production and its downstream signaling effects is crucial to understanding intestinal homeostasis.

IL-10-independent regulation of innate immune responses also contributes to intestinal homeostasis. One negative regulator of intestinal macrophage activation is paired immunoglobulin-like receptor B (PIR-B). PIR-B is expressed on colonic LP

macrophages, B cells, and neutrophils and contains several immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that activate intracellular phosphatases, negatively regulating TLR signaling (Munitz et al., 2010). PIR-B is highly upregulated on LP macrophages following DSS administration in mice. Furthermore, PIR-B-deficient (*Pirb*$^{-/-}$) macrophages produce significantly more TNF-α and IL-6 in response to *Escherichia coli*, and WT mice reconstituted with *Pirb*$^{-/-}$ macrophages demonstrate increased susceptibility to DSS colitis. PIR-B expression is also important in human intestinal biology, as LP mononuclear cells from both healthy controls and patients with UC express immunoglobulin-like transcript-2/leukocyte Ig-like receptor-1 (ILT-2/LIR-1), a human homologue of PIR-B. Our lab recently described spontaneous colitis development in mice harboring a kinase-dead PI3K catalytic subunit p110δ (p110δ^{KD}), a potent negative regulator of TLR responses in macrophages (Uno et al., 2010). CD11b^{+} LPMCs from p110δ^{KD} mice produced increased pro-inflammatory cytokines (IL-12p40, IL-23) and decreased anti-inflammatory IL-10 in response to enteric microbes compared to CD11b^{+} LPMCs from WT mice. Conversely, triggering receptor expressed on myeloid cells-1 (TREM-1) *amplifies* TLR-induced inflammatory responses in macrophages, and blocking its activity attenuates murine colitis (Bouchon et al., 2000; Schenk et al., 2005). Indeed, resident LP macrophages do not express TREM-1 but abundant TREM-1-expressing LP macrophages can be found in patients with IBDs (Schenk et al., 2007; Smith et al., 2001). Thus, unrestrained pro-inflammatory responses of LP macrophages and LPDCs participate in the induction of chronic inflammation by continued recruitment of inflammatory cells, inducing altered barrier function of the IEC layer, and promoting pathogenic adaptive immune responses.

The enteric microbiota interacts with host immune cells to induce protective anti-inflammatory responses and maintain intestinal homeostasis. Dysregulation of these protective pathways, either by enteric microbial dysbiosis or intrinsic defects in macrophage and DC responses to stimuli, may underlie IBD pathogenesis. Short chain fatty acids (SCFAs) are anti-inflammatory metabolites produced by specific phyla of enteric bacteria (Bacteroidetes, Firmicutes) (Cavaglieri et al., 2003). When DSS colitis is induced in immune cell-specific *Gpr43*$^{-/-}$ mice (a host receptor for SCFAs), colonic inflammation is exacerbated, pointing to the beneficial anti-inflammatory effect of SCFAs in the colon (Maslowski et al., 2009). Interestingly, bacteria also actively suppress intestinal inflammatory responses, although a bacterium can exploit this to promote its pathogenicity. *Citrobacter rodentium* and *Helicobacter pylori* express bacterial proteins with domains similar to host ITIMs (Yan et al., 2012). ITIMs negatively regulate immunoreceptor signaling pathways in immune cells, and bacterial ITIM-like-containing proteins dampen immune responses in murine colons. On the other hand, analysis of the enteric microbiota of patients with IBD demonstrates decreased biodiversity, decreased proportions of Firmicutes, and increased Gammaproteobacteria (Sokol and Seksik, 2010). While it is unknown whether enteric dysbiosis in IBD patients contributes to or is a consequence of colonic inflammation, researchers demonstrate reproducible increases in bacteria with unique abilities to adhere and invade mucosal cells in patients with IBD (i.e., adherent-invasive *E. coli*) (Boudeau et al., 1999), as well as decreases in bacteria capable of producing protective SCFAs (Morgan et al., 2012). Furthermore, it was recently shown that *E. coli* is especially adept at using nitrates as electron acceptors, supporting its selective growth during intestinal inflammation, when

nitrates are produced in abundance (Winter et al., 2013). This suggests that the interplay between host and bacteria actively shapes intestinal homeostasis and participates in IBD pathogenesis.

Both macrophages and DCs actively promote the transition from inflammation to the return to homeostasis after immune system activation, and non-resolving inflammation is associated with many chronic diseases, including IBDs (Nathan and Ding, 2010). A study found that the pro-resolution mediator prostaglandin D_2 was upregulated only in UC patients who had achieved long-term remission, suggesting that intact pro-resolution pathways are necessary to halt damaging intestinal inflammation (Vong et al., 2010). Additionally, a SNP associated with low expression of the immune cell ectonucleotidase CD39, which generates the pro-resolving mediator adenosine, is associated with CD (Friedman et al., 2009). Immune cells are major contributors of extracellular adenosine at inflammatory sites. Adenosine interacts with its receptor A_{2B} on macrophages and DCs to inhibit pro-inflammatory cytokine production, expression of co-stimulatory molecules, and induction of T cell proliferation while increasing IL-10 production (Hasko et al., 2009).

Other pro-resolving soluble mediators with diverse effects on macrophages and DCs are resolvins, lipoxins, protectins, and maresins (Uddin and Levy, 2011). These mediators are derived from polyunsaturated fatty acids (PUFAs), and both CD and UC patients have demonstrated deficiencies in these resolving mediators (Kuroki et al., 1997; Weylandt et al., 2007). Interestingly, there was found to be a very low incidence of IBDs among a population in Northwest Greenland that consumes high amounts of PUFAs, suggesting that dietary precursors of pro-resolving factors help to prevent chronic

gastrointestinal inflammation (Kromann and Green, 1980). PUFA-derived mediators enhance the capacity of macrophages and DCs to promote the resolution of inflammation by inducing efficient phagocytosis of apoptotic granulocytes and debris, preventing further recruitment of neutrophils, inducing anergy or deletion of effector T cells, and promoting repair of local damage (Uddin and Levy, 2011). Treatment with resolvin E1 ameliorates pathology in two experimental murine models of colitis, illustrating the powerful effects of PUFA-derived mediators on resolving inflammation (Arita et al., 2005; Ishida et al., 2010).

Macrophages and DCs additionally respond to resolving mediators by switching to unique "resolution phase" phenotypes. DCs generated in the presence of resolvin E1 demonstrate decreased expression of co-stimulation molecules, TNF-α, and IL-12, while inducing antigen-specific $CD4^+$ T cell apoptosis via IDO production and activation (Vassiliou et al., 2008). A defining distinction of resolution phase DCs from tolerogenic DCs is the continued expression of CCR5, which enhances chemotaxis toward inflammatory sites, without upregulation of CCR7, which induces chemotaxis to lymph nodes, on resolution phase DCs (Vassiliou et al., 2008). Similarly, resolution phase macrophages demonstrate a distinct phenotype from both M1 and M2 macrophages. Like M2 macrophages, resolution phase macrophages express high levels of molecules associated with the recognition and clearance of apoptotic cells, TGF-β, IL-10, and arginase 1 (Bystrom et al., 2008; Stables et al., 2011). However, resolution phase macrophages also possess features of M1 macrophages, such as expression of iNOS, COX2, and CCR5 (Bystrom et al., 2008; Stables et al., 2011). It is likely that local factors condition both macrophages and DCs to switch phenotypes and promote the resolution of

inflammation, and that generation of these local factors or innate immune cell responses to these factors are defective in IBDs.

1.6.2 Human IBDs

In human IBDs, inflammatory lesions demonstrate an increase in accumulation of macrophages that display enhanced expression of co-stimulatory molecules (CD80, CD86) and macrophage activating receptors (CD40) (Rugtveit et al., 1997), TLRs (Hausmann et al., 2002), TREM-1 (Schenk et al., 2005), and CD14 (Kamada et al., 2008; Smith et al., 2001). Likewise, there are higher frequencies of LPDCs positive for markers of mature DCs (CD83, S-100, CD40) (Baumgart et al., 2009; Hart et al., 2005; te Velde et al., 2003; Verstege et al., 2008) and for PRRs (CD209, TLR2/4) found in patients with IBDs (Hart et al., 2005; Verstege et al., 2008). Interestingly, IECs from patients with CD secrete less TSLP, suggesting that the conditioning factors produced by IECs and stromal cells in the intestine that are necessary for inducing hemostatic LPDCs are deficient in IBDs (Rimoldi et al., 2005). Indeed, LPDCs from IBD patients also produce significantly more pro-inflammatory cytokines (IL-12, IL-6, IL-8, TNF-α) compared to those from healthy controls (Baumgart et al., 2009; Hart et al., 2005). Furthermore, there is an increase in frequency of LP pDC from IBD patients (Baumgart et al., 2011). However, stimulated peripheral blood pDC from IBD patients secrete significantly less IFN-α compared to those from healthy controls, suggesting that a decrease in functional tolerogenic pDC in IBD patients contributes to disease pathogenesis (Baumgart et al., 2011; Baumgart et al., 2005).

There is accumulating evidence that inappropriate macrophage and DC responses to the enteric microbiota contribute to human IBD pathogenesis (Xavier and Podolsky, 2007). These include both inadequate protective and enhanced pathogenic responses to such stimuli. Macrophages isolated from both CD and UC patients demonstrate altered cytokine production in response to bacterial challenge: CD macrophages produce more pro-inflammatory IL-23 but less of the protective cytokine IL-10, whereas UC macrophages constitutively produce high levels of the pro-inflammatory cytokine IL-12 (Campos et al., 2011). This may be in part due to impaired regulation of TLR-induced inflammatory responses in macrophages. For instance, patients with IBDs demonstrate significantly decreased expression of intestinal NFIL3, an IL-10- and microbiota-induced transcriptional repressor of IL-12p40 expression, compared to tissue from healthy, non-inflamed control patients (Kobayashi et al., 2011). Additionally, increased numbers of TREM-1-expressing LP macrophages are found in intestinal tissue from patients with IBDs compared to tissue from control patients (Schenk et al., 2007). TREM-1 critically amplifies TLR-induced inflammatory responses of macrophages and is implicated in IBD pathogenesis. Conversely, LP macrophages from IBD patients produce less of the cytokine G-CSF, which is protective in experimental models of colitis, in response to the probiotic *Lactobacillus rhamnosus* GR-1 compared to those from healthy controls (Martins et al., 2009).

There has long been evidence that patients with IBDs demonstrate impaired ability to eradicate bacteria (Rahman et al., 2008), and antibiotic therapy in certain clinical situations is efficacious for the induction and maintenance of remission in IBD (Khan et al., 2011; Pineton de Chambrun et al., 2012). The human IBD susceptibility

polymorphisms associated with *NOD2* and *ATG16L1* encode proteins involved in the autophagy pathway and lead to defective bacterial clearance (Travassos et al., 2010). Macrophages isolated from patients with CD demonstrate decreased ROS production and impaired eradication of bacteria (Palmer et al., 2009; Smith et al., 2009). Additionally, peripheral blood monocytes isolated from patients with both CD and UC demonstrate decreased phagocytosis and killing of bacteria (Caradonna et al., 2000). Perhaps the most compelling evidence of the link between bacterial persistence and IBD is the long list of primary immunodeficiencies, such as chronic granulomatous disease (CGD), associated with IBD-like clinical manifestations (Diez et al., 2010; Ishii et al., 1987; Marks et al., 2009; Marks et al., 2010; Yamaguchi et al., 2001). Approximately 50% of patients with CGD, in which phagocyte ROS production and bacterial clearance are greatly impaired, develop IBD-like manifestations that share clinical and pathological features of CD (Marks et al., 2009; Segal et al., 2009). Furthermore, a SNP within the first intron of *NCF4* (p40phox) is associated with enhanced susceptibility to IBD (Rioux et al., 2007). Bacterial persistence and chronic stimulation of macrophages and DCs may contribute to IBD development by producing increased pro-inflammatory cytokines that shape pathogenic adaptive immune responses. Indeed, defects in how macrophages and DCs respond to enteric antigens, eradicate bacteria and induce resolution of inflammation underlie IBD pathogenesis (See Figure 1.1 for summary of pathways and phenotypes).

1.7 Phosphoinositide 3-kinases in Immune Responses

PI3Ks are a group of kinases that regulate diverse cell functions, including growth, proliferation, survival, migration, glucose homeostasis and membrane trafficking

(Vanhaesebroeck et al., 2010). Indeed, dysregulation of PI3K signaling is implicated in various human diseases including diabetes and cancer (Falasca and Maffucci, 2012). PI3Ks initiate intracellular signaling cascades by phosphorylating the 3'-position of the inositol ring on select phosphoinositide molecules embedded in the cell membrane. The consequent phosphoinositide phosphate (PIP), either PI(3)P (e.g., PIP), PI(3,4)P (e.g., PIP_2) or PI(3,4,5)P (e.g., PIP_3), recruits proteins with recognition domains for PIP, PIP_2, or PIP_3 to the cell membrane signaling scaffold.

The PI3K heterodimer consists of a catalytic and regulatory subunit; the regulatory subunit protects the catalytic subunit from degradation, prevents its promiscuous signaling and acts as a protein-binding scaffold (Vanhaesebroeck et al., 2010). PI3K catalytic subunit isoforms are grouped into three classes based on substrate specificity and structure: Class I, II and III. The most abundant phosphoinositide generated by Class I PI3Ks is PIP_3. Class I PI3Ks are further divided into Class I_A and I_B. There are three members of the Class I_A PI3Ks: p110α, p110β and p110δ. The Class I_A PI3Ks propagate signals downstream of receptor tyrosine kinases, such as TLRs and cytokine receptors. Class I_A PI3Ks may also be activated downstream of G protein-coupled receptors (GPCRs) by Ras (Vanhaesebroeck et al., 2010). The regulatory subunits of Class I_A PI3Ks include p85α, p55α, p50α, p85β and p55γ. The sole member of Class I_B PI3Ks is p110γ and is associated with signaling downstream of GPCRs. PI3K p110γ associates with p101 or p84/p87 regulatory subunits. Whereas p110α and p110β are ubiquitously expressed, p110δ and p110γ are highly enriched in leukocytes (Koyasu, 2003; Papakonstanti et al., 2008), suggesting dominant roles for these subunits in immune functions. Class II PI3Ks consist of only catalytic subunits, PI3K-C2α, PI3K-

C2β and PI3K-C2γ and preferentially generate PIP. Class II PI3Ks are implicated in cell growth, survival, migration, membrane trafficking and insulin signaling (Falasca and Maffucci, 2012). The Class III PI3K, vacuolar protein sorting 34 (Vps34), associates with the Vps15 regulatory subunit to preferentially generate PIP. Vps34 has been associated with regulation of endocytosis, autophagy, and nutrient homeostasis (Backer, 2008).

1.7.1 Structure and Signaling Downstream of the Class I_A PI3Ks

Class I PI3Ks contain a Ras binding domain (RBD), a C2 domain, a helical domain, and a catalytic domain (Vanhaesebroeck et al., 2001). Class I_A PI3Ks additionally have a p85-binding domain. Upon activation of receptor tyrosine kinases or GPCRs, Class I_A PI3Ks are recruited to the activated receptor by either Ras or phosphorylated tyrosine residues, which are recognized by Src homology 2 (SH2) domain of the regulatory p85α subunit (Vanhaesebroeck et al., 2010). Recruitment to the membrane releases p110 from inhibition by p85α, allowing generation of PIP_3. Proteins containing a pleckstrin homology (PH) domain, such as Akt and phosphoinositide-dependent kinase-1 (PDK1), recognize and are recruited to newly generated PIP_3. PIP_3 is rapidly dephosphorylated by phosphatase and tensin homolog (PTEN), attenuating signaling (Vanhaesebroeck et al., 2010). Akt is a major signaling molecule activated downstream of Class I PI3K. In turn, Akt regulates multiple signaling pathways, including mammalian target of rapamycin (mTOR), glycogen synthase kinase-3β (GSK-3β), forkhead box protein O1 (FOXO1), and mouse double minute 2 homolog (MDM2) (Hemmings and Restuccia, 2012).

1.7.2 PI3K p110δ in Innate Immune Cells

The cell-type distribution of the Class I_A PI3Ks p110δ and p110γ suggests that these enzymes regulate immune responses. Indeed, the promoter of *PIK3CD*, the gene encoding p110δ, contains binding motifs for several immune-specific transcription factors (Kok et al., 2009). PI3K p110δ has emerged as an important negative regulator of TLR signaling in innate immune cells (Fukao and Koyasu, 2003). This was first shown in DCs, as p85α-deficient DCs produced significantly more IL-12p40 and IL-12p70 in response to TLR ligands compared to WT DCs (Fukao et al., 2002). The increased generation of IL-12p70 by DCs led to enhanced Th1 responses. Additionally, p110δ signaling in DCs induces IL-6 production, which limits Th1 responses (Krishnamoorthy et al., 2008). Recently, Aksoy *et al*., demonstrated p110δ regulates the transition from pro-inflammatory MyD88/TIR domain-containing adaptor protein (TIRAP) signaling downstream of TLRs to anti-inflammatory TRIF/TRIF-related adaptor molecule (TRAM) signaling (Aksoy et al., 2012). The switch to TRIF/TRAM signaling allows the potent anti-inflammatory cytokine IL-10 to be produced. In macrophages, the different Class I_A isoforms perform distinct, non-redundant functions, and p110δ is the dominant isoform responsible for Akt activation downstream of cytokine receptor activation (Papakonstanti et al., 2008). PI3K p110δ signaling in macrophages downstream of TLRs also negatively regulates IL-12p40, IL-12p70 and IL-23 through increased MAPK p38 and JNK activation (Uno et al., 2010). Furthermore, p110δ positively regulates bactericidal activity in macrophages but does not affect phagocytosis of opsonized bacteria (FcγR-dependent phagocytosis) or apoptotic cell debris (Leverrier et al., 2003; Uno et al., 2010). PI3K p110δ additionally regulates colony stimulating factor-1 (CSF-1) induced cell spreading

and migration of monocytes and macrophages (Mouchemore et al., 2013). Further demonstrating a role for p110δ in regulating vesicular trafficking, macrophage trafficking and secretion of TNF-α requires p110δ signaling (Low et al., 2010).

PI3K p110δ also regulates functions of other innate immune cell populations. PI3K p110δ mediates rolling, adhesion, migration of and activation-induced cell morphology changes and cytokine production in eosinophils in circulation, thus regulating allergic immune responses *in vivo* (Kang et al., 2012; Tanemura et al., 2009). Neutrophil cytokine production elicited by LPS or TNF-α stimulation and neutrophil migration into tissues is regulated by p110δ (Fortin et al., 2011; Randis et al., 2008). PI3K p110δ signaling in neutrophils recruits p40phox and p47phox, subunits of NADPH oxidase, to the membrane signaling complex, implicating p110δ activation in ROS production (Kanai et al., 2001). Interestingly, pharmacologic inhibition of p110δ in neutrophils and other cell types decreases production of ROS and the respiratory burst (Yamamori et al., 2004). Furthermore, p110δ negatively regulates LPS-induced IL-1β production, but enhances TNF-α and IL-6 production, in mast cells (Hochdorfer et al., 2011). In natural killer cells, p110δ positively regulates maturation as well as secretion of IFN-γ, TNF-α and GM-CSF (Kim et al., 2007).

1.7.3 PI3K p110δ in Adaptive Immune Cells

Additionally, p110δ regulates adaptive immune cell functions, although its functional output is often the opposite of responses in innate immune cells. PI3K p110δ regulates differentiation, survival, chemotaxis, antigen presentation and both T cell-dependent and -independent generated antibody responses of B cells (Al-Alwan et al.,

2007; Haylock-Jacobs et al., 2011; Jou et al., 2002; Okkenhaug et al., 2002; Reif et al., 2004). Furthermore, p110δ positively signals through the T cell receptor (TCR) and B cell receptor (BCR) to induce antigen-specific proliferation of T and B cells (Garcon et al., 2008; Jou et al., 2002; Okkenhaug et al., 2002; Okkenhaug et al., 2006; Ying et al., 2012). PI3K p110δ signaling is also necessary for $CD4^{+}CD25^{+}Foxp3^{+}$ Treg function (Patton et al., 2006; Patton et al., 2011), antigen-specific cytokine production by both naïve and memory T cells (Liu and Uzonna, 2010; Okkenhaug et al., 2006; Soond et al., 2010), development and function of T follicular helper cells involved in induction of B cell responses (Rolf et al., 2010; So et al., 2013) and regulates antigen-specific T cell homing to inflamed tissues (Jarmin et al., 2008; Liu and Uzonna, 2010; Sinclair et al., 2008). Thus, whereas p110δ negatively regulates many responses to receptor tyrosine kinase activation in innate immune cells, p110δ positively regulates functions downstream of the TCR and BCR in adaptive immune cells.

1.8 PI3K p110δ in Intestinal Homeostasis

Paradoxically, mice with a germline knock-in of *Pik3cd* harboring an inactivating point mutation (p110δ kinase-dead; hereafter referred to as "$p11\delta^{KD}$") demonstrate *enhanced* Th1 responses and develop spontaneously occurring experimental colitis (Liu et al., 2009a; Okkenhaug et al., 2002; Uno et al., 2010). Enhanced Th1 responses, despite impaired TCR-induced proliferation and cytokine production, can be partially explained by the reduction in numbers of functional Treg cells (Liu et al., 2009a). Conversely, $p110\delta^{KD}$ macrophages produce increased IL-12p40, IL-12p70 and IL-23 in response to microbial products and demonstrate impaired bacterial clearance *in vitro* and *in vivo* (Uno

et al., 2010). Intriguingly, a case study recently described a patient with homozygous germline loss of full-length p85α who lacked B cells and had colitis but did not demonstrate other pathologic inflammatory processes (Conley et al., 2012). This patient demonstrated normal expression of immune cell p50α and p55α, but greatly reduced expression of p110δ. Both human and murine studies strongly implicate p110δ signaling in the maintenance of intestinal homeostasis. Contrary to prevailing paradigms where p110δ inhibition is a strategic approach in inflammatory diseases driven by adaptive immune defects (Durand et al., 2013; Haylock-Jacobs et al., 2011; Matteoli et al., 2010; Ying et al., 2012), blockade of p110δ in diseases where innate immune processes are central drivers of pathogenesis, such as IBDs, may actually be harmful.

Given the role of the PI3K p110δ subunit in innate immune processes fundamental to the pathogenesis of IBD, we further characterized host-enteric microbiota and APC-T cell interactions in $p110\delta^{KD}$ mice. We describe a requirement for the enteric microbiota to drive intestinal inflammation in $p110\delta^{KD}$ mice. Microbial-innate immune interactions maintain homeostasis through regulation of both protective (IL-10) and inflammatory (IL-12p40) cytokines, and p110δ is a central regulator of this balance. Additionally, p110δ positively regulates eradication of intracellular bacteria in macrophages. Persistence of intracellular bacteria and chronic stimulation in intestinal $p110\delta^{KD}$ macrophages propagates the imbalance of cytokines. Furthermore, p110δ orchestrates innate immune cell regulation of pathogenic adaptive immune responses. Importantly, in human CD, decreased intestinal *PIK3CD* gene expression and an inverse

correlation with intestinal *IL12B*:*IL10* ratios are demonstrated. Thus, p110δ appears to be a central homeostatic switch in the intestine, governing the critical balance between IL-12/23 and IL-10 induced by the microbiota that determines the subsequent T cell response.

1.9 Figures

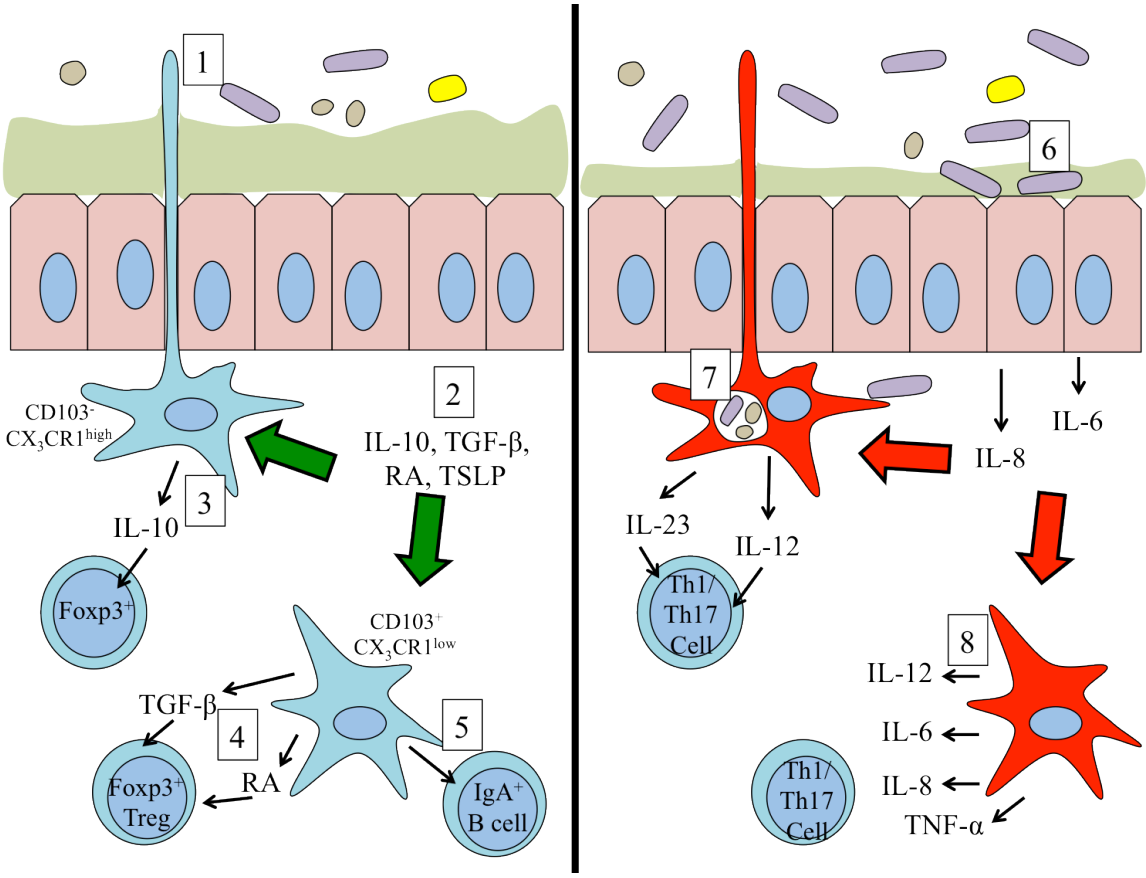


Figure 1.1. Lamina propria mononuclear cells affect intestinal homeostasis in health and disease. LPMCs participate in maintaining intestinal homeostasis and in initiating disease when homeostasis is perturbed. [1] $CD103^{-}CX_3CR1^{high}$ LPMCs extend dendrites across the IEC barrier to sample luminal bacteria and antigens. [2] IECs and stromal cells produce local factors that condition LPMCs to be tolerant. [3] LP macrophages constitutively produce high levels of IL-10, which is necessary for the maintenance of Foxp3 expression in LP Treg cells. [4] $CD103^{+}CX_3CR1^{low}$ LPDCs produce TGF-β and retinoic acid (RA) to induce Treg cells and imprint gut homing receptors in adaptive immune cells. [5] $CD103^{+}CX_3CR1^{low}$ LPDCs induce IgA class switching in B cells. IgA is important in controlling the growth and composition of the enteric microbiota. [6] During perturbation of intestinal homeostasis, the enteric microbiota demonstrates dysbiosis. Additionally, the mucous layer just superficial to the IEC layer can break down, exposing IECs to the microbiota and inducing IECs to produce inflammatory cytokines. [7] Defects in intracellular bacterial clearance leads to persistent stimulation of LPMCs and induction of pro-inflammatory cytokines. IL-12 and IL-23 support the maintenance and differentiation of Th1 and Th17 cells, respectively. [8] $CD103^{+}CX_3CR1^{low}$ cells become inflammatory, producing increased amounts of IL-12, IL-6, IL-8 and TNF-α, supporting the differentiation of pathogenic T cells and the recruitment of inflammatory cells to the intestines.

CHAPTER 2

ALTERED MACROPHAGE FUNCTION CONTRIBUTES TO COLITIS IN MICE DEFECTIVE IN THE PHOSPHOINOSITIDE 3-KINASE SUBUNIT p110δ[1]

2.1 Personal Contributions to Manuscript

I am a co-author on the manuscript entitled, "Altered macrophage function contributes to colitis in mice defective in the phosphoinositide 3-kinase subunit p110δ," published in *Gastroenterology* in 2010 (Uno et al., 2010). I contributed to the manuscript by performing the bacterial assays in which we infected WT and $p110\delta^{KD}$ bone marrow-derived macrophages (BMDMs) with K12 *Escherichia coli*, NC101 *E. coli* or *Salmonella typhimurium* and measured bacterial survival and phagocytosis and IL-12p40 produced by the BMDMs. Additionally, I isolated bacterial DNA from various tissues from WT and $p110\delta^{KD}$ mice and quantified total bacteria by quantitative RT-PCR. This was a significant contribution to the manuscript, as my work contributed to most of Figure 5.

2.2 Overview

Background and Aims: Innate immune responses are crucial for host defense against pathogens, but need to be tightly regulated to prevent chronic inflammation. Initial

[1]Jennifer K. Uno, Kavitha N. Rao, Katsuyoshi Matsuoka, Shehzad Z. Sheikh, Taku Kobayashi, Fengling Li, Erin C. Steinbach, Antonia R. Sepulveda, Bart Vanhaesebroeck, R. Balfour Sartor, Scott E. Plevy. 2010. Altered macrophage function contributes to colitis in mice defective in the phosphoinositide 3-kinase subunit p110δ. *Gastroenterology* 139(5):1642-1653. doi: 10.1053/j.gastro.2010.07.008

characterization of mice with a targeted inactivating mutation in the p110δ subunit of phosphoinositide 3-kinase (PI3K p110δ^{KD}) reveal defects in B- and T-cell signaling and chronic colitis. Here, we further characterize features of inflammatory bowel diseases (IBD) in these mice and investigate underlying innate immune defects.

Methods: Colons and macrophages from PI3K p110δ^{KD} mice were evaluated for colonic inflammation and innate immune dysfunction. Colonic p110δ mRNA expression was examined in IL-10-deficient (*Il10*$^{-/-}$) and wild type (WT) germ free (GF) mice during transition to a conventional microbiota. To assess polygenic impact on colitis development, p110δ^{KD} mice were backcrossed to *Il10*$^{-/-}$ mice.

Results: A mild spontaneous colitis was demonstrated in p110δ^{KD} mice at 8 weeks with inflammation increasing with age. An inflammatory mucosal and systemic cytokine profile was characterized by expression of IL-12/23. In p110δ^{KD} macrophages, augmented toll-like receptor signaling and defective bactericidal activity were observed. Consistent with an important homeostatic role for p110δ, WT mice raised in a GF environment markedly upregulated colonic p110δ expression with the introduction of the enteric microbiota, however colitis-prone *Il10*$^{-/-}$ mice do not. Moreover, PI3K p110δ^{KD} mice crossed to *Il10*$^{-/-}$ mice developed severe colitis at an early age.

Conclusions: This study describes a novel model of experimental colitis that highlights the importance of PI3K p110δ in maintaining mucosal homeostasis and could provide insight into the pathogenesis of human IBD.

2.3 Introduction

The pathogenesis of the human inflammatory bowel diseases (IBD) Crohn's disease (CD) and ulcerative colitis (UC) is complex, with abnormal immune responses in genetically susceptible individuals eliciting uncontrolled intestinal inflammation (Xavier and Podolsky, 2007). Genetic variants that confer CD susceptibility highlight the importance of innate immune interactions with the enteric microbiota in controlling inflammation (Xavier and Podolsky, 2007). Commensal and pathogenic bacteria are recognized through conserved molecular microbial patterns by pattern-recognition receptors (PRRs), of which toll-like receptors (TLRs) form integral components (Abreu, 2010). Signaling through TLRs leads to the activation of NF-κB, culminating in the induction of inflammatory cytokines including IL-12/23 and TNF-α. This inflammatory response is essential for the eradication of infectious microorganisms; however, excessive and prolonged activation can be detrimental to the host. Although mechanisms by which the host distinguishes commensal from pathogenic bacteria are not well defined, under normal conditions TLR signaling initiated by the enteric microbiota is protective (Rakoff-Nahoum et al., 2004).

Phosphoinositide 3-kinases (PI3Ks) have emerged as important regulators of TLR signaling (Fukao and Koyasu, 2003; Liew et al., 2005). Class I_A PI3Ks are a family of heterodimeric enzymes consisting of a regulatory subunit (p85α, p55α or p50α) and a catalytic subunit (p110α, p110β, p110δ) (Vanhaesebroeck et al., 2001). While p110α and p110β are expressed ubiquitously, the p110δ isoform is highly expressed in leukocytes (Vanhaesebroeck et al., 1997). The clearest role of PI3K in chronic inflammation is described in a mouse harboring a point mutation in the p110δ catalytic subunit of PI3K

(p110δ kinase-dead; hereafter referred to as "p110δ^{KD}") (Okkenhaug et al., 2002). These mice demonstrate B- and T-cell defects including improper maturation, defective antigen receptor signaling and impaired humoral immune responses. Notably, these mice spontaneously develop chronic segmental colonic inflammation. However, effects of the p110δ subunit on innate immune responses in mucosal inflammation remain uncharacterized. In this study, we further describe the development of chronic IBD in p110δ^{KD} mice and investigate the role of p110δ in the regulation of TLR signaling and bactericidal pathways in macrophages.

2.4 Results

2.4.1 PI3K p110δ^{KD} mice develop chronic colitis

Macroscopically, colons from 16-week-old PI3K p110δ^{KD} mice were shorter in length and thicker than those from wild type (WT) with ten percent of mutant mice developing rectal prolapse (data not shown). To characterize histological features and progression of colitis, colonic sections were examined from 6 to 45 weeks of age (Figure 2.1A). A histological scoring system was developed based on features of this model to assess the severity of inflammation (see Section 2.6 Materials and Methods). Colitis was characterized by increased colonic epithelial apoptotic bodies and a marked increase in the number of mitoses in the colonic crypts. There was an increase in lamina propria lymphocytes and neutrophils (Figure 2.1C, left). The colonic crypt architecture was generally well preserved with focal disruption of the tubular architecture associated with crypt abscesses (Figure 2.1C, middle, black arrow). Histologic inflammation was detected starting at 8 weeks of age (Figure 2.1B). The percentage of fields demonstrating

no histological inflammation (grade 0) significantly decreased and the percentage of fields with marked inflammation (grade 3 to 4) significantly increased (Figure 2.1A,B) with age. A reduction in goblet cells was observed in older mice (Figure 2.1C, right). A unique feature was the presence of numerous intraepithelial lymphocytes (IELs) in the colonic epithelium (Figure 2.1C, right, white arrows). Immunohistochemical analysis revealed the presence of numerous $CD3^+$ IELs in the colonic crypts of mutant mice compared to WT mice (Supplemental Figure 2.1).

2.4.2 PI3K p110δ^{KD} mice display an exaggerated mucosal and systemic Th1/Th17 cytokine profile

Colonic explants from p110δ^{KD} mice secreted significantly elevated amounts of inflammatory cytokines IL-12p40, IL-12p70, TNF-α, IFN-γ and IL-17 (Figure 2.1D), as well as the growth factors and chemokines G-CSF, MIP1α, RANTES and KC (Supplemental Figure 2.2). LPS-stimulated PI3K p110δ^{KD} splenocytes secreted elevated levels of IL-12p40 (Figure 2.2A) and TNF-α (Supplemental Figure 2.3) compared to WT splenocytes.

$CD11b^+$ lamina propria mononuclear cells (LPMCs), comprising macrophages and dendritic cells, were isolated from colons of p110δ^{KD} and WT mice. PI3K p110δ^{KD} $CD11b^+$ colonic LPMCs produced lower basal levels of IL-10 relative to WT LPMCs (Figure 2.2B). Moreover, p110δ^{KD} $CD11b^+$ LPMCs activated with heat killed *E. coli* expressed increased levels of IL-12p40 and decreased levels of IL-10 compared to WT $CD11b^+$ LPMCs (Figure 2.2B,C). Furthermore, $CD11b^+$ LPMCs from p110δ^{KD} mice demonstrated upregulation of numerous activation markers, TLR4, and CD14

(Supplemental Figure 2.4B) compared to WT CD11b^{+} LPMCs, consistent with *in vivo* activation and/or recruitment of macrophages during the development of colitis.

2.4.3 PI3K p110δ^{KD} macrophages are hyperresponsive to TLR signaling

The role of PI3K p110δ in the regulation of IL-12p40 gene expression was next studied as a biologically relevant target of TLR signaling in macrophages. Bone marrow-derived macrophages (BMDMs) from p110δ^{KD} mice secreted significantly greater amounts of IL-12p40 protein with LPS stimulation compared to WT BMDMs (Figure 2.2D). Although the kinetics of IL-12p40 induction were similar between WT and p110δ^{KD} BMDMs, the magnitude of induction at each time point was significantly greater in the latter. PI3K p110δ^{KD} and WT BMDMs revealed similar kinetics of IL-12p40 mRNA (*Il12b*) expression that peaked 4 hours post-stimulation and was significantly attenuated by 12 hours. However, there was increased magnitude of expression at all time points until 12 hours in p110δ^{KD} BMDMs (Figure 2.2E).

Next, BMDMs from WT and p110δ^{KD} mice were stimulated with TLR9 (CpG), TLR2 (synthetic bacterial lipoprotein, sBLP) or TLR5 (flagellin) ligands. IL-12p40, IL-12p70, IL-23 and nitric oxide (NO) production were assessed. PI3K p110δ^{KD} BMDMs produced enhanced amounts of inflammatory cytokines and nitric oxide in response to multiple TLR ligands (Figure 2.3). There were no differences in cell surface phenotypic or activation marker expression between p110δ^{KD} and WT BMDMs, including TLR4 and CD14 (Supplemental Figure 2.4A), suggesting that augmented TLR signaling in p110δ^{KD} BMDMs is secondary to intrinsic defects in TLR signaling pathways and not a result of a heightened activation state or increased expression of TLRs or TLR co-receptors.

2.4.4 PI3K p110δ^{KD} macrophages display enhanced MAP kinase activation

PI3K signaling is significantly diminished in PI3K p110δ^{KD} BMDMs as demonstrated by decreased phosphorylation of the PI3K downstream target Akt in LPS or sBLP activated p110δ^{KD} BMDMs compared to WT BMDMs (Figure 2.4A). TLR signaling in macrophages is positively regulated by the MAP kinases p38 and JNK (Feng et al., 1999; Zhu et al., 2001) and negatively regulated by the ERK MAP kinase pathway (Feng et al., 1999). LPS-activated p110δ^{KD} BMDMs displayed a different kinetic pattern of JNK and p38 MAP kinase activation compared to WT BMDMs (Figure 2.4B), with earlier activation and enhanced phosphorylation of p38 MAP kinase. There were no significant differences in ERK activation between p110δ^{KD} and WT BMDMs. Likewise there was no difference in magnitude or kinetics of NF-κB p65 phosphorylation in LPS-stimulated WT and p110δ^{KD} BMDMs (Supplemental Figure 2.5A).

2.4.5 PI3K p110δ^{KD} macrophages demonstrate decreased bactericidal activity

To determine whether p110δ^{KD} macrophages are defective in eradicating intracellular bacteria, gentamicin protection assays were performed with the commensal enteric bacteria K12 *E. coli*, NC101 *E. coli*, and the invasive enteric organism *S. typhimurium*. NC101 *E. coli* is a colitogenic bacterial strain isolated from *Il10*$^{-/-}$ mice (Kim et al., 2005). PI3K p110δ^{KD} BMDMs display decreased bactericidal activity when infected with K12 *E. coli,* NC101 *E. coli*, and *S. typhimurium* (Figure 2.5A, right). Moreover, bacterial colonies recovered one hour following infection were not significantly different from WT BMDMs (Figure 2.5A, left). Additionally, WT and

p110δ^{KD} BMDMs were infected for one hour with K12 *E. coli,* washed and permeabilized, and then immunostained with anti-*E. coli* LPS antibodies. No immunoreactivity was demonstrated in non-permeabilized cells, and WT and p110δ^{KD} BMDMs demonstrated similar numbers of intracellular bacteria, demonstrating that uptake/phagocytosis is not defective in p110δ^{KD} BMDMs (Supplemental Figure 2.6). Culture supernatants showed a marked increase in IL-12p40 protein in p110δ^{KD} BMDMs that inversely correlated with bactericidal activity (Figure 2.5B).

Bacterial products such as LPS and inflammatory cytokines like IFN-γ activate macrophages and augment bactericidal responses (Rada and Leto, 2008; Yap et al., 2007). Bactericidal activity was significantly enhanced in WT BMDMs treated with LPS and IFN-γ. This augmentation was completely absent in p110δ^{KD} BMDMs (Figure 2.5C).

To obtain *in vivo* evidence of defective enteric bacterial clearance in p110δ^{KD} mice, the presence of bacterial DNA in WT and p110δ^{KD} spleens and mesenteric lymph nodes (MLNs) was determined using universal bacterial 16S ribosomal RNA (rDNA) gene primers. Markedly increased bacterial rDNA was detected in spleens and MLNs of p110δ^{KD} mice compared to WT mice, consistent with defective bacterial clearance and/or increased bacterial translocation (Figure 2.5D).

2.4.6 The enteric microbiota induces colonic PI3K p110δ expression in WT but not in colitis-prone Il10$^{-/-}$ mice

Colonic expression of p110δ was studied in WT and colitis-prone *Il10*$^{-/-}$ mice raised in a GF environment and then colonized with a conventional microbiota. PI3K p110δ mRNA (*Pik3cd*) (Figure 2.6A) expression increased in WT mice beginning 7 days following colonization and was most strongly upregulated 14 days following transition.

This increase was specific for the p110δ isoform, as colonic mRNA expression for the p55α (*Pik3r3*) and p85α (*Pik3r1*) subunits were not significantly altered (Figure 2.6B). This robust increase in colonic *Pik3cd* was not observed in GF *Il10*$^{-/-}$ mice transitioned to a conventional microbiota (Figure 2.6A). These results, in a well-established model of experimental colitis, support the hypothesis that p110δ is an important homeostatic pathway limiting the extent and duration of intestinal inflammation.

Additionally, this result suggested that IL-10 might be an important cofactor for the induction of p110δ expression by enteric bacteria. Therefore, induction of p110δ was assessed in LPS-activated WT and *Il10*$^{-/-}$ BMDMs. LPS-activated *Il10*$^{-/-}$ BMDMs demonstrated decreased *Pik3cd* (Figure 2.6C, left) and p110δ protein expression compared to WT BMDMs (Figure 2.6C, right). Moreover, *Pik3cd* induction in LPS-activated BMDMs is MyD88-dependent, as significantly less *Pik3cd* and p110δ protein was observed in *MyD88*$^{-/-}$ BMDMs compared to WT BMDMs (Supplemental Figure 2.5B), confirming that p110δ induction is through the canonical TLR signaling pathway.

2.4.7 Il10$^{-/-}$*/p110δ*^{KD} *mice exhibit severe colitis at an early age*

The phenotype of murine and human IBDs is influenced by polygenic contributions. In p110δ^{KD} and *Il10*$^{-/-}$ mice on the C57BL/6 background, in contrast to other backgrounds, the phenotype of colitis is relatively mild. Moreover, partial but not complete abrogation of colonic p110δ expression was observed in GF *Il10*$^{-/-}$ mice transitioned to a conventional microbiota (Figure 2.6A). Therefore, to address whether a combined genetic defect in p110δ and IL-10 alters the phenotype of colitis, *Il10*$^{-/-}$ mice were crossed with p110δ^{KD} mice to create *Il10*$^{-/-}$/p110δ^{KD} mice. While 4-week-old

p110δ^{KD} and *Il10*$^{-/-}$ mice do not demonstrate colonic inflammation, *Il10*$^{-/-}$/p110δ^{KD} mice developed severe colitis and were notably smaller in size than either parent strain. Gross colonic appearance showed 100% disease penetrance with all mice developing colitis by 4 weeks of age and over 50% displaying severe inflammatory changes (Supplemental Figure 2.7). Colitis scores from *Il10*$^{-/-}$/p110δ^{KD} mice were significantly higher than age-matched p110δ^{KD}, *Il10*$^{-/-}$ and WT mice (Figure 2.7A). Intestinal explant cultures demonstrated increased secretion of IL-12p40, IL-12p70 and IL-23 in *Il10*$^{-/-}$ /p110δ^{KD} mice compared to age-matched *Il10*$^{-/-}$ and p110δ^{KD} mice (Figure 2.7B-D).

2.5 Discussion

This study describes an important role for the activity of the PI3K p110δ isoform in the regulation of TLR signaling and bactericidal pathways in macrophages. Notably, PI3K p110δ plays a critical role in intestinal homeostasis in experimental colitis models. Class I_A PI3Ks are heterodimers consisting of a catalytic subunit (p110α, p110β, or p110δ) which complexes with one of five p85 isoform regulatory units. While p110α and p110β are ubiquitously expressed, p110δ expression is low or absent in most cell types but is abundantly expressed in leukocytes (Koyasu, 2003). PI3K p110δ^{KD} mice have significant defects in B cell antigen receptor signaling, substantial declines in immunoglobulin levels and diminished numbers of immature and mature B cells. Interestingly, in B cells, p110δ regulates TLR-induced proliferation (Al-Alwan et al., 2007). PI3K p110δ also suppresses TLR9-induced IL-12 production in B cells, inhibiting a Th1-skewed response (Dil and Marshall, 2009). T cell functional abnormalities have also been described in p110δ^{KD} mice, including defects in T cell signaling through the T

cell receptor, and defective $CD4^+/CD25^+/Foxp3^+$ T regulatory cell function, recently demonstrated in an adoptive transfer model of colitis (Patton et al., 2006).

We provide the first detailed characterization of spontaneously occurring colitis in $p110\delta^{KD}$ mice. Immunologically, an exuberant inflammatory Th1/Th17 cytokine profile was observed systemically and in the colon. Several characteristics of colitis are reminiscent of features of human IBD, including leukocytic and neutrophilic infiltrates, intestinal epithelial cell damage, and goblet cell depletion. Although older $p110\delta^{KD}$ mice had more significant histologic inflammation, the majority of mice demonstrated colonic inflammatory changes that were not severe in nature. However, genetic background is an important modifier of phenotype in murine experimental colitis and human IBD and could account for this observation. For instance, *Il10*$^{-/-}$ mice on the C57BL/6 background have a relatively mild colitis phenotype. In fact, in our comparative studies, the incidence and severity of histological inflammation and intestinal inflammatory cytokine secretion were similar in $p110\delta^{KD}$ mice and age-matched *Il10*$^{-/-}$ mice on the C57BL/6 background. As polygenic contributions and genetic background can alter phenotype, *Il10*$^{-/-}$ mice were backcrossed to $p110\delta^{KD}$ mice. Using a scoring system devised for *Il10*$^{-/-}$ mice, *Il10*$^{-/-}$ /$p110\delta^{KD}$ mice developed severe colitis and an exuberant mucosal inflammatory cytokine response at an early age compared to each of the founder strains. This finding implicates IL-10 and p110δ as two important, non-redundant, homeostatic pathways that function in normal physiology to suppress intestinal inflammation directed against the enteric microbiota.

The importance of the enteric microbiota in the initiation of IBD is illustrated by *Il10*$^{-/-}$ mice, where the development of spontaneous colitis is dependent on the presence

of the microbiota (Sellon et al., 1998). We show dramatically increased levels of p110δ mRNA (*Pik3cd*) in the colon of WT GF mice transitioned to a conventional microbiota. Importantly, augmented expression was not seen in transitioned colitis-prone *Il10*$^{-/-}$ mice, which correlated with the development of intestinal inflammation. Strikingly similar findings were observed in LPS-activated BMDMs from *Il10*$^{-/-}$ and WT mice. These findings suggest that p110δ regulation may be an important homeostatic pathway in other models of intestinal inflammation. Based on our results, p110δ induced through TLR signaling is an event that limits the extent and duration of TLR-activated pro-inflammatory responses. With IL-10 deficiency, one mechanism for exuberant and prolonged inflammatory responses may be loss of induction of p110δ (See Supplemental Figure 2.8 for model).

A prominent histological feature in p110δ^{KD} mice that is not characteristic of human CD or UC is the presence of numerous IELs. However, intraepithelial lymphocytosis is characteristic of three rare forms of human IBD: celiac disease, lymphocytic colitis and collagenous colitis (Moayyedi et al., 1997; Sollid, 2004). As small intestinal inflammation is characteristic of these microscopic colitides (Green and Cellier, 2007; Moayyedi et al., 1997), we extensively searched but were not able to identify any inflammation in the small bowel of p110δ^{KD} mice. Although the purpose of this study was to correlate the development of colitis with defects in innate immunity in p110δ^{KD} mice, a goal of future research will be to characterize the role of IELs in this model.

PI3K p110δ^{KD} macrophages demonstrate heightened sensitivity to stimulation by TLR ligands. This finding underscores the importance of p110δ in dampening TLR

signaling and also suggests that aberrant regulation of innate immune responses could contribute to the development of colitis in p110δ^{KD} mice. Accumulating evidence has established the role of PI3K in the attenuation of TLR signaling (Aksoy et al., 2005; Fukao et al., 2002; Kuo et al., 2006; Yu et al., 2006). For instance, mice with genetic deletion of the PI3K p85α subunit display altered balance of Th1/Th2 responses (Fukao et al., 2002), and dendritic cells produce enhanced levels of IL-12 in response to TLR2 (PGN), TLR4 (LPS) and TLR9 (CpG) ligands. However, these mice do not develop chronic colonic inflammation. It is possible that mice deficient in PI3K subunits demonstrate compensatory changes in expression and availability of regulatory subunits, which could affect phenotypic and functional analyses (Okkenhaug et al., 2002). As we demonstrate in p110δ^{KD} mice, TLR activation in macrophages elicits an exuberant inflammatory response. However, TLR signaling in intestinal epithelial cells is protective against inflammation (Abreu, 2010). We speculate that the genetic defects in p110δ^{KD} mice that lead to the development of colitis are limited to the hematopoietic compartment, as p110δ is not highly expressed in epithelial cells (Papakonstanti et al., 2008).

Genetic variants linked to CD include genes that mediate autophagy and phagosomal function (Xavier and Podolsky, 2007). Recent studies indicate the importance of PI3K signaling in phagosomal maturation and acidification, essential for optimal bacterial killing (Booth et al., 2003; Levine and Deretic, 2007). Here, we show that p110δ^{KD} macrophages are less efficient than WT macrophages at eliminating enteric commensal and pathogenic bacteria. Our results indicate that p110δ is not necessary for phagocytosis of bacteria into the cell, as there is no difference in bacterial survival

following one hour of incubation with bacteria between WT and p110δ^{KD} macrophages. We also demonstrated that defective bactericidal activity in p110δ^{KD} macrophages is associated with increased inflammatory cytokine production. Additionally, p110δ^{KD} mice are defective at clearing enteric bacteria *in vivo*, suggesting that the inability of p110δ^{KD} macrophages to efficiently kill and clear microbes may contribute to prolonged inflammatory responses. However, macrophage function is not globally compromised in p110δ^{KD} mice. Indeed, the ability of macrophages and dendritic cells from p110δ^{KD} mice to produce NO and destroy intracellular *Leishmania* parasites was recently reported to be similar to WT mice (Liu et al., 2009a).

In summary, the PI3K p110δ^{KD} mouse is an interesting model for understanding the pathogenesis of human IBD as it provides an example of how a genetic defect in a specific intracellular signaling molecule can lead to global defects in innate and adaptive homeostatic pathways in the intestine. Furthermore, polygenic contributions alter the phenotype of colitis as *Il10*$^{-/-}$/p110δ^{KD} mice develop severe colitis at a young age compared to the parent strains. This study describes aberrant innate immunity including exuberant TLR signaling and defective bactericidal activity in macrophages that contribute to the pathogenesis of colitis in this model.

2.6 Materials and Methods

Mice. PI3K p110δ^{KD} mice were on the C57BL/6 background. C57BL/6 WT and *Il10*$^{-/-}$ mice were obtained from Jackson Laboratories. Mice were housed in conventional housing in accordance with guidelines from the American Association for Laboratory Animal Care and Research. Germ free (GF) 8-week-old 129 Sv/Ev WT and *Il10*$^{-/-}$ mice

were provided by the University of North Carolina Gnotobiotic Facility. Mice were colonized with conventional enteric microbiota at 8 weeks of age with a microbiota isolated from WT mice raised in conventionalized conditions (Sellon et al., 1998). PI3K p110δ^{KD} and *Il10*$^{-/-}$ homozygous mice were crossed and offspring were genotyped for *Pik3cd* and *Il10* mutations. For F2 breeding, mice homozygous for one mutation and heterozygous for the other mutation were bred and mice homozygous for both p110δ^{KD} and *Il10*$^{-/-}$ were identified. All experimental mice were genotyped by PCR screening prior to tissue collection with littermates used as controls. The Institutional Animal Care and Use Committee of the University of Pittsburgh and the University of North Carolina approved all methods used in this study.

Reagents. Flagellin was purchased from Invivogen (San Diego, CA). CpG DNA was obtained from Integrated DNA Technologies (Coralville, IA). Synthetic bacterial lipoprotein (sBLP) was purchased from EMC Microcollections (Tübingen, Germany). Peptidoglycan (PGN) and Lipopolysaccharide (LPS) from *Salmonella enteritidis* was purchased from Sigma (St. Louis, MO). LPS was repurified by modified phenol extraction as previously described (Hirschfeld et al., 2000). GM-CSF, and M-CSF were obtained from Peptotech, Inc (Rocky Hill, NJ) and IFN-γ was purchased from R&D Systems (Minneapolis, MN).

Cell isolation. Splenocytes and bone marrow-derived macrophages (BMDMs) were cultured as described (Xiong et al., 2004). Lamina propria mononuclear cells (LPMCs) were isolated from mouse colons by an enzymatic method as previously described

(Kamada et al., 2005). LPMCs were separated into $CD11b^+$ and $CD11b^-$ cells using anti-CD11b microbeads from Miltenyi Biotec (Auburn, CA).

ELISAs. Murine IL-12p40, IL-12p70, IL-10, IFN-γ and TNF-α from R&D Systems (Minneapolis, MN) and IL-23 from eBioscience (San Diego, CA) immunoassay kits were used according to manufacturers' instructions. IL-17, MIP1α, RANTES, KC and G-CSF levels were determined by multiplex ELISA from Luminex (Austin, TX). Phosphorylation levels of p-Akt was determined using a cell-based ELISA from SA Biosciences (Valencia, CA).

Western blot. Western blot analyses were performed on whole cell extracts as described (Xiong et al., 2004). Antibodies to p-JNK, p-p38, p-ERK, JNK2, p38, ERK, NF-κB p65, and p110δ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and p-p65 was obtained from Cell Signaling (Danvers, MA).

Nitrite Determination. Nitrite was assayed by a standard Greiss Reaction adapted to a microplate system.

Real-time RT-PCR analysis. Quantitative real-time RT-PCR was performed as described (Hegazi et al., 2005). Primer sequences are available on request.

Colonic tissue explant cultures and histology. Colonic explant cultures were performed as described previously (Hegazi et al., 2005). Slides were prepared for hematoxylin and

eosin staining and histologic analysis was performed by a pathologist blinded to the study groups (ARS) using established criteria for *Il10*$^{-/-}$ mice (and *Il10*$^{-/-}$/p110δ^{KD} mice) (Hegazi et al., 2005).

Histologic scoring of PI3K p110δ^{KD} mice. A histological scoring system was developed to assess colonic inflammation based on the characteristic of this model. The criteria used to classify histology into grades 0 to 4 were as follows: *Grade 0* was defined as (a) presence of 1 or less mitosis in the colonic crypts per 10 high power fields (HPF), (b) no epithelial hyperplasia, and (c) no neutrophils in the lamina propria. *Grade 1* was established if less than two of the following criteria were found: (a) presence of epithelial hyperplasia, (b) presence of more than 2 mitosis/10 HPF in the colonic crypts, (c) any apoptotic body in the colonic crypts, (d) Infiltration by neutrophils in the lamina propria, (e) infiltration by lymphocytes and/or plasma cells in the lamina propria, (d) infiltration by lymphocytes and/or plasma cells and neutrophils in the lamina propria, (g) less than 30% of colonic crypts showing intraepithelial lymphocytes (IELs). If there were two or more of the criteria for grade 1, *grade 2* was attributed. *Grade 3* was defined as (a) any of the criteria for grade 2 was present and there were more than 30% of IELs involving the colonic crypts per 10 HPF, or (b) there was submucosal inflammation. *Grade 4* was attributed when any of the criteria for grade 3 was identified together with the presence of crypt abscesses and/or mucosal ulcers. Histopathologic analysis of WT mice revealed no or minimal mucosal inflammation with one or less mitosis per 10 HPF, and was scored as grade 0 in the majority of mice, or 1.

Gentamicin Protection Assay. Bacterial invasion was measured by gentamicin protection assays (Darfeuille-Michaud et al., 2004). Briefly, BMDMs were infected with bacteria with a multiplicity of infection of 10 bacteria per cell in antibiotic-free media. Cells were incubated with the bacteria for 2 hours at 37°C with 5% CO_2. LPS (100 ng/ml) and IFN-γ (10 ng/ml) were added 2 hours prior to bacteria where indicated. Cells were then washed twice with PBS and fresh media containing 100 μg/ml of gentamicin was added for one hour. Cells were lysed with 1% Triton-X, and samples were diluted and plated on LB agar plates to determine the number of colony-forming units.

Bacterial DNA Isolation. Total DNA was extracted from splenocytes and mesenteric lymph nodes as outlined previously (Frank et al., 2007). Universal bacteria primer sequences were obtained from Horz HP, *et al*. (Horz et al., 2005). Bacterial DNA expression was determined by real-time RT-PCR and expression was normalized to host *Gapdh* and represented as relative expression to control.

Statistical Analysis. Statistical significance from experiments in cells was determined using student t-test or one-way ANOVA. Statistical significance for *in vivo* data was assessed by the Mann-Whitney U test (SPSS, Chicago, IL, USA) with Bonferroni correction.

2.7 Figures

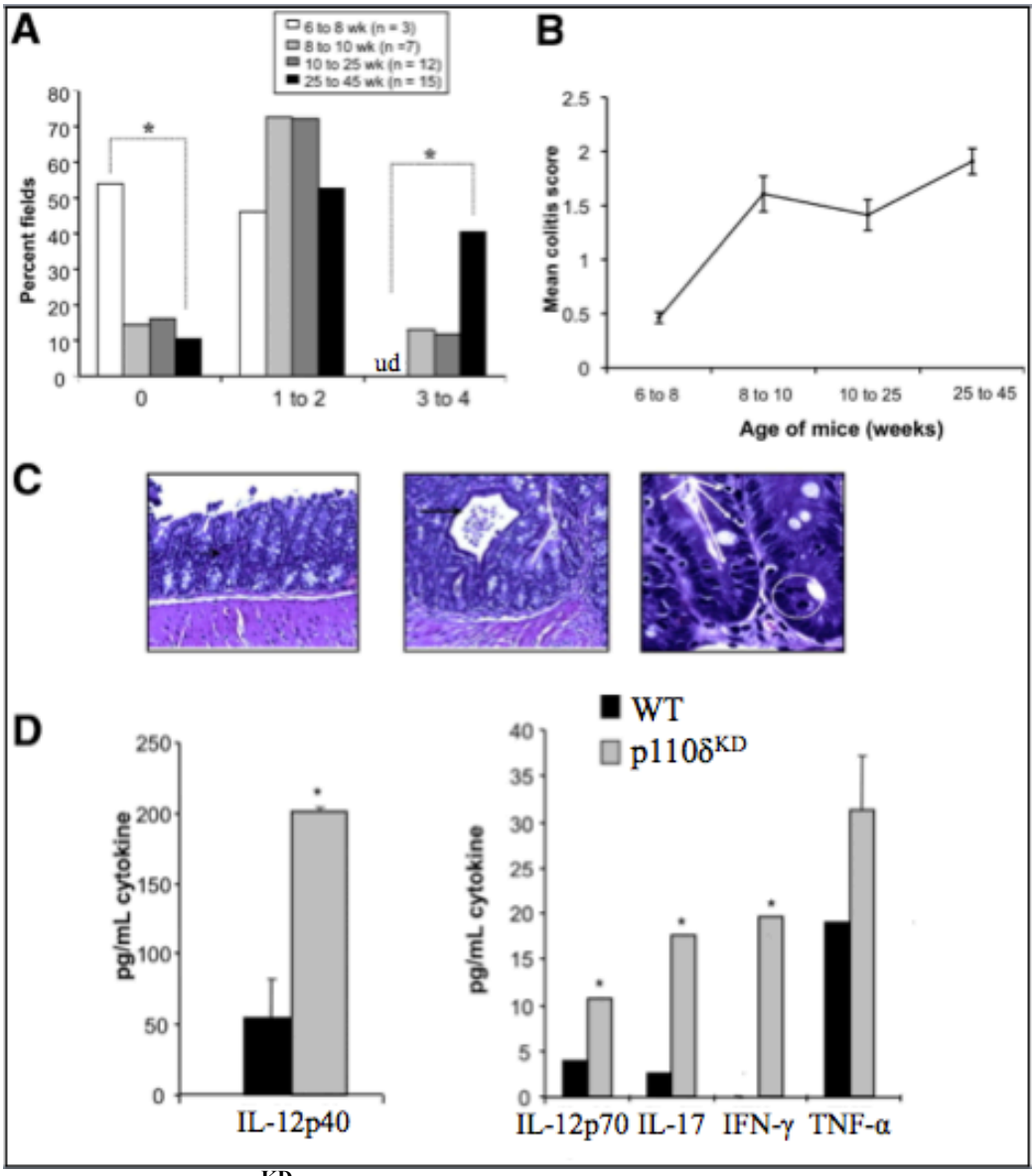


Figure 2.1. PI3K p110δ^{KD} mice develop colitis. (A,B) Histological scores of colonic sections from WT and p110δ^{KD} mice at different ages. Results are represented as percentage of microscopic fields in each age group with score 0, 1 to 2 or 3 to 4 (A); or mean colitis scores (B). (ud, undetermined; *, $p<0.05$ versus WT 25-45-week-old mice) (C) Colonic sections from 10-week-old p110δ^{KD} mice demonstrate leukocytic infiltration of the lamina propria (white circle) and intraepithelial lymphocytes (white arrows) in the crypts. Focal crypt abscesses were observed (black arrow). (D) Colonic explants from WT (black bars) and p110δ^{KD} (grey bars) mice were assayed for spontaneous secretion of cytokines. Error bars represent mean ± SEM of three independent experiments.

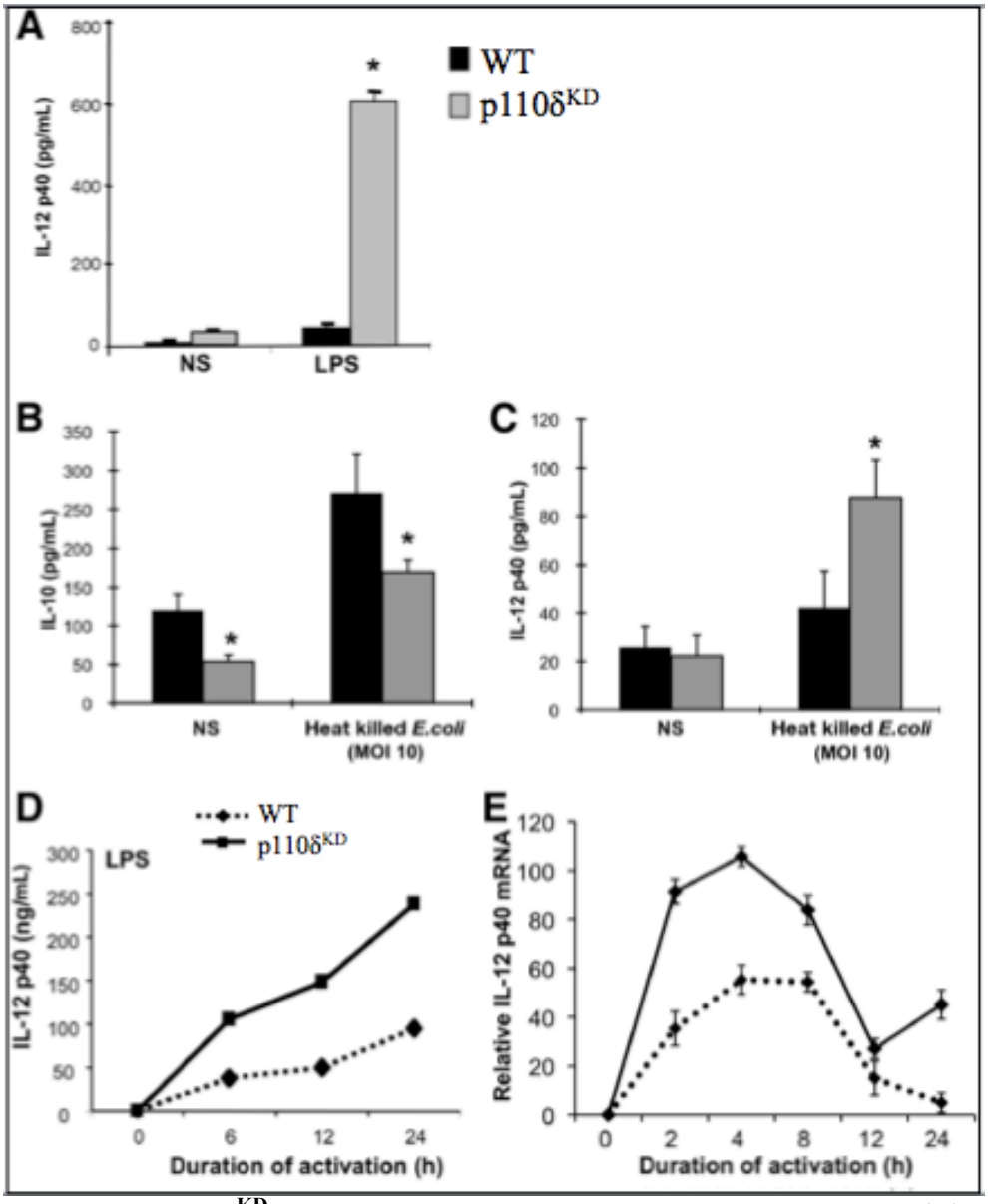


Figure 2.2. PI3K p110δ^{KD} mice display enhanced expression of IL-12p40. (A) Splenocytes from WT (black bars) and p110δ^{KD} (grey bars) mice were not stimulated (NS) or stimulated with LPS (1 μg/ml) for 24 hours. IL-12p40 was measured by ELISA. (B,C) Colonic macrophages from WT or p110δ^{KD} mice were stimulated with heat killed *E. coli* (multiplicity of infection=10) for 24 hours. ELISAs were performed to assess IL-12p40 (B) and IL-10 (C) levels. (D,E) Bone marrow-derived macrophages (BMDMs) from WT (black bars) and p110δ^{KD} (grey bars) mice were not stimulated (NS) or stimulated with LPS (1 μg/ml) and supernatants analyzed for IL-12p40 (D). PI3K p110δ^{KD} BMDMs were harvested at each time point and IL-12p40 mRNA (*Il12b*) (E) levels were assessed by real-time RT-PCR. Results are expressed as fold induction normalized to β-actin. Error bars represent mean ± SEM of three independent experiments (*, $p<0.05$ versus WT).

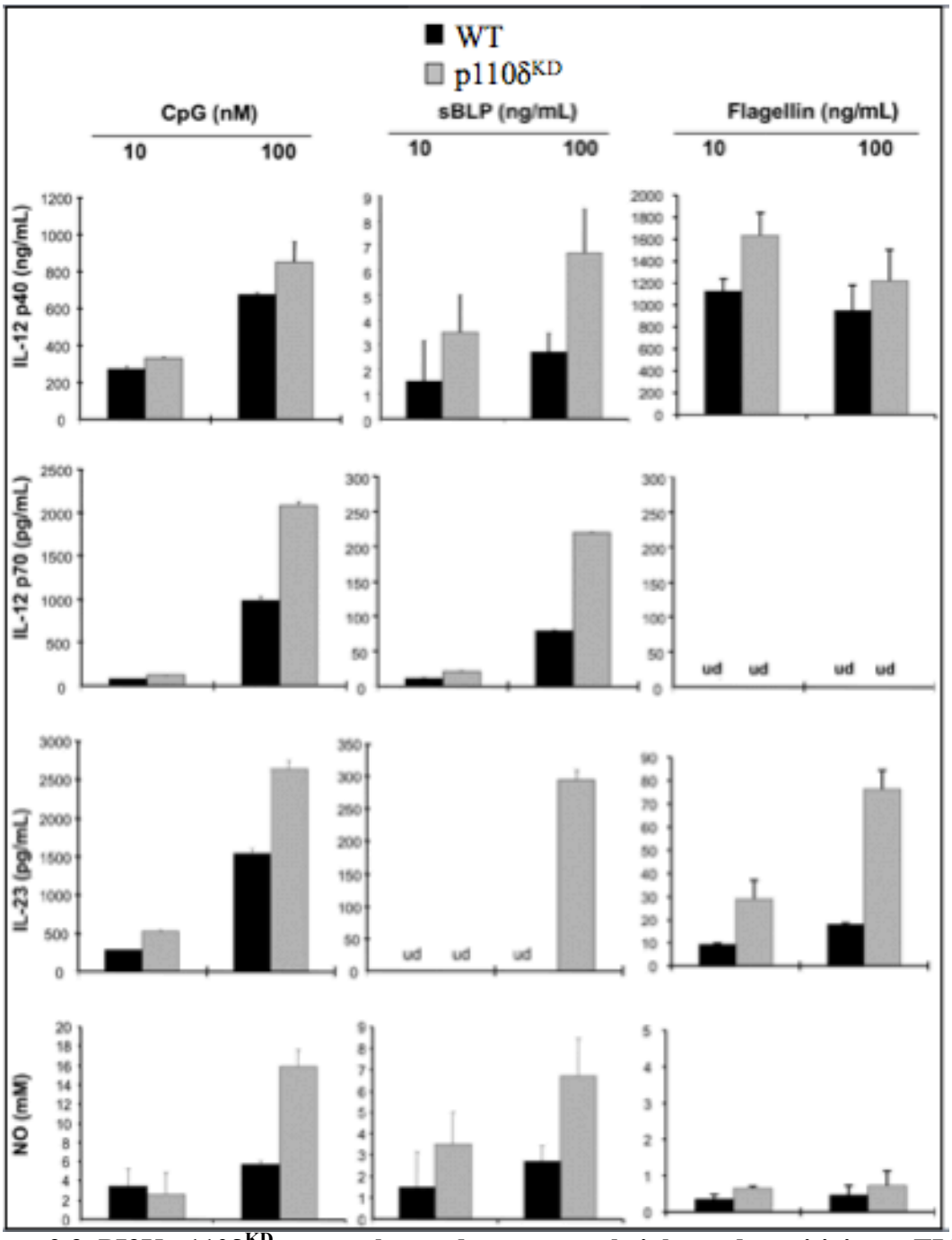


Figure 2.3. PI3K p110δ^{KD} macrophages demonstrate heightened sensitivity to TLR stimulation. BMDMs from WT (black bars) and p110δ^{KD} (grey bars) mice were stimulated with TLR9 (CpG), TLR2 (sBLP) or TLR5 (Flagellin) ligands for 24 hours. Supernatants were analyzed for IL-12p40, IL-12p70 or IL-23 secretion by ELISA and nitric oxide secretion by Greiss reaction. Error bars represent mean ± SEM of three independent experiments (ud, undetermined).

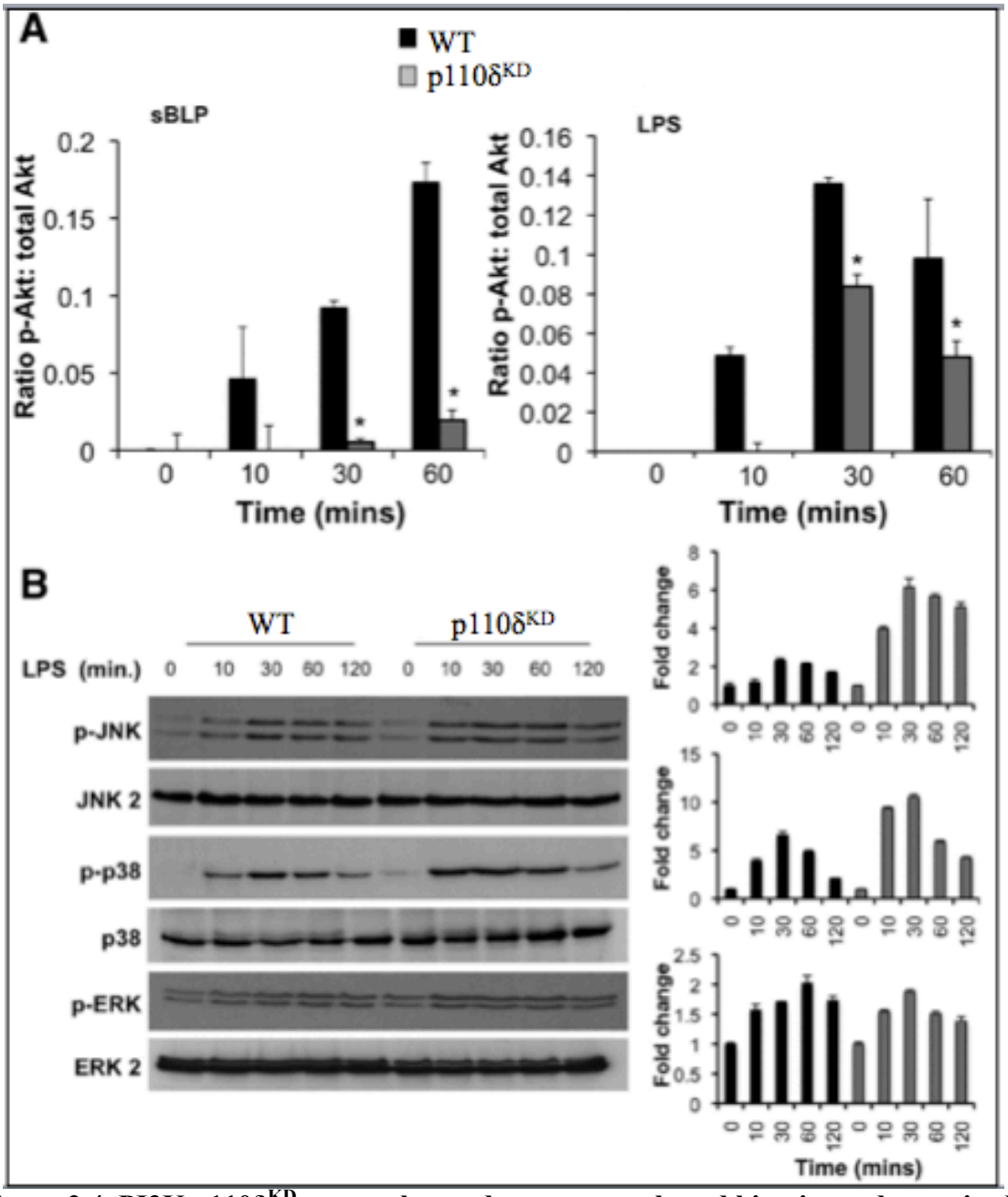


Figure 2.4. PI3K p110δ^{KD} macrophages demonstrate altered kinetics and magnitude of MAPK activation. (A) BMDMs from WT or p110δ^{KD} mice were stimulated with sBLP (100 ng/ml) (left) or LPS (100 ng/ml) (right) for the indicated periods of time and phosphorylation of Akt (p-Akt) was assayed by ELISA. Results are presented as a ratio of p-Akt to total Akt. (B) BMDMs from WT and p110δ^{KD} mice were stimulated with LPS (1 μg/ml) for the indicated times. Whole cell extracts were analyzed for phosphorylation of MAPK (JNK, ERK, p38) by western blot. Results represent mean ± SEM of three independent experiments (*, $p<0.05$ versus WT).

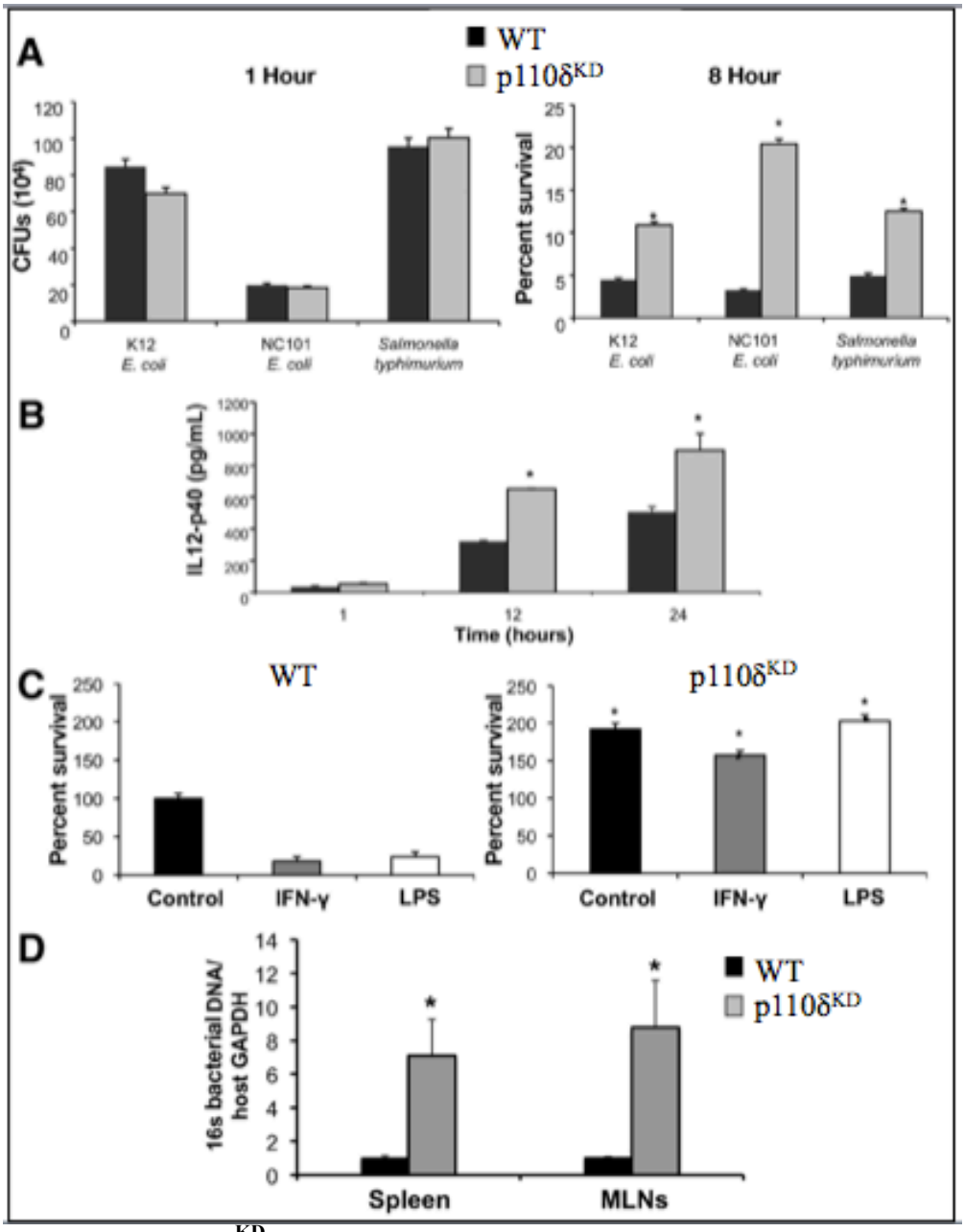


Figure 2.5. PI3K p110δ^{KD} BMDMs demonstrate defective bactericidal activity. (A) WT and p110δ^{KD} BMDMs were cultured with K12 *E. coli.*, NC101 *E. coli* or *S. typhimurium.* Bacteria were recovered one hour post-infection (left panel) and 8 hours post-infection (right panel). (B) IL-12p40 production by ELISA was assessed in p110δ^{KD} BMDMs infected with K12 *E. coli.* (C) BMDMs were treated with LPS (100 ng/ml) or IFN-γ (10 ng/ml) prior to bacterial infection, and bacteria was recovered from lysed cells 8 hours post-infection. (D) Total bacterial DNA in spleen and mesenteric lymph nodes was detected by real-time RT-PCR using primers for total 16S rDNA genes normalized to host *Gapdh*. Error bars represent mean ± SEM of three independent experiments (*, $p<0.05$ versus WT).

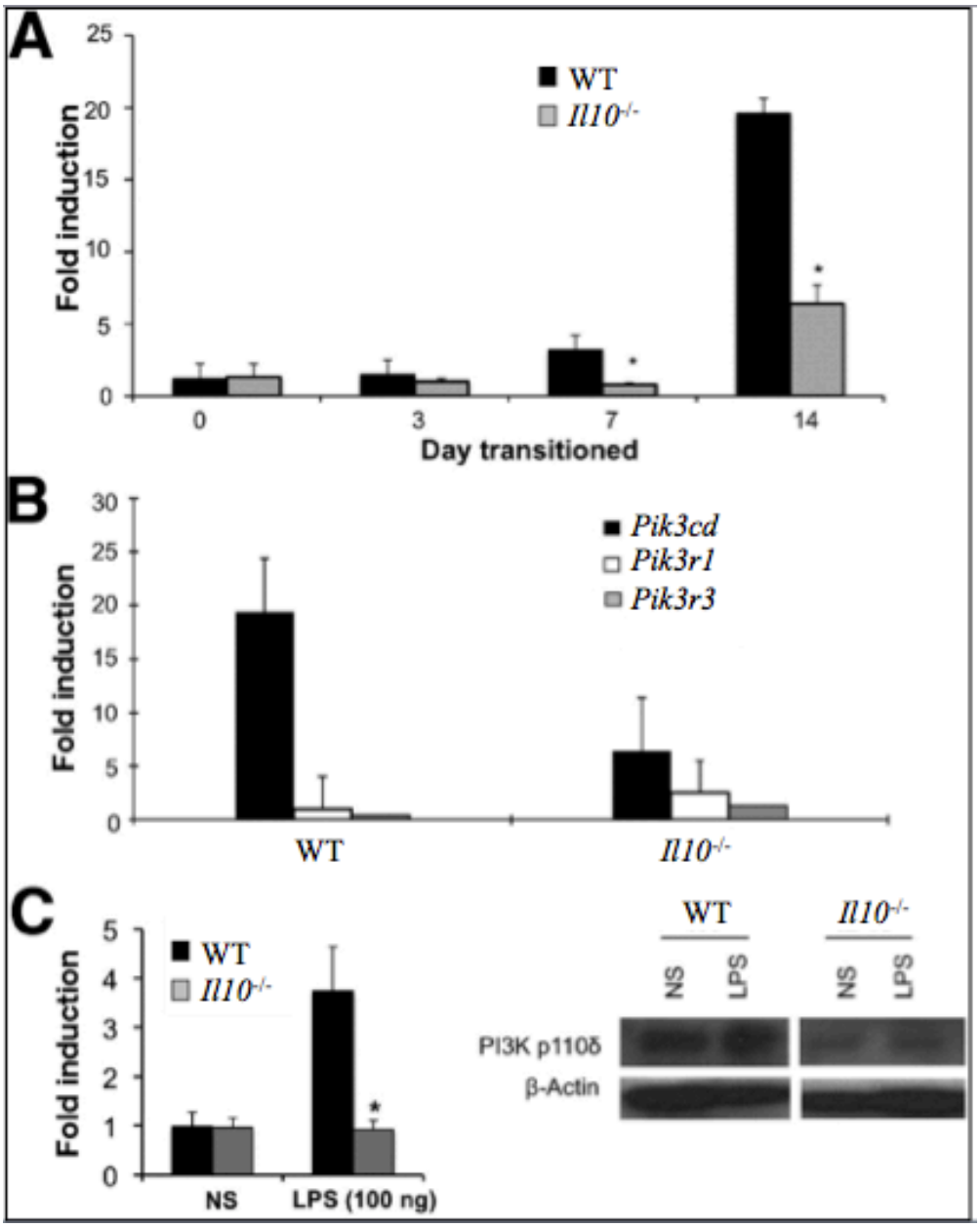


Figure 2.6. The enteric microbiota induces colonic p110δ expression in WT but not colitis-prone *Il10*$^{-/-}$ mice. (A,B) Germ-free (GF) WT and *Il10*$^{-/-}$ mice were transitioned to a conventional microbiota. Colonic mRNA was isolated, and expression of *Pik3cd* (p110δ), *Pik3r1* (p85α), and *Pik3r3* (p55α) mRNA was assessed by real-time RT-PCR. (A) Colonic *Pik3cd* was determined in WT and *Il10*$^{-/-}$ mice at 0, 3, 7, and 14 days post-colonization of GF mice with conventional microbiota (*, $p<0.05$ versus WT). (B) Colonic expression of *Pik3cd*, *Pik3r1*, and *Pik3r3* was examined 14 days post-transition of GF mice to conventional microbiota. Results are expressed as fold induction normalized to *β-actin*. Error bars represent mean ± SEM of three independent experiments. (C) BMDMs from WT and *Il10*$^{-/-}$ mice were stimulated with LPS (100 ng/ml) for the indicated times. *Pik3cd* levels were assessed by real-time RT-PCR (left panel). Results are expressed as fold induction normalized to *β-actin* and represent mean

± SEM of three independent experiments (*, $p<0.05$ versus WT). BMDMs from WT and *Il10*$^{-/-}$ mice were stimulated with LPS (100 ng/ml) for 16 hours. Whole cell extracts were analyzed for p110δ by western blot (right panel). Results are representative of three independent experiments.

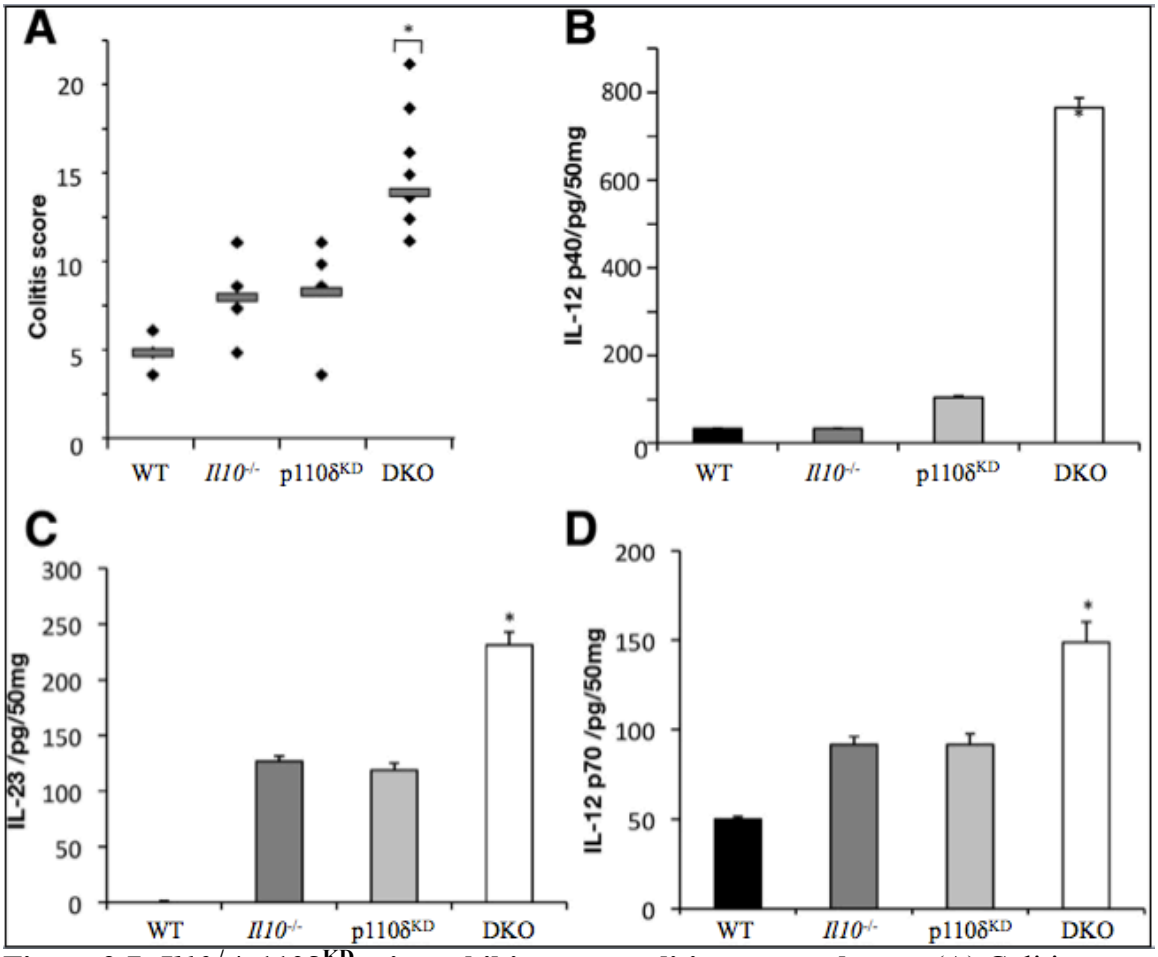
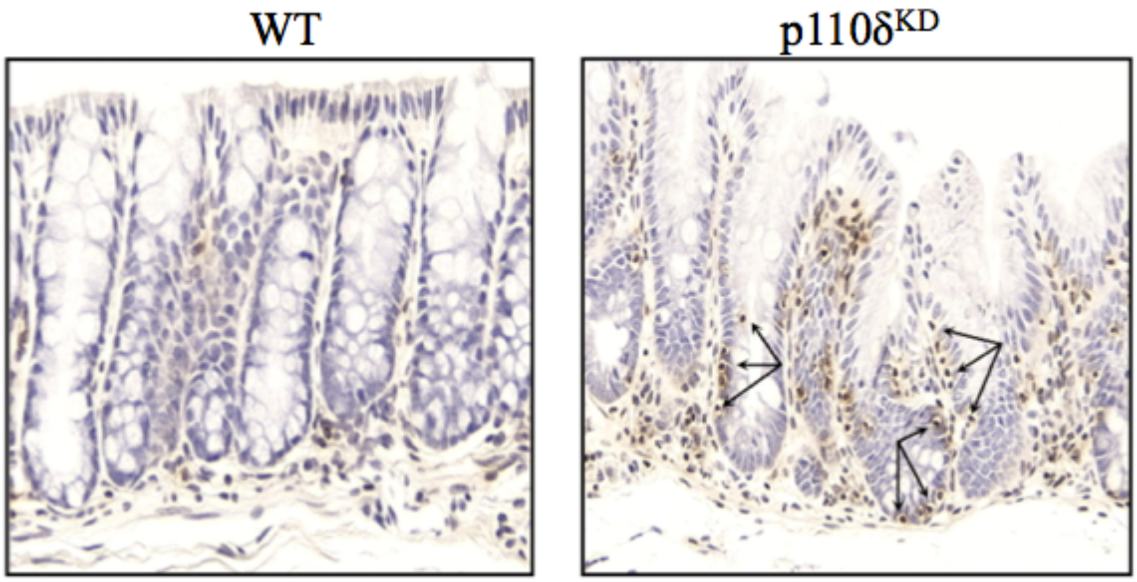
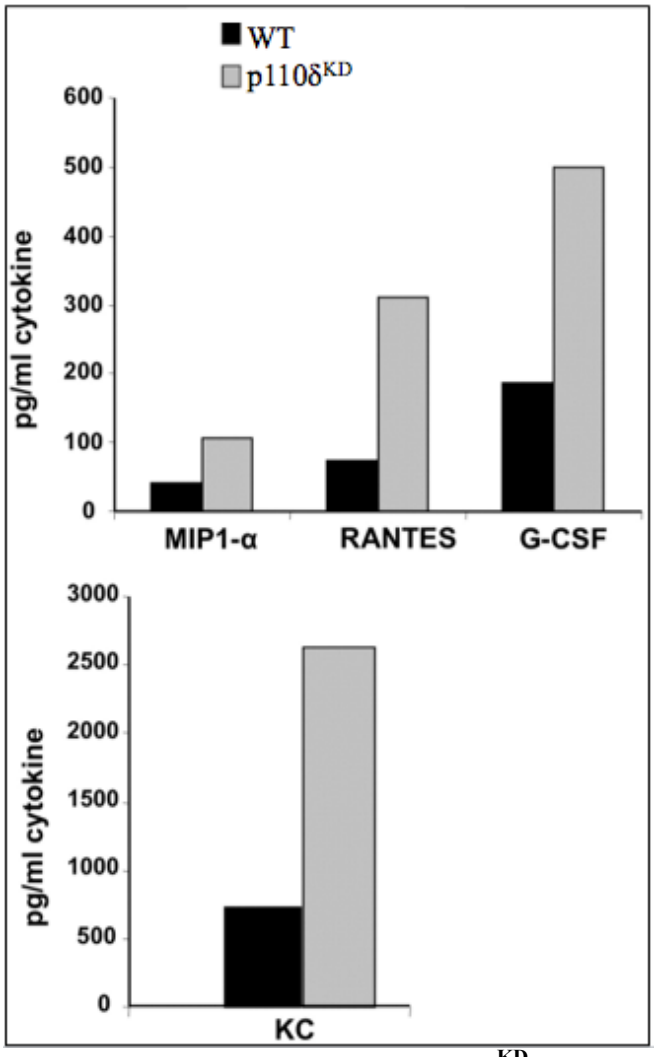


Figure 2.7. *Il10*$^{-/-}$/p110δ^{KD} mice exhibit severe colitis at an early age. (A) Colitis scores were determined for 4-week-old *Il10*$^{-/-}$/p110δ^{KD} (DKO), WT, *Il10*$^{-/-}$, and p110δ^{KD} mice using criteria established for *Il10*$^{-/-}$ mice (Hegazi et al., 2005) by a pathologist (ARS) blinded to experimental groups (*, $p<0.05$ versus WT). IL-12p40 (C), IL-12p70 (E) and IL-23 (D) protein in supernatants from colon explant cultures from DKO, WT, *Il10*$^{-/-}$ and p110δ^{KD} mice were analyzed by ELISA (*, $p<0.05$ versus WT). Error bars represent mean ± SEM of three independent experiments.

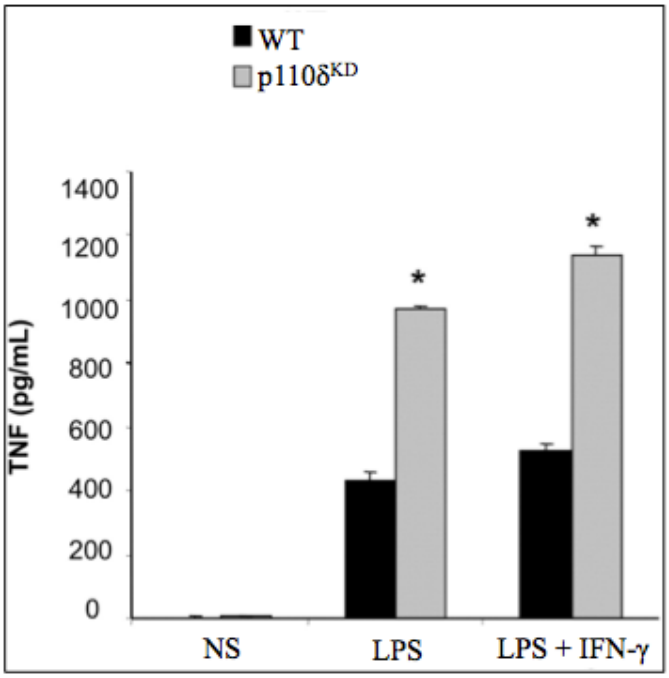
2.8 Supplemental Figures



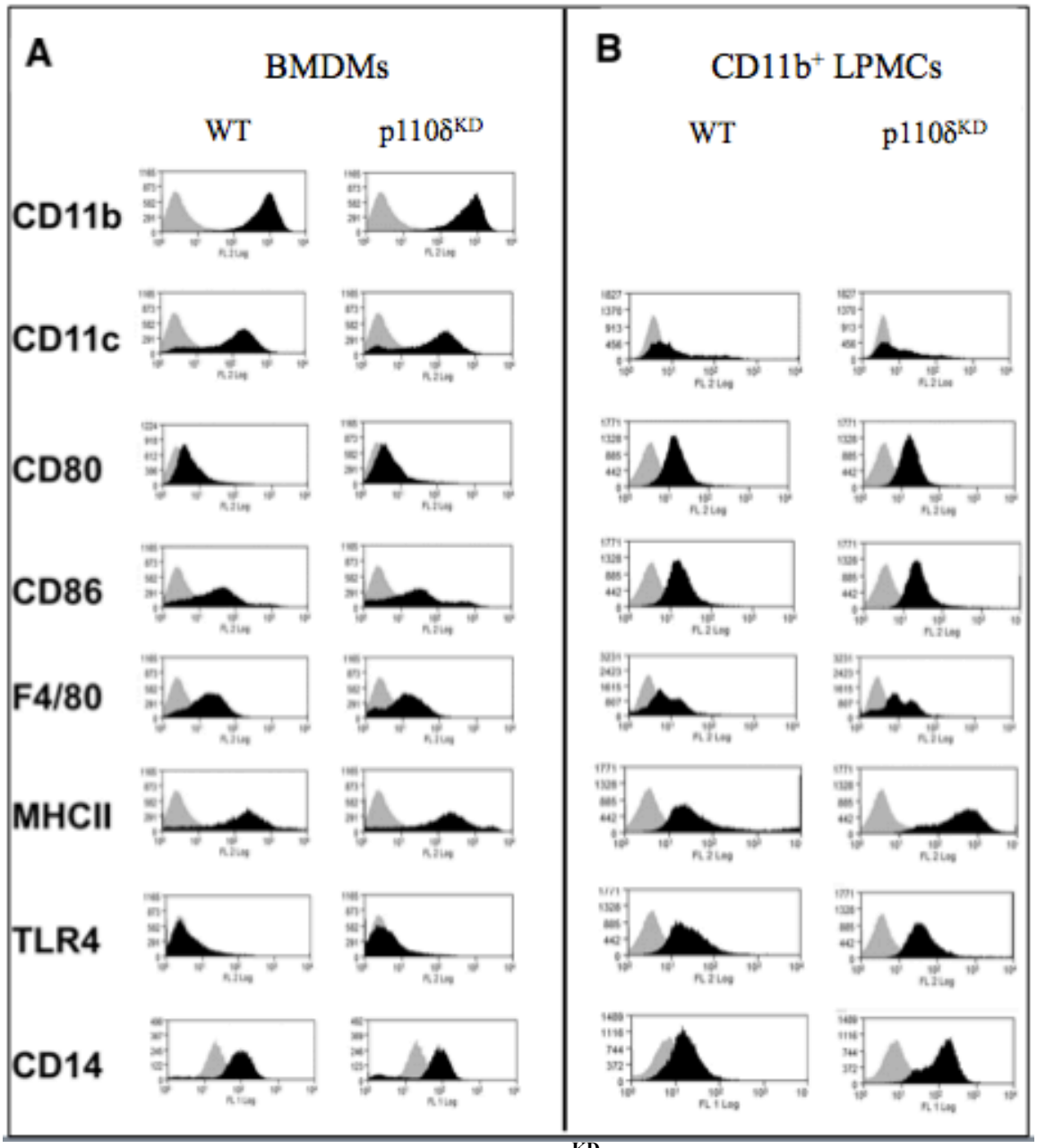
Supplemental Figure 2.1. Increased $CD3^+$ IELs in the colonic crypts of $p110\delta^{KD}$ mice. Representative immunohistochemical analysis of $CD3^+$ cells in paraffin embedded colonic sections from WT and p110δ mice. A marked increase in $CD3^+$ IELs prominent at the base of the crypts (black arrows) is observed in $p110\delta^{KD}$ colonic sections, compared to WT colons.



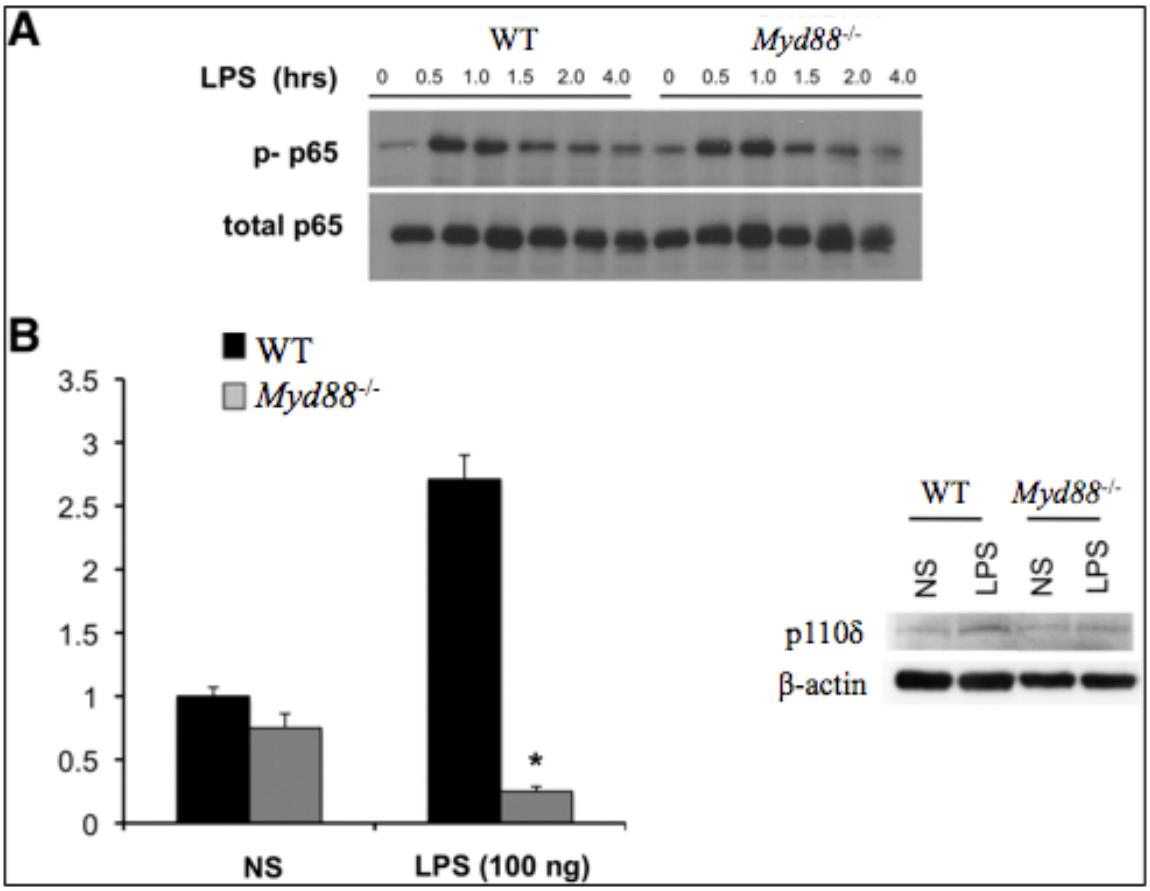
Supplemental Figure 2.2. Colonic explants from p110δ^{KD} secrete elevated chemokines. Intestinal explants from WT (black bars) and p110δ^{KD} (grey bars) mice were cultured for 24 hours and cell-free supernatants were assayed for spontaneous secretion of the indicated chemokines by ELISA. Results are representative of a Luminex multiplex array analysis replicated independently three times.



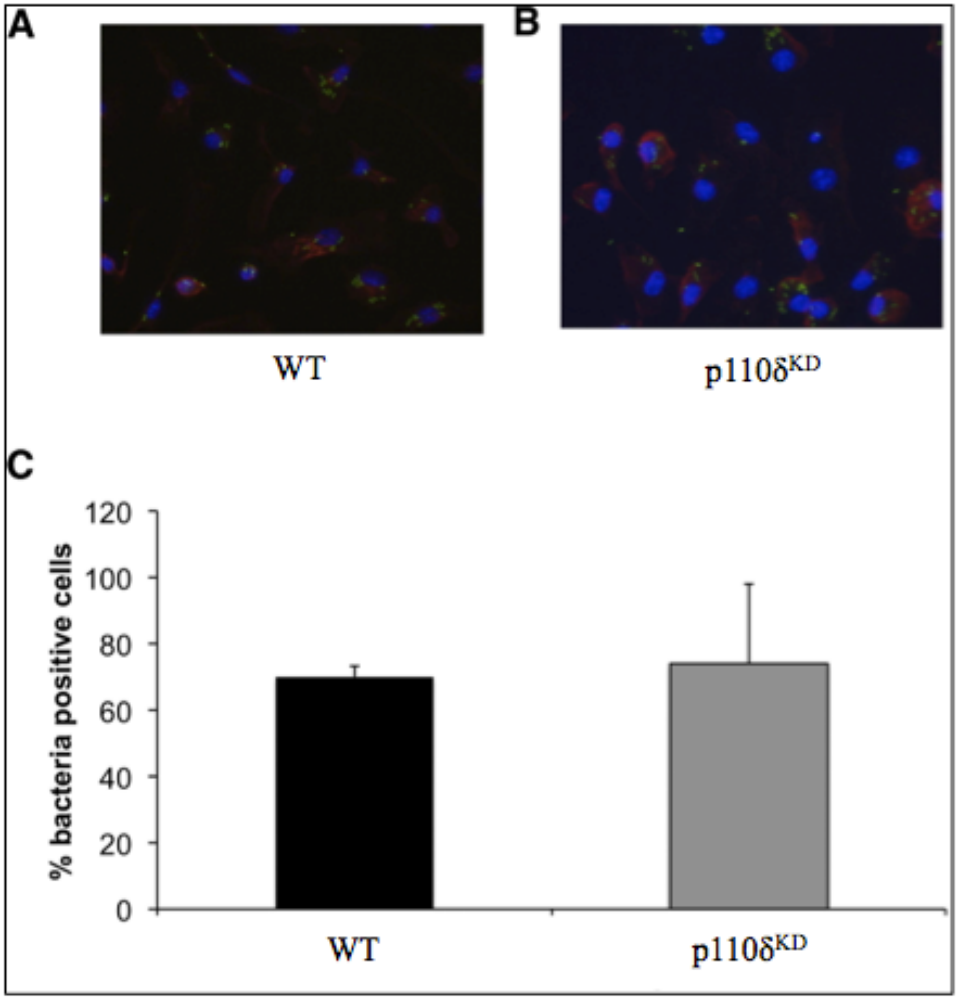
Supplemental Figure 2.3. PI3K p110δ^{KD} splenocytes secrete elevated levels TNF-α. Splenocytes from WT (black bars) and p110δ^{KD} (grey bars) mice were not stimulated (NS) or stimulated with LPS (1 μg/ml) or LPS (1 μg/ml) plus IFN-γ (10 ng/ml) for 24 hours. TNF-α levels were measured by ELISA in cell-free supernatants. Error bars represent mean ± SEM of three independent experiments (*, $p<0.05$ versus WT).



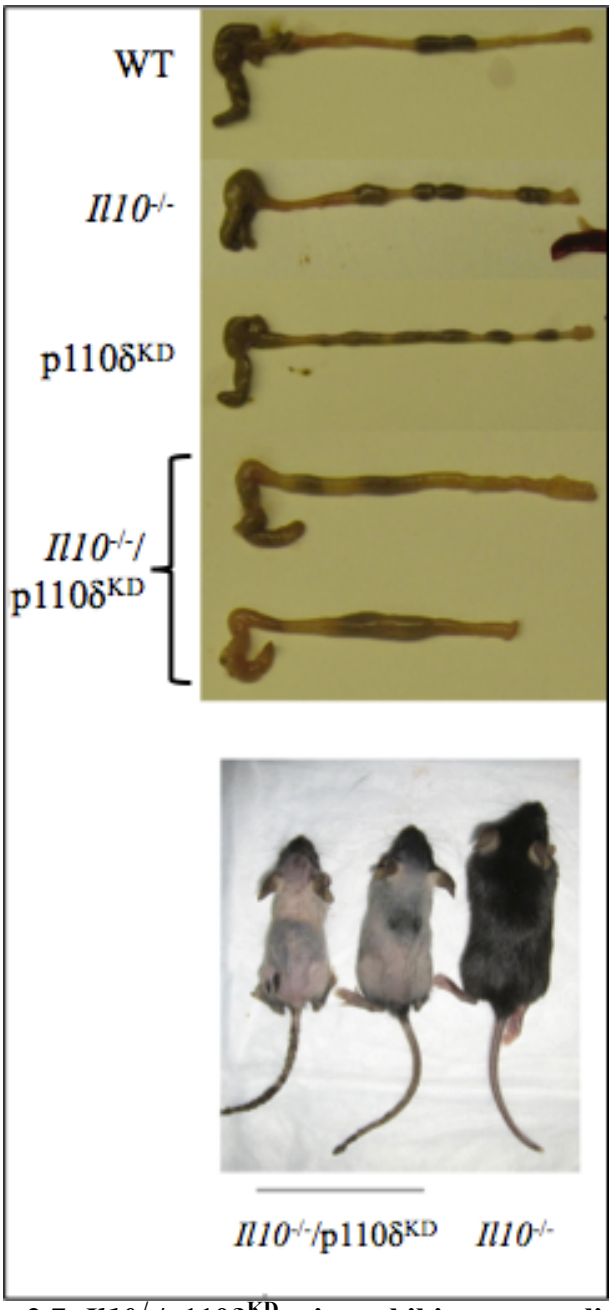
Supplemental Figure 2.4. WT and PI3K p110δ^{KD} BMDMs and LPMCs phenotypic and activation marker expression. BMDMs (A) or colonic LPMCs (B) from 8- to 10-week-old WT and p110δ^{KD} mice were isolated and labeled with antibodies against macrophage lineage and activation markers (CD11b, F4/80, CD80, CD86, MHCII, TLR4, and CD14), and analyzed by flow cytometry for each antibody (black histograms) and the isotype controls (grey histograms). Macrophages were gated using forward scatter and side scatter to exclude contaminating cells. Histograms are representative patterns from three independent experiments.



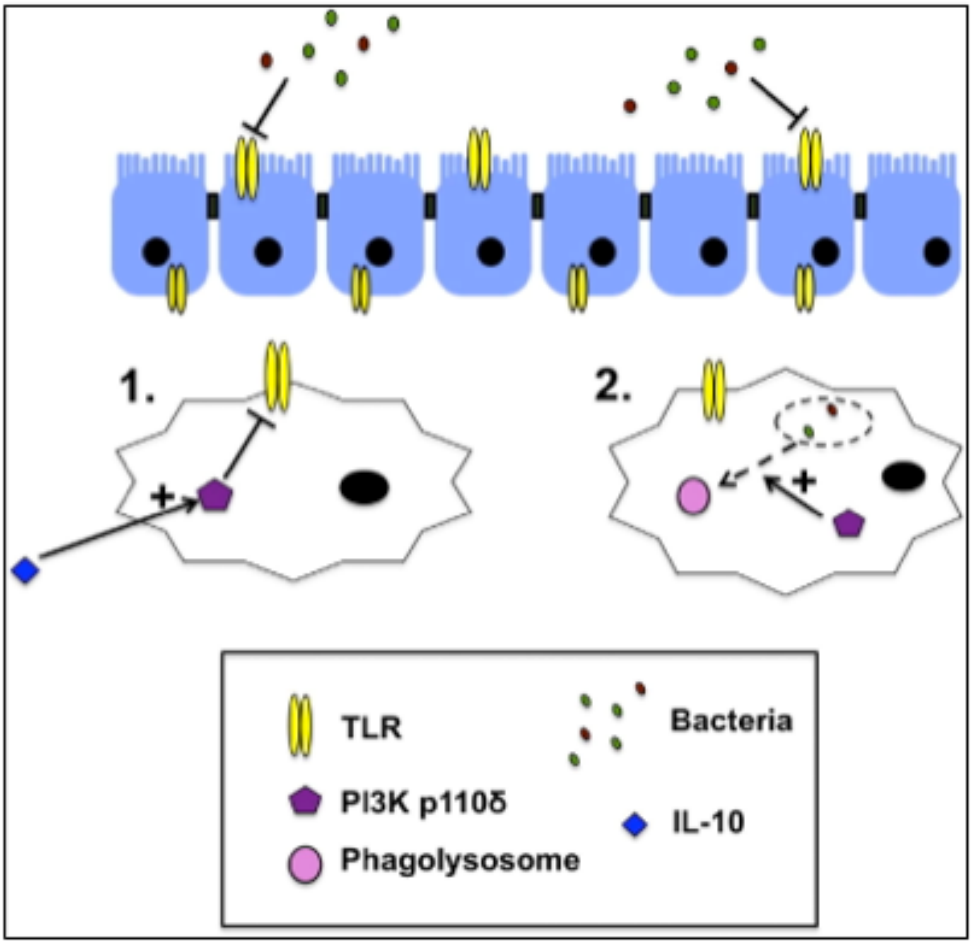
Supplemental Figure 2.5. Further characterization of signal transduction pathways in p110δ^{KD} macrophages. (A) BMDMs from WT and p110δ^{KD} mice were stimulated with LPS (100 ng/ml) for the indicated periods of time. Whole cell extracts were analyzed for p-p65 (NF-κB) and total p65 by Western blot. Results are representative of three independent experiments. (B) BMDMs from WT and *MyD88*$^{-/-}$ mice were stimulated with LPS (100 ng/ml) for 4 hours. Expression of p110δ mRNA (*Pik3cd*) was assessed by quantitative real-time RT- PCR at the indicated time points. Results are expressed as fold induction normalized to *β-actin*. Results represent mean ± SEM of three independent experiments (*, $p<0.05$ versus WT). BMDMs from WT and *MyD88*$^{-/-}$ mice were stimulated with LPS (100 ng/ml) for 16 hours. Whole cell extracts were analyzed for p110δ by western blot. Results are representative of three independent experiments.



Supplemental Figure 2.6. Bacterial uptake/phagocytosis is intact in p110δ^{KD} BMDMs. WT (A) and p110δ^{KD} (B) BMDMs were infected for one hour with K12 *E. coli*, extensively washed to remove adherent bacteria, permeabilized, and then immunostained with anti-*E. coli* LPS antibodies. Results are representative of three independent experiments. (C) Infected cells were quantitated in 12 fields from three independent experiments and expressed as percentage of cells infected per total number of cells counted.



Supplemental Figure 2.7. *Il10*$^{-/-}$/p110δ^{KD} mice exhibit severe colitis at an early age. (A) Colons from 1-month-old *Il10*$^{-/-}$/p110δ^{KD} are shorter and thicker relative to age-matched WT, *Il10*$^{-/-}$ and p110δ^{KD} mice. (B) *Il10*$^{-/-}$/p110δ^{KD} mice were significantly smaller in size relative to age-matched *Il10*$^{-/-}$ mice.



Supplemental Figure 2.8. Pathogenesis of IBD in p110δ^{KD} mice. (1) PI3K p110δ dampens TLR signaling, suggesting that dysregulation of innate immune responses contributes to the development of colitis in p110δ^{KD} mice. This is opposed to TLR function on intestinal epithelial cells, which has been postulated to be protective against inflammation (Abreu, 2010). Furthermore IL-10 contributes to induction of p110δ expression. (2) Defective bactericidal activity is demonstrated in p110δ^{KD} macrophages. The inability of p110δ^{KD} macrophages to efficiently kill and clear microbes may contribute to prolonged inflammatory responses.

CHAPTER 3

INNATE PI3K p110δ REGULATES TH1/TH17 DEVELOPMENT AND MICROBIOTA-DEPENDENT COLITIS[2]

3.1 Overview

The p110δ subunit of class I_A phosphoinositide 3-kinase modulates signaling in innate immune cells. We previously demonstrated that mice harboring a kinase-dead p110δ subunit ($p110\delta^{KD}$) develop spontaneous colitis. Macrophages contributed to the Th1/Th17 cytokine bias in $p110\delta^{KD}$ mice through increased IL-12 and IL-23 expression. Here, we show that the enteric microbiota is required for colitis development in germ free $p110\delta^{KD}$ mice. Colonic tissue and macrophages from $p110\delta^{KD}$ mice produced significantly less IL-10 compared to wild type (WT) mice. $p110\delta^{KD}$ APCs co-cultured with naïve $CD4^+$ antigen-specific T cells also produce significantly less IL-10 and induce more IFN-γ- and IL-17A-producing $CD4^+$ T cells compared to WT APCs. Illustrating the importance of APC-T cell interactions in colitis pathogenesis *in vivo*, *Rag1*$^{-/-}$/$p110\delta^{KD}$ mice develop mild colonic inflammation and produced more colonic IL-12p40 compared to *Rag1*$^{-/-}$ mice. However, $CD4^+CD45RB^{high/low}$ T cell recipient *Rag1*$^{-/-}$/$p110\delta^{KD}$ mice develop severe colitis with increased percentages of IFN-γ- and IL-17A-producing

[2] Erin C. Steinbach, Taku Kobayashi, Steven M. Russo, Shehzad Z. Sheikh, Gregory R. Gipson, Jennifer K. Uno, Yoshiyuki Mishima, Luke B. Borst, Bo Liu, Hans Herfarth, Jenny P. Y. Ting, R. Balfour Sartor, Scott E. Plevy. 2013. Innate PI3K p110δ regulates Th1/Th17 development and microbiota-dependent colitis. *Journal of Immunology* (In resubmission).

lamina propria $CD3^+CD4^+$ T cells compared to recipient *Rag1*$^{-/-}$ mice. Intestinal tissue samples from patients with Crohn's disease revealed significantly lower expression of *PIK3CD* compared to intestinal samples from non-IBD control subjects ($p<0.05$). *PIK3CD* expression inversely correlated with the ratio of *IL12B*:*IL10* expression. In conclusion, the PI3K subunit p110δ controls homeostatic APC-T cell interactions by altering the balance between IL-10 and IL-12p40. Defects in p110δ expression and/or function may underlie the pathogenesis of human IBD and lead to new therapeutic strategies.

3.2 Introduction

Genetic variants that confer susceptibility to the human inflammatory bowel diseases Crohn's disease (CD) and ulcerative colitis highlight the importance of innate immune interactions with the enteric microbiota in both initiating and controlling inflammation (Rioux et al., 2007). Commensal and pathogenic microorganisms are recognized through conserved molecular microbial patterns by pattern-recognition receptors, of which toll-like receptors (TLRs) form integral components (Abreu, 2010). Although mechanisms by which the host distinguishes commensal from pathogenic bacteria are not well defined, under normal conditions TLR signaling initiated by the enteric microbiota is protective (Rakoff-Nahoum et al., 2004). Phosphoinositide 3-kinases (PI3Ks) have emerged as important regulators of TLR signaling (Fukao and Koyasu, 2003; Liew et al., 2005). Class I_A PI3Ks have five different regulatory subunits and three p110 catalytic subunits: p110α and p110β are expressed ubiquitously in many tissues whereas p110δ is enriched in leukocytes (Koyasu, 2003). Agents that activate

macrophages to produce IL-12p40, the common subunit of the proximal inflammatory cytokines IL-12 and IL-23, also activate Class I_A PI3K (Fukao et al., 2002). Activation of PI3K in turn blocks the expression of IL-12p40 mRNA (*Il12b*) (Fukao et al., 2002). Although inflammatory responses are essential for eradicating pathogenic microbes, excessive/prolonged activation of innate immunity is harmful to the host. PI3K-mediated negative feedback of IL-12p40 is important to prevent excessive innate immune responses.

The clearest role of PI3K in chronic inflammation is described in a mouse harboring a kinase-dead p110δ catalytic subunit of PI3K ($p110\delta^{D910A/D910A}$ kinase-dead; here on referred to as "$p110\delta^{KD}$") (Okkenhaug et al., 2002). These mice demonstrate B and T cell defects including defective antigen receptor signaling and impaired humoral responses. Notably, the occurrence of spontaneous colitis was demonstrated in PI3K $p110\delta^{KD}$ mice (Uno et al., 2010). Expression of IL-12p40, Th1 and Th17 cytokines was described in the intestinal and systemic immune compartments. Consistent with a homeostatic role for p110δ in the intestine, wild type (WT) mice raised in a germ free (GF) environment markedly upregulated colonic p110δ (*Pik3cd*) expression when the enteric microbiota were introduced, but colitis-prone *Il10*$^{-/-}$ mice did not (Uno et al., 2010).

Given the role of the PI3K p110δ subunit in innate immune processes fundamental to the pathogenesis of IBD, we further characterized host-enteric microbiota and APC-T cell interactions in $p110\delta^{KD}$ mice. We describe a requirement for the enteric microbiota to drive intestinal inflammation in $p110\delta^{KD}$ mice. Microbial-innate immune interactions maintain homeostasis through regulation of both protective (IL-10) and

inflammatory (IL-12p40) cytokines, and p110δ is a central regulator of this balance. Furthermore, p110δ orchestrates innate immune cell regulation of pathogenic adaptive immune responses. Importantly, in human CD, decreased intestinal *PIK3CD* gene expression and an inverse correlation with intestinal *IL12B*:*IL10* ratios are demonstrated. Thus, p110δ appears to be a central homeostatic switch in the intestine, governing the critical balance between IL-12p40 and IL-10 induced by the microbiota that determines the subsequent T cell response. Counter to prevailing paradigms where p110δ inhibition is a strategic approach in inflammatory diseases (Maxwell et al., 2012; So et al., 2013), strategies to induce p110δ gene expression could be a potential therapeutic approach in human IBD.

3.3 Results

3.3.1 Presence of the enteric microbiota is necessary for the development of colitis in p110δ^{KD} mice

To determine whether the microbiota is necessary for the development of colitis, p110δ^{KD} mice were derived germ free (GF). GF p110δ^{KD} mice up to 30 weeks of age did not develop histological colitis (Fig. 3.1A, left, Supplemental Fig. 3.1A). Interestingly, GF p110δ^{KD} mice produced significantly less colonic IL-10 compared to GF WT mice. GF p110δ^{KD} and WT mice were then transitioned to conventionalized housing (CNV), and colonic inflammation was assessed at days 7 and 14 after transfer. Compared to GF to CNV WT mice, colons from GF to CNV p110δ^{KD} mice demonstrated increased colitis scores (Fig. 3.1A, middle, right, Supplemental Fig. 3.1A). However, GF to CNV p110δ^{KD} mice gained weight similarly to GF to CNV WT mice (Supplemental Fig. 3.1B).

Furthermore, colonic explants from day 7 and 14 GF to CNV p110δ^{KD} mice produced significantly less IL-10 (Fig. 3.1B, middle, right) compared to GF to CNV WT mice. At day 14, GF to CNV p110δ^{KD} mice produced significantly elevated IL-12p40 (Fig. 3.1C, middle, right) compared to GF to CNV WT mice. IL- 10 is important for the maintenance of intestinal homeostasis in part through inhibition of IL-12p40 (Sheikh and Plevy, 2010). The ratio of colonic IL-12p40 to IL-10 protein production therefore reflects the overall balance of intestinal pro- and anti-inflammatory cytokines. Indeed, colons from days 7 and 14 GF to CNV p110δ^{KD} mice demonstrated significantly higher ratios of IL-12p40:IL-10 production (Fig. 3.1F) compared to GF to CNV WT mice.

3.3.2 PI3K p110δ regulates macrophage production of IL-10 in response to TLR ligands

WT and p110δ^{KD} bone marrow-derived macrophages (BMDMs) were exposed to TLR agonists (LPS (TLR4), 5 ng/ml; Pam3CSK4 (TLR2/1), 5 ng/ml; Zymosan A (TLR2/6), 5 μg/ml), and cytokine production was measured. BMDMs from p110δ^{KD} mice produced less IL-10 in response to all TLR agonists tested compared to WT BMDMs (Fig. 3.2A). Additionally, p110δ^{KD} BMDMs exposed to TLR agonists produced significantly more IL-12p40 compared to WT BMDMs (Fig. 3.2B), in agreement with our previously published data (Uno et al., 2010). Consequently, the ratio of IL-12p40:IL-10 production in TLR ligand treated p110δ^{KD} BMDMs was consistently increased compared to WT BMDMs (Fig. 3.2C). LPS or Zymosan A stimulated $CD11b^{+}$ and $CD11c^{+}$ splenocytes from p110δ^{KD} mice also produced less IL-10 and more IL-12p40 than WT splenic cells (Supplemental Fig. 3.2).

To further validate these findings, LPS stimulated WT BMDMs were treated with p110 isoform-specific chemical inhibitors. LPS activated WT BMDMs demonstrated a dose-dependent decrease in IL-10 (Fig. 3.3A, left) and increase in IL-12p40 (Fig. 3.3B, left) production with specific chemical inhibition of p110δ (IC87114). PI3K p110α and p110β inhibition (PIK-90 and TGX-221, respectively) did not alter LPS stimulated IL-10 (Supplemental Fig. 3.3A,B, left) or IL-12p40 (Supplemental Fig. 3.3C,D, left) production in WT BMDMs, in agreement with reported results in dendritic cells (Aksoy et al., 2012). As a control, p110δ-specific inhibition of LPS-activated $p110\delta^{KD}$ BMDMs did not alter IL-10 or IL-12p40 expression (Fig. 3.3, right panels). However, in $p110\delta^{KD}$ BMDMs, p110β inhibition decreased IL-10 production (Supplemental Fig. 3.3B, right), and p110α or p110β inhibition modestly enhanced LPS induced IL-12p40 expression (Supplemental Fig. 3.3C,D, right) suggesting that in the absence of p110δ function other isoforms may have modest effects on IL-10/IL-12p40 regulation.

Chemical inhibition of p110δ in *Il10*$^{-/-}$ BMDMs led to a dose-dependent increase in IL-12p40 production (Fig. 3.3C, right), suggesting that p110δ-mediated decreases in IL-12p40 are in part independent of the inhibitory actions of IL-10. Relevant to mucosal innate inflammatory responses, WT $CD11b^{+}$ colonic lamina propria mononuclear cells (LPMCs) treated with heat killed *E. coli* (HKEC) demonstrated diminished *Il10* (Fig. 3.3D) and enhanced *Il12b* (Fig. 3.3E) expression in the presence of the p110δ-specific inhibitor. As a control, expression of neither cytokine was altered in $CD11b^{+}$ colonic LPMCs from $p110\delta^{KD}$ mice treated with the p110δ-specific inhibitor.

3.3.3 mTOR and GSK-3β act downstream of p110δ in macrophages to regulate cytokine production

PI3Ks modulate multiple downstream signaling pathways, of which mammalian target of rapamycin (mTOR) and glycogen synthase kinase-3β (GSK-3β) have been previously shown to regulate cytokine secretion in macrophages (Wang et al., 2011a). BMDMs from WT and p110δ^{KD} mice were exposed to mTOR or GSK-3β inhibitors (rapamycin or SB-216763, respectively) prior to LPS stimulation. Rapamycin decreased IL-10 (Fig. 3.4A,C) and increased IL-12p40 (Fig. 3.4B,D) protein and mRNA expression in WT and p110δ^{KD} TLR stimulated BMDMs. These same trends were observed in WT and p110δ^{KD} CD11b^{+} and CD11c^{+} splenocytes (Supplemental Fig. 3.4A-D). Inhibition of GSK-3β in p110δ^{KD} BMDMs and splenocytes increased IL-10 protein (Fig. 3.4E, Supplemental Fig. 3.4E,F) and mRNA expression (Fig. 3.4G) and decreased IL-12p40 protein (Fig. 3.4F, Supplemental Fig. 3.4G) and mRNA (Fig. 3.4H) expression. Hence, mTOR and GSK-3β are downstream of p110δ and are relevant for regulation of IL-10 and IL-12p40.

3.3.4 Antigen presenting cell p110δ regulates T cell cytokine production

To begin to determine whether resident antigen presenting cells (APCs) regulate intestinal T cell phenotype and function, T cell cytokines and lineage markers were measured in GF to CNV p110δ^{KD} colons. Colonic *Tbx21* and *Rorc* transcripts (Fig. 3.5A,B, middle, right), the hallmark transcription factors of Th1 and Th17 cells, respectively, were increased in GF to CNV p110δ^{KD} mice at days 7 and 14 post-transition compared to matched GF to CNV WT mice. Likewise, increased *Ifng* and *Il17a*

transcripts (Fig. 3.5C,D, middle, right) were detected in cecal tissue from days 7 and 14 GF to CNV p110δ^{KD} compared to matched GF to CNV WT mice.

Consequently, we next investigated whether T cell dependent IL-12p40 and IL-10 expression was altered in p110δ^{KD} APCs. Splenic CD4^{+}CD62L^{+} T cells from WT mice were cultured with either WT or p110δ^{KD} splenic APCs pulsed with cecal bacterial lysate (CBL). CBL pulsed p110δ^{KD} APCs cultured with naïve WT CD4^{+} T cells produced decreased levels of IL-10 (Fig. 3.5E, middle) and increased levels of IL-12p40 (Fig. 3.5F, middle) compared to WT APCs. WT and p110δ^{KD} APCs cultured with *Il10*$^{-/-}$ CD4^{+} T cells demonstrate that IL-10 expression is largely derived from APCs (Fig. 3.5E, right). As expected, CBL pulsed p110δ^{KD} APCs also produced significantly less IL-10 (Fig. 3.5E, left) and more IL-12p40 (Fig. 3.5F, left) compared to WT APCs in the absence of CD4^{+} T cells.

Next, to study antigen-specific APC-T cell interactions, splenic CD4^{+} T cells from OVA specific transgenic T cell receptor mice (OT-II mice) were co-cultured with OVA pulsed and LPS activated WT and p110δ^{KD} APCs. OVA-loaded p110δ^{KD} APCs induced significantly more IFN-γ- (Fig. 3.5H,I) and IL-17A-producing (Fig. 3.5H,J) CD4^{+} T cells compared to OVA-loaded WT APCs (Fig. 3.5G,I,J). However, WT and p110δ^{KD} APCs induced the same amount of T cell proliferation (Supplemental Fig. 3.5A-C). These data suggest that cytokine production by p110δ^{KD} APCs directs differentiation of antigen specific Th1 and Th17 CD4^{+} T cells.

These results suggest a model where defective p110δ signaling, through regulation of IL-10 and IL-12p40, leads to inflammatory effector T cell development. To test this model *in vivo,* we generated *Rag1*$^{-/-}$/p110δ^{KD} mice (RKO/δ^{KD}). Interestingly,

colitis was present but attenuated in the absence of an adaptive immune system (Fig. 3.6A,B). Colonic explant cultures from RKO/δ^{KD} mice produced significantly decreased IL-10 (Fig. 3.6C) and increased IL-12p40 (Fig. 3.6D) compared to colonic tissue explant cultures from *Rag1*$^{-/-}$ mice.

It was previously reported that p110δ^{KD} CD4^{+} T cells adoptively transferred into *Rag1*$^{-/-}$ recipients induce colitis owing to impaired T regulatory cell function (Patton et al., 2006). To study how p110δ inactivation in non-lymphocyte populations affects T cell differentiation, admixed WT CD4^{+}CD45RB^{high} and CD4^{+}CD45RB^{low} T cells were adoptively transferred into *Rag1*$^{-/-}$ and RKO/δ^{KD} mice (CD45RB recipients), and recipients monitored for colitis development. Total body weight of recipients was recorded until the experiment was terminated at day 24 due to severe clinical manifestations in the CD45RB RKO/δ^{KD} recipients. Clinical colitis activity scores (Fig. 3.7A) and quantitative colonic histologic analysis (Fig. 3.7B,C) from CD45RB recipient RKO/δ^{KD} mice were increased compared to the respective recipient *Rag1*$^{-/-}$ mice. Colonic IL-10 production was significantly lower (Fig. 3.7D) and IL-12p40 production higher (Fig. 3.7E) in CD45RB recipient RKO/δ^{KD} mice compared to CD45RB recipient *Rag1*$^{-/-}$ mice. Consequently, ratios of colonic IL-12p40:IL-10 production from CD45RB recipient RKO/δ^{KD} mice were significantly higher (Fig. 3.7F) than ratios from recipient *Rag1*$^{-/-}$ mice. Furthermore, more IFN-γ-producing (Fig. 3.7G), but not IL-17A-producing (Fig. 3.7H), CD4^{+} T cells were isolated from mesenteric lymph nodes (MLNs) of CD45RB recipient RKO/δ^{KD} mice compared to recipient *Rag1*$^{-/-}$ mice. Finally, a greater percentage of lamina propria CD3^{+}CD4^{+} T cells from recipient RKO/δ^{KD} mice produced IFN-γ (Fig. 3.7I) and IL-17A (Fig. 3.7J), compared to recipient *Rag1*$^{-/-}$ mice.

3.3.5 Intestinal PIK3CD expression correlates with IL12B:IL10 ratios from patients with CD

Expression of p110δ (*PIK3CD*), IL-12p40 (*IL12B*) and IL-10 (*IL10*) mRNA was determined in human intestinal tissue from control subjects without intestinal inflammation and patients with CD or UC. Significantly higher levels of *PIK3CD* mRNA were detected in non-inflamed intestinal samples from control subjects compared to tissue from patients with CD, but not UC (Fig. 3.8A). Paired macroscopically inflamed and non-inflamed intestinal resections were obtained from 14 CD patients. There was lower expression of *PIK3CD* in inflamed intestinal tissues compared to non-inflamed tissues obtained from the same patient (Fig. 3.8B,C). Furthermore, ratios of *IL12B*:*IL10* expression from individual CD patients demonstrated a strong and statistically significant inverse correlation with *PIK3CD* expression (Fig. 3.8D).

3.4 Discussion

We previously described the development of spontaneously occurring Th1 and Th17 mediated colitis in p110δ^{KD} (Uno et al., 2010). In the present series of experiments, we further elucidate intestinal host-microbial and APC-T cell interactions mediated by p110δ. Colitis in p110δ^{KD} mice is dependent on host responses to the enteric microbiota, as has been described in other murine colitis models (Nell et al., 2010). In the absence of the enteric microbiota, p110δ^{KD} mice did not develop intestinal inflammation, whereas after reconstitution with commensal enteric microbiota, colons from p110δ^{KD} mice demonstrated histological inflammation and impaired IL-10 and increased IL-12p40

production (Fig. 1). Consequently, altered IL-10 and IL-12p40 production by p110δ^{KD} APCs in response to microbial products and cognate interactions with T cells orchestrate pathogenic adaptive immune responses contributing to intestinal inflammation.

Class I_A PI3Ks regulate macrophage and DC responses to bacteria (Fukao and Koyasu, 2003). Taken together, our results and those of others (Aksoy et al., 2012) elucidate a model where p110δ is an intracellular integrator of environmental signals that is involved in the restoration of inflammatory responses to homeostasis, mediated in part by IL-10. Regulation of IL-10 expression involves both PI3K-dependent and -independent pathways. Moreover, IL-10 signaling in macrophages has been shown to activate the PI3K pathway (Antoniv and Ivashkiv, 2011). Indeed, we have shown that colonic p110δ expression is attenuated in colitis-prone *Il10*$^{-/-}$ mice, suggesting that IL-10 regulation of IL-12p40 occurs in part via induction of p110δ (Uno et al., 2010). Aksoy *et al.* recently demonstrated that p110δ signaling in dendritic cells dampens responses to LPS by sequestering TLR4 signaling components and facilitating the switch from toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP)/MyD88-dependent inflammatory cytokine production (IL-12, IL-6, TNF-α) to TIR domain containing adaptor inducing interferon-β (TRIF)-related adaptor molecule (TRAM)/TRIF-dependent anti-inflammatory cytokine production (IFN-β, IL-10) (Aksoy et al., 2012). It is possible that LPS induced p110δ signaling in macrophages also facilitates the switch to TRAM-TRIF signaling, leading to the enhanced production of IL-10 and IFN-β. Indeed, this agrees with our finding that p110δ^{KD} macrophages produce less LPS induced IL-10. However, we have previously shown that CD11b^{+} LPMCs from *Trif*$^{-/-}$ mice produce higher levels of basal and bacterially stimulated IL-10 compared to

WT mice (Kobayashi et al., 2012; Onyiah et al., 2013). Conversely, BMDMs from *Trif*$^{-/-}$ mice produce less LPS-induced IL-10 compared to WT mice (Boonstra et al., 2006). These findings suggest that the TRIF pathway negatively regulates IL-10 production uniquely in intestinal macrophages. Furthermore, CD11b^{+} LPMCs produce high levels of IL-10 in GF conditions (Kobayashi et al., 2012), suggesting that microbial signals are not necessary for driving constitutive expression of IL-10. However, TLR signaling, perhaps through the recognition of endogenous ligands, remains vital for IL-10 production in intestinal macrophages, as HKEC stimulated *MyD88*$^{-/-}$ CD11b^{+} LPMCs do not produce detectable levels of IL-10 (Kobayashi et al., 2012). While we did not investigate IFN-β expression as the prototype TRAM-TRIF regulated gene, it is possible that IFN-β and IL-10 demonstrate differential regulation in CD11b^{+} LPMCs, in contrast to the recent study demonstrating convergent regulation in LPS stimulated bone marrow-derived DCs (BMDCs) (Aksoy et al., 2012). Indeed, Kaiser *et al.* demonstrated cell type-specific differences in IL-10 and IFN-β production in response to LPS; BMDMs and splenic macrophages did not make detectable amounts of IFN-β but did make significant amounts of IL-10 in response to LPS, whereas BMDCs induced both IFN-β and IL-10 (Kaiser et al., 2009), suggesting that macrophages utilize distinct pathways to regulate IFN-β and IL-10. Thus, further studies are necessary to elucidate specific intestinal macrophage signaling pathways required for IL-10 production.

In macrophages, mTOR and GSK-3β are central regulators of IL-12p40 and IL-10 downstream of PI3K. Bacterial products induce MyD88-dependent PI3K activation, leading to phosphorylation of its downstream effector molecule Akt. Akt inactivates tuberous sclerosis complex (TSC), a negative regulator of mTOR (Weichhart et al.,

2008). Both Akt and PI3K dependent mTOR activation modulate IL-12p40 and IL-10 production by suppressing GSK-3β activity (Wang et al., 2011a; Zhang et al., 2006). GSK-3β constitutively represses IL-10 by blocking cAMP response element-binding (CREB) binding to and activation of the *Il10* promoter (Martin et al., 2005). PI3K- and mTOR-mediated inhibition of GSK-3β thus releases IL-10 from suppression by GSK-3β (Martin et al., 2005). Interestingly, mTOR activation targets were not altered in LPS stimulated p110δ^{KD} BMDCs compared to WT BMDCs, suggesting that compensatory activation pathways sustain mTOR signaling in p110δ^{KD} BMDCs (Aksoy et al., 2012). Indeed, mTOR is activated through many pathways, including cellular energy sensing and Wnt signaling (Weichhart and Saemann, 2009). Additionally, the balance of IL-10 and IL-12p40 production, as well as other cytokines, is regulated by MAPK signaling downstream of TLR signaling (Bode et al., 2012). Furthermore, other cytokines such as TNF-α are affected by p110δ, which regulates tubule fusion in TNF-α containing vesicles bound for secretion (Low et al., 2010). Indeed, we have previously shown dysregulation of IL-12p70, IL-23, and NO in TLR-activated p110δ^{KD} macrophages (Uno et al., 2010). Thus, p110δ may regulate cytokine secretion in multiple ways. Here we showed that inhibition of GSK-3β rescues LPS induced IL-10 production in p110δ^{KD} BMDMs (Fig. 4). Our results therefore suggest that GSK- 3β activity may be a therapeutic target in IBDs to induce IL-10 production. Indeed, GSK-3β inhibition has previously been shown to ameliorate colitis in mice (Hofmann et al., 2010).

PI3K p110δ^{KD} B and T cells demonstrate impaired proliferative signaling through the B cell and T cell receptors (Okkenhaug et al., 2002). PI3K p110δ^{KD} mice demonstrate impaired intrinsic T regulatory cell function, and p110δ^{KD} CD4^{+}CD45RB^{low} cells co-

transferred with colitogenic WT $CD4^+CD45RB^{high}$ cells did not protect *Rag1*$^{-/-}$ mice from T cell-mediated colitis (Patton et al., 2006). To determine how APC p110δ influences T cell subset differentiation and colitis development, APC-$CD4^+$ T cell co-culture experiments were performed (Fig. 5). $CD4^+$ T cells induced greater production of IL-12p40 by APCs, while APCs from p110δ^{KD} mice induced more antigen-specific IFN-γ- and IL-17A-producing T cells. Because only WT $CD4^+$ T cells were used in co-culture with APCs from both WT and p110δ^{KD} mice, T cell phenotype can be attributed to the defect in p110δ^{KD} APCs. As an *in vivo* correlate, *Rag1*$^{-/-}$ and RKO/δ^{KD} mice were reconstituted with admixed WT $CD4^+CD45RB^{high}$ and $CD45RB^{low}$ T cells (Fig. 7). Compared to respective recipient *Rag1*$^{-/-}$ mice, recipient RKO/δ^{KD} mice demonstrated significantly increased clinical and histology scores. More IFN-γ-producing T cells were isolated from MLNs and colonic lamina propria of recipient RKO/δ^{KD} mice compared to recipient *Rag1*$^{-/-}$ mice. IL-17A-producing $CD4^+$ T cells are rarely found in MLNs and other secondary lymphoid tissues but are found in abundant quantities at mucosal surfaces (Atarashi et al., 2008). Indeed, recipient RKO/δ^{KD} mice contained significantly higher percentages of IL-17A-producing T cells in the colonic lamina propria compared to the respective recipient *Rag1*$^{-/-}$ mice. We previously showed that bacterially stimulated p110δ^{KD} macrophages produce significantly more IL-23, a cytokine necessary for the differentiation and maintenance of Th17 cells (Uno et al., 2010).

While we extensively studied the development of pathogenic $CD4^+$ T cells in CD45RB recipient mice, we did not study Treg cell differentiation and function in these mice. During GF to CNV transition, colons from p110 δ^{KD} mice demonstrated significantly increased transcription of *Foxp3*, correlating with increased inflammation,

compared to colons from WT mice (data not shown). However, this does not rule out functional defects in p110 δ^{KD} Treg cells. It is entirely possible, and in fact likely, akin to human IBD pathogenesis, that innate and adaptive immune defects interact to drive the colitis phenotype. Thus it would be appropriate to expand our current studies in the future to include evaluation of Treg cell development and maintenance by p110 δ^{KD} non-lymphocyte populations in the intestines.

Interestingly, in the absence of T cells, RKO/δ^{KD} mice developed mild histopathologic colonic inflammation. The development of mild colonic inflammation in RKO/δ^{KD} mice could be explained by the presence non-hepaticus *Helicobacter* species in our mouse colony (data not shown). The ability of *H. hepaticus* to induce innate immune driven colonic inflammation in the absence of adaptive immune cells has been well described (Kullberg et al., 2001; Kullberg et al., 1998).

Recently, p110δ inhibition has been targeted for the treatment of chronic rejection of tissue transplants, systemic lupus erythematosus, and certain lymphoid cell malignancies (Maxwell et al., 2012; So et al., 2013; Ying et al., 2012). While preliminary clinical results are promising, this study highlights a potentially untoward consequence of p110δ inhibition – enhanced intestinal and innate inflammatory processes initiated by APCs.

3.5 Materials and Methods

Mice. All mice were maintained on a C57BL/6 background in conventional or GF housing. PI3K p110$\delta^{D910A/D910A}$ (p110δ^{KD}) mice were previously obtained from Dr. Bart Vanhaesebroeck (Queen Mary University of London, London, England). GF p110δ^{KD}

mice were Caesarian derived as previously described (MacDonald and Carter, 1978) and were maintained according to standard techniques in the University of North Carolina National Gnotobiotic Resource Center. OT-II (C57BL/6- Tg(TcraTcrb)425Cbn/J) male mice were provided by JPY Ting (UNC, Chapel Hill). All animal experiments were in compliance with protocols approved by the International Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Reagents. LPS from *Escherichia coli* was purchased from Invivogen (San Diego, CA). Zymosan A from *Saccharomyces cerevisiae* was purchased from Sigma (St. Louis, MO). Synthetic bacterial lipopeptide Pam3CSK4 (sBLP) was purchased from EMC Microcollections (Germany). Inhibitors IC87114, Rapamycin, and SB-216763 were purchased from Selleck Chemicals (Houston, TX). Cecal bacterial lysates (CBL) C57BL/6 mice were prepared as described previously (Cong et al., 2002). The peptide corresponding to residues 323-339 of ovalbumin (OVA) was purchased from AnaSpec (Fremont, CA).

Colonic Tissue Explant Culture. Colonic tissue explant cultures were performed as described previously (Hegazi et al., 2005).

Histology. Slides were prepared for H&E staining and a pathologist (LBB) blinded to the study groups performed histological analysis using established criteria for p110δ^{KD} mice (Uno et al., 2010). In T cell adoptive transfer studies, the following scoring system was utilized: Tissue changes were categorized into inflammatory and epithelial changes and

graded for severity (0= normal, 1= mild, 2= moderate, 3= marked and 4= severe); the sum of the two grades comprises the histopathology score. For inflammation, a score of 0 (normal) signified rare small lymphoplasmacytic aggregates confined to the lamina propria; scores of 1 (mild) and 2 (moderate) represented increasing numbers of multifocal inflammatory aggregates which were predominantly confined to the lamina propria, with occasional submucosal infiltration; a score of 3 (marked) was assigned if inflammatory infiltrates frequently extended into the submucosa and muscular layers; a score of 4 (severe) was designated if transmural inflammation was common. Epithelial changes, characterized by hypertrophy, were scored (0-4) with increasing severity and prevalence of the observed change.

Cell Isolation. Bone marrow-derived macrophages (BMDMs) were cultured as described previously (Xiong et al., 2004). Splenocytes were isolated as described (Murali-Krishna et al., 1998) and further separated into $CD11c^+$ and $CD11c^-/Cd11b^+$ cells by MACS with anti-CD11c and anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA). Lamina propria mononuclear cells (LPMCs) were isolated from mouse colons as described previously (Kamada et al., 2005). LPMCs were further separated into $CD11b^+$ and $CD11b^-$ cells by MACS with anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA).

Cell culture experiments. BMDMs or splenocytes were cultured at $1x10^6$/ml in the presence of LPS (1 ng/ml), Zymosan A (10 μg/ml), sBLP (20 ng/ml) or PBS, and supernatants were harvested after 4 hours or 24 hours (BMDMs or splenocytes, respectively). Inhibitors IC87114 (0.1 or 1 μM), Rapamycin (1 or 10 μM), SB-216763 (1

or 10 μM), or DMSO were added 1 hour prior to stimulation with LPS or Zymosan A. $CD11b^+$ LPMCs were treated with IC87114 (10 μM) for 30 minutes prior to exposure to heat killed *E. coli* (HKEC, MOI=100) for 3 hours. Total RNA was assessed for *Il12b* and *Il10* expression by quantitative PCR.

Quantitative RT-PCR. Quantitative real time RT-PCR was performed on total RNA as described (Hegazi et al., 2005). Murine primer sequences will be provided upon request. The following human primer sequences were used: *PIK3CD,* forward, 5'-GCGCCGGGACGATAAGGAGTC-3', reverse, 5'-GCTGCCCACAGGGGTCTACCT-3'; *IL10,* forward, 5'-GCCTAACATGCTTCGAGATC-3', reverse, 5'-TGATGTGTGGGTCTTGGTTC-3'; *IL12B,* forward, 5'-GCTCTTGCCCTGGACCTGAACGC-3', reverse, 5'-CGTAGAATTGGATTGGTATCCGG-3'; *GAPDH,* forward, 5'-GGTGAAGGTCGGAGTCAACGGA-3', reverse, 5'-GAGGGATCTCGCTCCTGGAAGA-3'.

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

APC-$CD4^+$ T cell Co-culture. Splenic APCs from WT or $p110\delta^{KD}$ mice were isolated by negative selection using CD90.2 microbeads (Miltenyi Biotec, Auburn, CA). Splenic APCs were incubated overnight with CBL (50 ng/ml), and, after washing several times to remove extracellular antigen, APCs were co-cultured with negatively-selected $CD4^+$ T

cells (CD8α/B220/MHC II microbeads, Miltenyi Biotec, Auburn, CA) from WT or *Il10*$^{-/-}$ mice at a 3:2 ratio (APCs:T cells) for 72 hours. For antigen specific studies, APCs were incubated overnight with LPS (10 ng/ml) and OVA peptide (323-339, 5 μM) (Cong et al., 2002). After washing to remove extracellular antigen, APCs were co-cultured with negatively-selected CD4^{+}CD62L^{+} T cells (CD8α/B220/MHC II microbeads, Miltenyi Biotec, Auburn, CA) from mice expressing a transgenic TCR that recognizes OVA epitope residues 323-339 (OT-II mice) at a 3:2 ratio (APCs:T cells) for 72 hours. CD4^{+} T cells were analyzed for intracellular cytokine expression (IFN-γ and IL-17A) by flow cytometry.

Flow Cytometry. CD4^{+} T cells were stimulated for 4 hours with PMA (100 ng/ml) and ionomycin (1 μg/ml) in the presence of GolgiStop™ (BD Biosciences, San Jose, CA). Cells were then washed and stained with APC-conjugated anti-CD3 (Clone 17A2, eBioscience, San Diego, CA). After fixing and permeabilizing the cells with BD Cytofix/Cytoperm™ (BD Biosciences, San Jose, CA), staining for intracellular PE-conjugated anti-IFN-γ (Clone XMG1.2, eBioscience, San Diego, CA) and FITC-conjugated anti-IL-17A (Clone eBio17B7, eBioscience, San Diego, CA) was performed. Flow cytometry samples were run on a CyAn™ ADP Analyzer (Beckman Coulter, Brea, CA) and analyzed using Summit v4.3 (Beckman Coulter, Brea, CA).

CD4^{+}CD45RB$^{high/low}$ T cell adoptive transfer colitis. T cell mediated colitis was induced in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$/p110δ^{KD} (RKO/δ^{KD}) mice at 8 weeks of age as described previously (Read and Powrie, 2001). CD4^{+} T cells were isolated by negative selection

(CD8α/B220/MHC II microbeads, Miltenyi Biotec, Auburn, CA) and stained with FITC-conjugated anti-CD4 (Clone GK1.5, eBioscience, San Diego, CA) and PE-conjugated anti-CD45RB (Clone 16A, BD Pharmingen, San Jose, CA). $CD4^+$ T cells were sorted into $CD45RB^{high}$ and $CD45RB^{low}$ populations using a MoFlo™ XDP Cell Sorter (Beckman Coulter, Brea, CA). Mice were i.p. injected with 4×10^5 $CD4^+CD45RB^{high}$ cells admixed with 2×10^5 $CD4^+CD45RB^{low}$ cells as described (Read and Powrie, 2001). Clinical scores were assigned as described (Maillard et al., 2007).

Human intestinal samples. Intestinal samples were obtained from surgical resections from CD patients and subjects requiring surgical intervention for non-inflammatory conditions (e.g., colon cancer). In CD patients, when available, paired inflamed and non-inflamed intestinal segments, as determined by gross appearance by the processing pathologist, were obtained for analysis. The University of North Carolina Institutional Review Board approved collection of de-identified samples, and written informed consent was obtained from all patients.

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

3.6 Figures

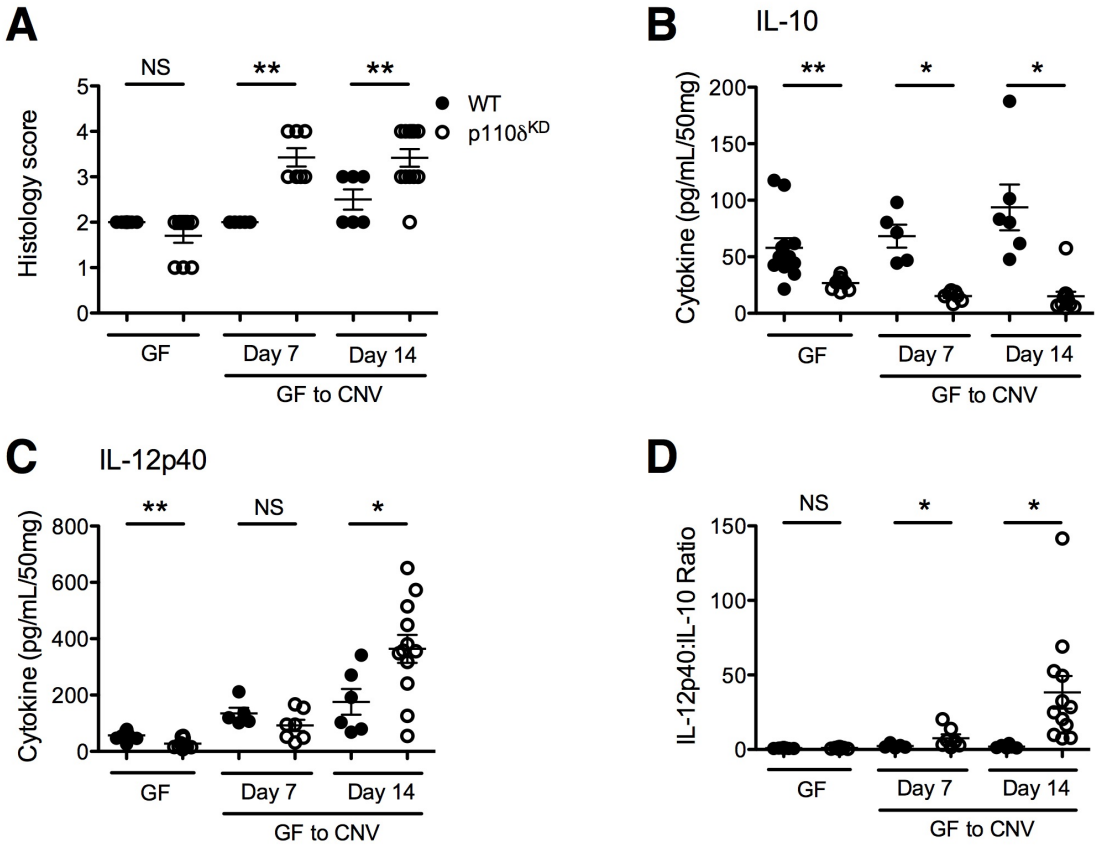


Figure 3.1. The enteric microbiota is required for the development of colitis in p110δ^{KD} mice. Germ-free (GF) p110δ^{KD} (n = 10) and age-matched WT (n = 12) mice were monitored for colitis up to 30 weeks of age. Additionally, GF WT and p110δ^{KD} mice were transferred to CNV housing and monitored for colitis at days 7 (n = 5 and 7, respectively) and 14 (n = 6 and 12, respectively) after transfer. (A) H&E slides of colonic tissue were scored for colitis severity using criteria described in the methods by a pathologist (LBB) blinded to the experimental groups. Error bars represent mean ± SEM (NS, not significant; **, $p<0.005$). (B, C) Colonic tissue explants were incubated in media for 24 hours. Supernatants were collected and assayed for IL-10 (B) and IL-12p40 (C) production by ELISA, and are expressed as the amount of cytokine (pg/ml) per 50 mg colonic tissue weight. Error bars represent mean ± SEM (NS, not significant; *, $p<0.05$; **, $p<0.005$). (D) IL-12p40 and IL-10 protein levels from colonic tissue explant cultures in individual mice were used to determine the ratio of IL-12p40 to IL-10 production. Error bars represent mean ± SEM (NS, not significant; *, $p<0.05$).

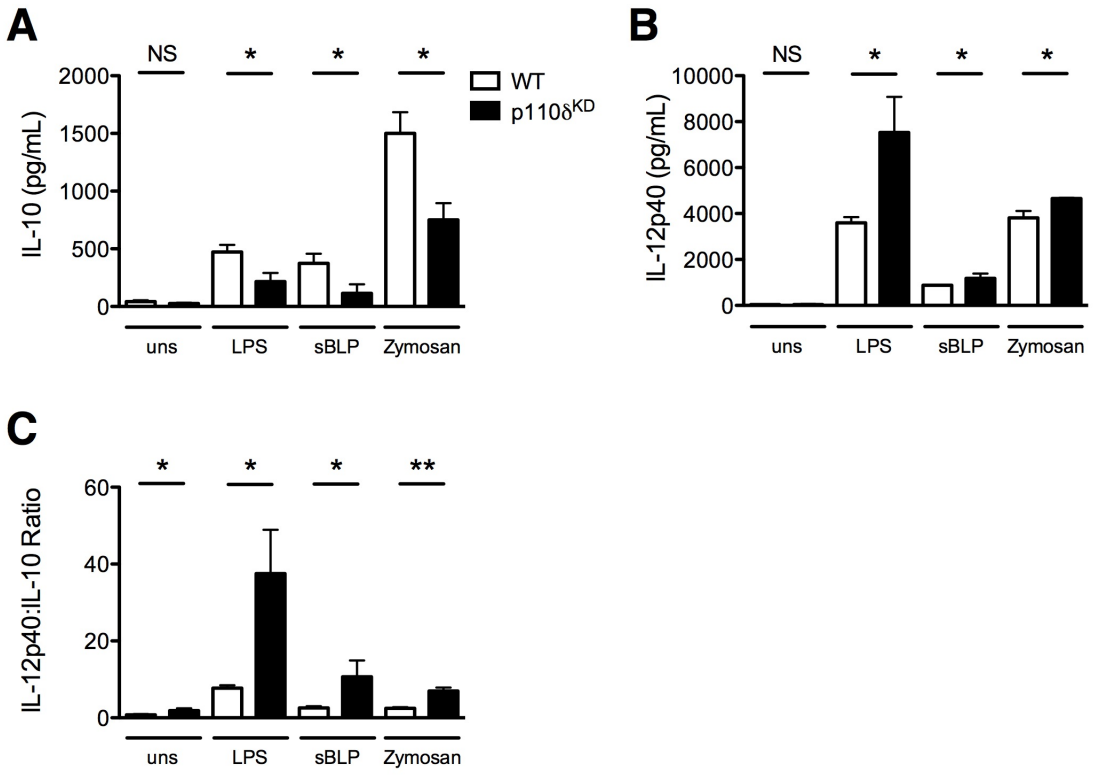


Figure 3.2. Defective p110δ activity alters macrophage production of IL-10 and IL-12p40 in response to bacterial products. BMDMs were stimulated with LPS (5 ng/ml), sBLP (5 ng/ml) or Zymosan A (5 μg/ml) for 8 hours. Supernatants were collected and assayed for IL-10 (A) and IL-12p40 (B) production by ELISA. Error bars represent mean ± SEM from three independent experiments (NS, not significant; *, $p<0.05$). (C) The ratio of IL-12p40 to IL-10 from individual experiments was calculated. Error bars represent mean ± SEM from three independent experiments (*, $p<0.05$; **, $p<0.005$).

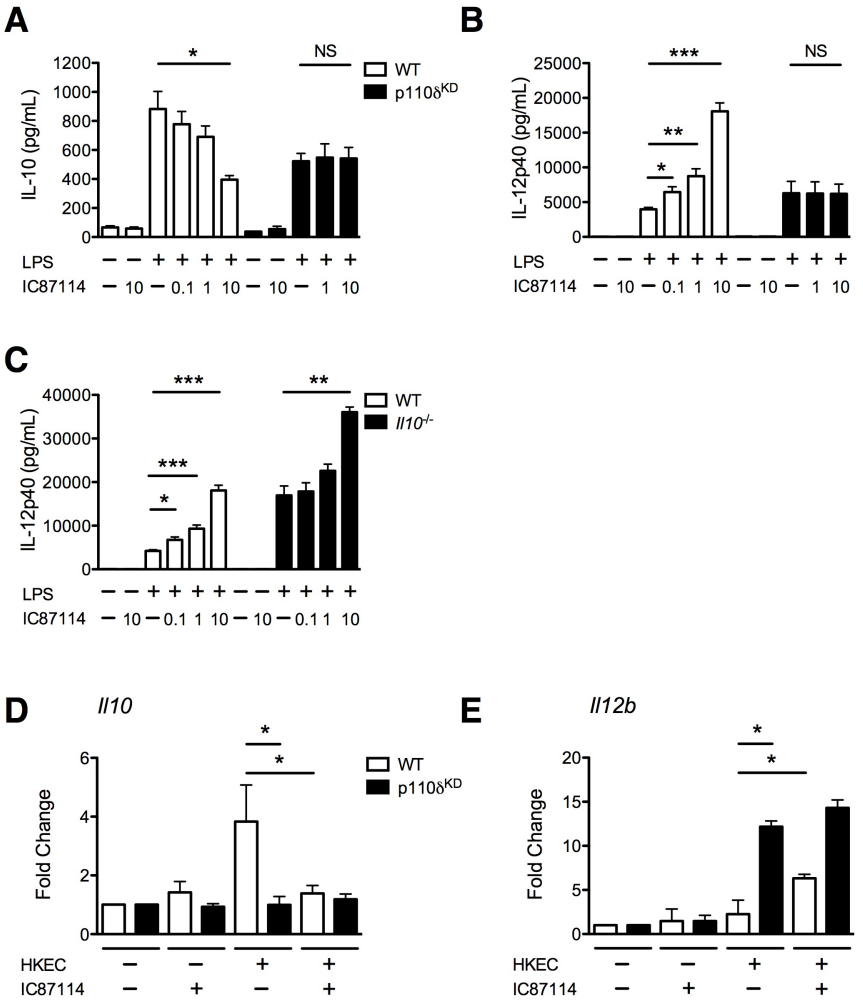


Figure 3.3. A p110δ-specific inhibitor decreases IL-10 and augments IL-12p40 production in WT macrophages stimulated with bacterial products. (A-C) WT, p110δ^{KD} and *Il10*$^{-/-}$ BMDMs were cultured with a p110δ-specific inhibitor (IC87114, 0.1, 1 or 10 μM) for 1 hour prior to stimulation with LPS (1 ng/ml). Supernatants from WT and p110δ^{KD} BMDMs were collected after 8 hours of culture and assayed for IL-10 (A) and IL-12p40 (B) production by ELISA. Error bars represent mean ± SEM from three independent experiments (NS, not significant; *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$). (C) Supernatants from WT and *Il10*$^{-/-}$ BMDMs were collected after 8 hours of culture and assayed for IL-12p40. Error bars represent mean ± SEM from three independent experiments (*, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$). (D, E) WT and p110δ^{KD} CD11b^{+} LPMCs were incubated with a p110δ-specific inhibitor (IC87114, 10 μM) 1 hour prior to stimulation with HKEC (MOI=100) for 3 hours. Quantitative real time RT-PCR was performed in duplicate for *Il10* (D) and *Il12b* (E) expression levels normalized to *β-actin* expression and calculated as fold induction over unstimulated cells. Error bars represent mean ± SEM for three independent experiments (*, $p<0.05$).

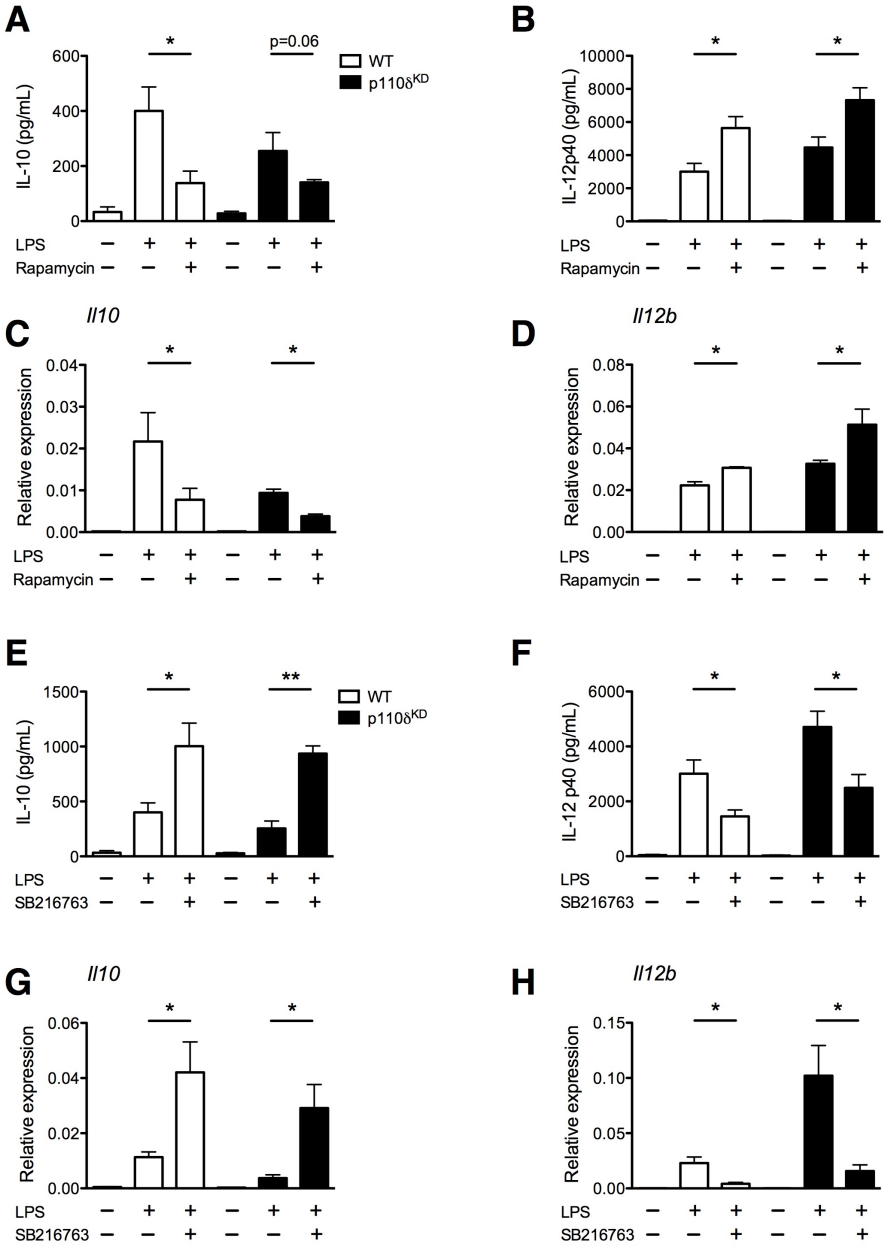


Figure 3.4. IL-10 and IL-12p40 production in macrophages is mTOR- and GSK-3β-dependent. WT and p110δ^{KD} BMDMs were cultured with the mTOR inhibitor rapamycin (A-D) or the GSK-3β inhibitor SB-216763 (E-H) for 1 hour prior to stimulation with LPS (1 ng/ml) for 4 (quantitative RT-PCR) or 8 (ELISA) hours. Supernatants were assayed for IL-10 (A, E) and IL-12p40 (B, F) production by ELISA. Error bars represent mean ± SEM from three independent experiments (*, $p<0.05$; **, $p<0.005$). Total RNA was assayed for *Il10* (C, G) and *Il12b* (D, H) expression normalized to *β-actin* expression by quantitative RT-PCR. Error bars represent mean ± SEM from three independent experiments (*, $p<0.05$).

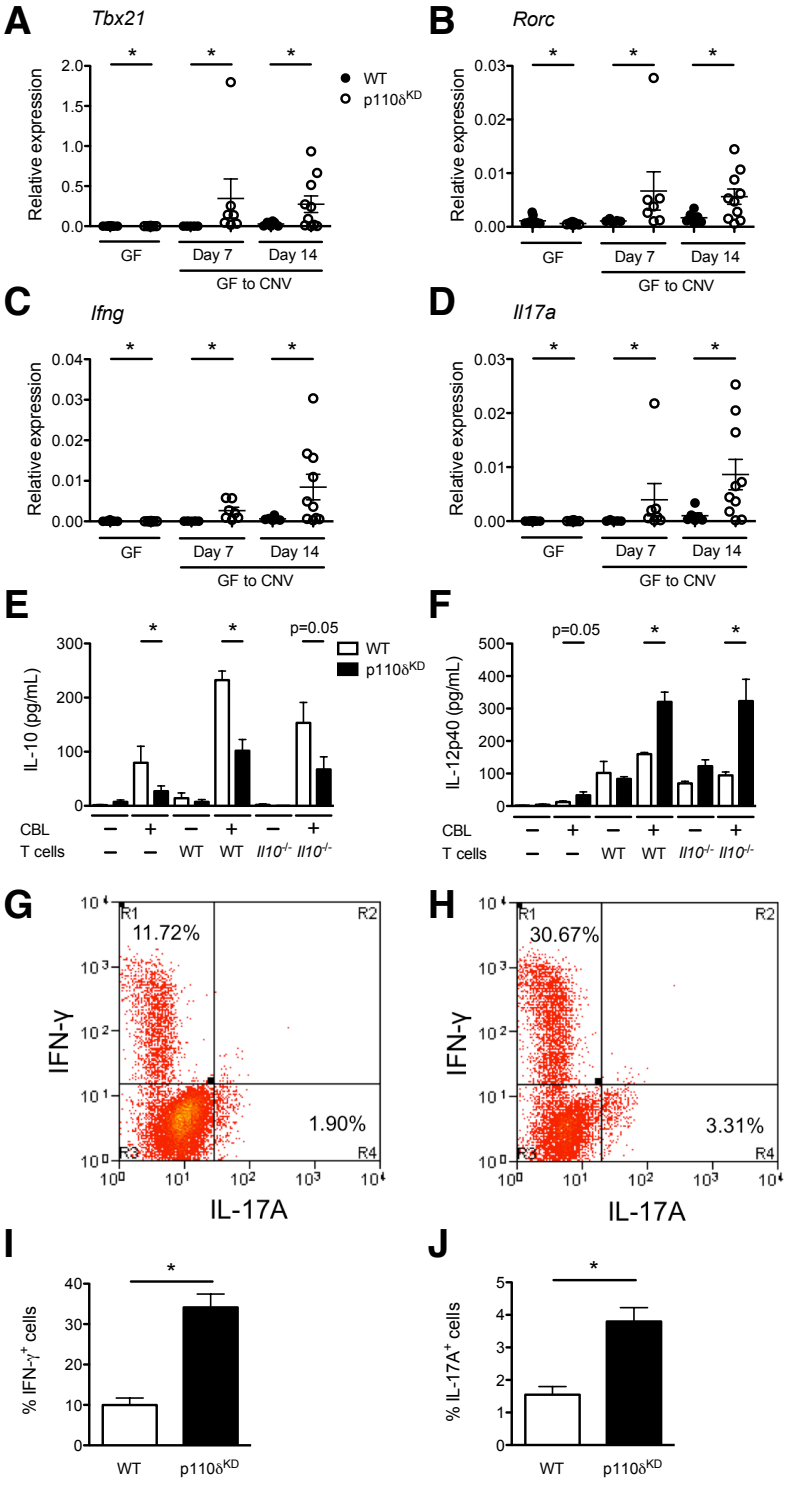


Figure 3.5. APC p110δ regulates T cell differentiation. (A-D) GF WT and p110δ^{KD} mice were transferred to CNV housing. Colonic tissue was collected from WT and p110δ^{KD} mice sacrificed at days 0 (n = 12 and 10, respectively), 7 (n = 5 and 7, respectively) and 14 (n = 6 and 12, respectively) after transfer. Quantitative real time RT-PCR was performed in duplicate for *Tbx21* (A), *Rorc* (B), *Ifng* (C), and *Il17a* (D)

expression normalized to *β-actin* expression. Error bars represent mean ± SEM (*, $p<0.05$). (E, F) WT and $p110\delta^{KD}$ APCs were cultured overnight with CBL (50 ng/ml) and then co-cultured with WT or *Il10*$^{-/-}$ $CD4^+$ T cells at a ratio of 3:2 for 72 hours. Supernatants were assayed for IL-10 (E) and IL-12p40 (F) production by ELISA. Error bars represent mean ± SEM for three independent experiments (*, $p<0.05$). (G-J) WT and $p110\delta^{KD}$ APCs were stimulated with LPS (10 ng/ml) and OVA peptide (5 μM) overnight and then co-cultured with WT $CD4^+CD62L^+$ OT-II T cells at a ratio of 3:2 for 72 hours. T cells were assayed for IL-17A and IFN-γ production by flow cytometry. $CD4^+$ lymphocytes were gated using forward and side scatter. Representative flow cytometry plots show IFN-γ- and IL-17A-producing WT $CD4^+$ OT-II T cells co-cultured with LPS and OVA stimulated WT (G) and $p110\delta^{KD}$ (H) APCs. Plots are representative of results from three independent experiments with similar results. Quantification of the percentage of total $CD4^+$ T cells producing IFN-γ (I) and IL-17A (J) was determined from the flow cytometry analysis. Error bars represent mean ± SEM from three independent experiments (*, $p<0.05$).

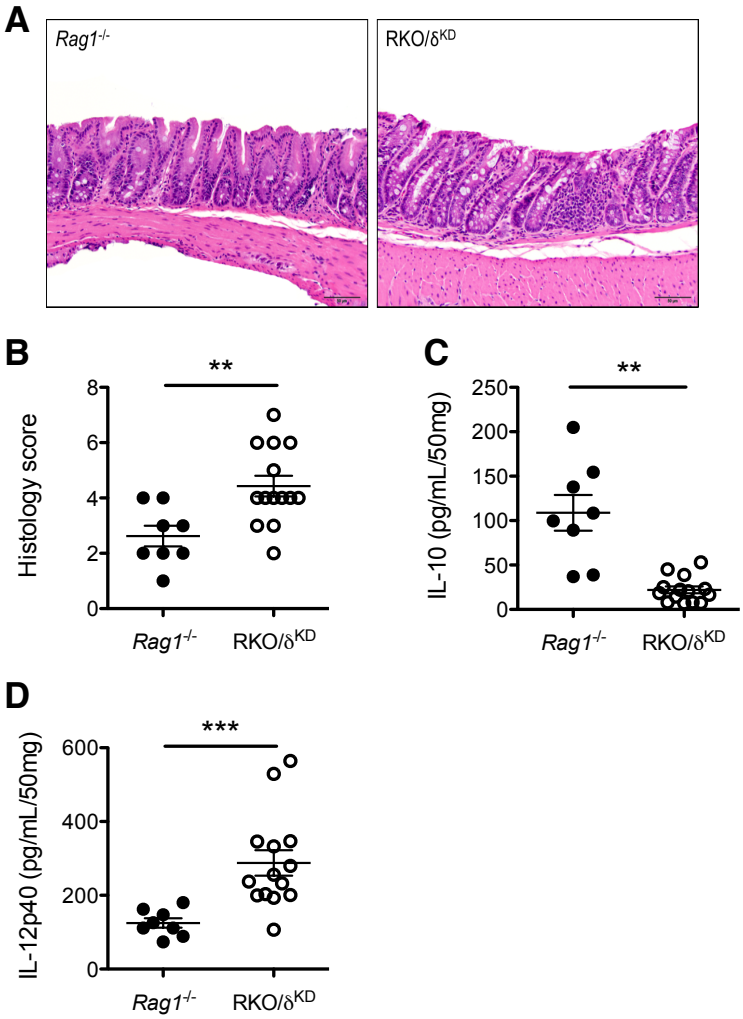


Figure 3.6. Mild innate mediated colitis develops in *Rag1*$^{-/-}$/p110δ^{KD} mice. 16-week-old *Rag1*$^{-/-}$ (n = 8) and *Rag1*$^{-/-}$/p110δ^{KD} (RKO/δ^{KD}, *n* = 14) mice were assessed for colitis severity by histopathology and cytokine production in colonic explant cultures. (A) (20X, H&E) Representative histology pictures are shown. (B) H&E slides of colonic tissue were scored for colitis severity using criteria described in the methods by a pathologist (LBB) blinded to the experimental groups. Error bars represent mean ± SEM (**, p<0.005). IL-10 (C) and IL-12p40 (D) production was determined by ELISA and expressed as the amount of cytokine (pg/ml) per 50 mg colonic tissue weight. Error bars represent mean ± SEM (**, p<0.005; ***, p<0.0005).

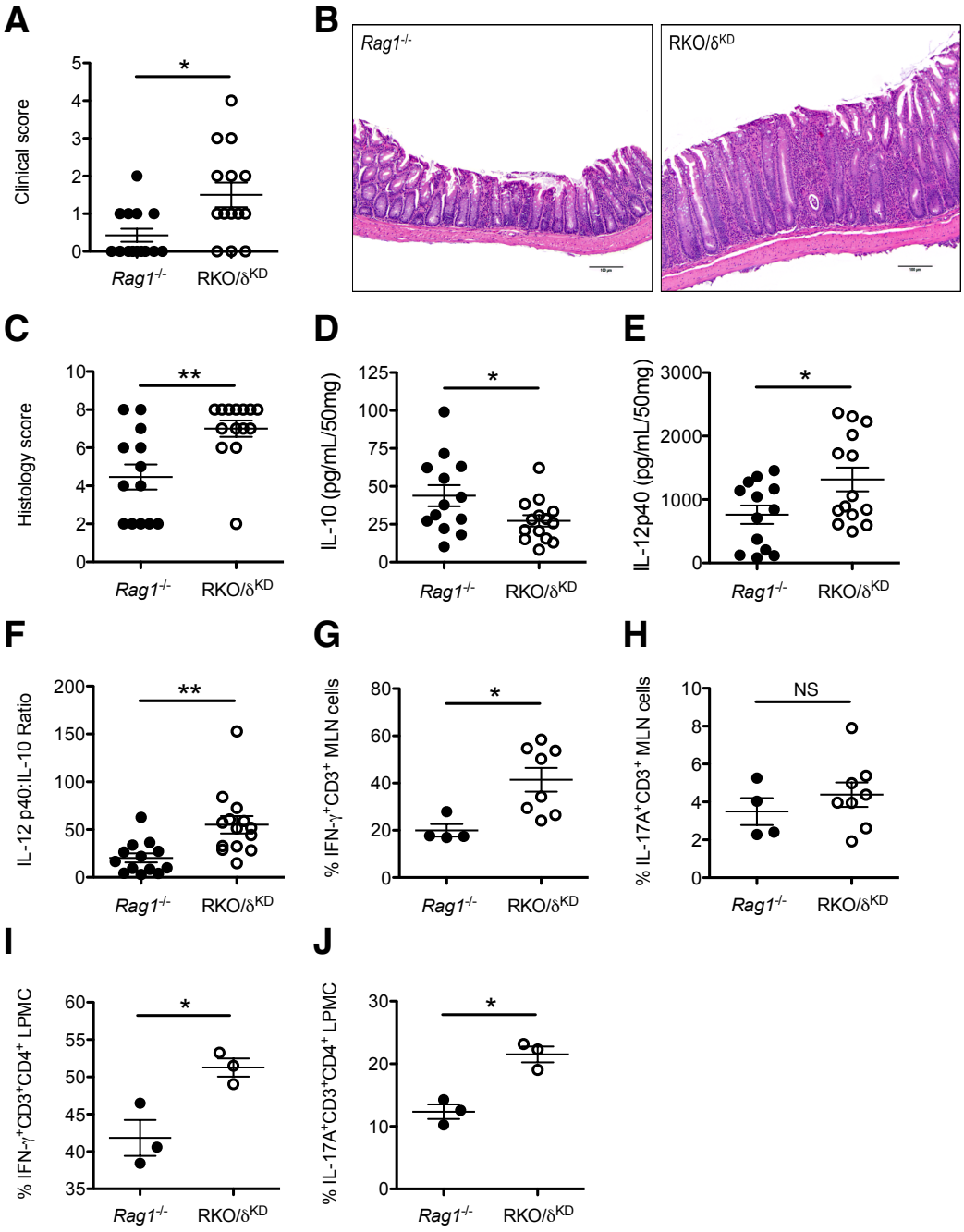


Figure 3.7. Adoptive transfer of CD4$^+$CD45RB T cells into *Rag1*$^{-/-}$/ p110δ^{KD} recipient mice leads to severe colitis. 8 week old *Rag1*$^{-/-}$ (n = 13) and RKO/δ^{KD} (n = 14) recipient mice were given 4x10^5 CD4$^+$CD45RB^{high} T cells admixed with 2x10^5 CD4$^+$CD45RB^{low} T cells by i.p. injection to induce colitis as described in the methods. Mice were assessed for colitis severity at 24 days after adoptive transfer. (A) Clinical disease activity scores were determined as described in the methods. Error bars represent mean ± SEM (*, $p<0.05$). (B) (20X, H&E) Representative histology pictures are shown. (C) H&E slides of colonic tissue were scored for colitis severity by a pathologist (LBB) blinded to the experimental groups as described in the methods. Error bars represent mean ± SEM (**,

p<0.005). (D-F) Supernatants from 24 hour colonic tissue explants were collected and assayed for IL-10 (D) and IL-12p40 (E) production by ELISA, and are expressed as the amount of cytokine (pg/ml) per 50 mg colonic tissue weight. Error bars represent mean ± SEM (*, p<0.05). (F) IL-12p40 and IL-10 protein levels from colonic tissue explant culture in individual mice were used to determine the ratio of IL-12p40 to IL-10. Error bars represent mean ± SEM (**, p<0.005). (G,H) Mesenteric lymph nodes (MLNs) from *Rag1*$^{-/-}$ and RKO/δ^{KD} recipient mice were analyzed by flow cytometry for intracellular IFN-γ (G) and IL-17A (H) expression in CD4^{+} T cells. Each point on the graphs represents MLN cells from one mouse. Error bars represent mean ± SEM (NS, not significant; *, p<0.05). (I,J) LPMCs were analyzed by flow cytometry for IFN-γ (I) and IL-17A (J) expression in CD3^{+}CD4^{+} T cells. Each point on the graphs represents pooled LPMCs from three mice. Error bars represent mean ± SEM (*, p<0.05).

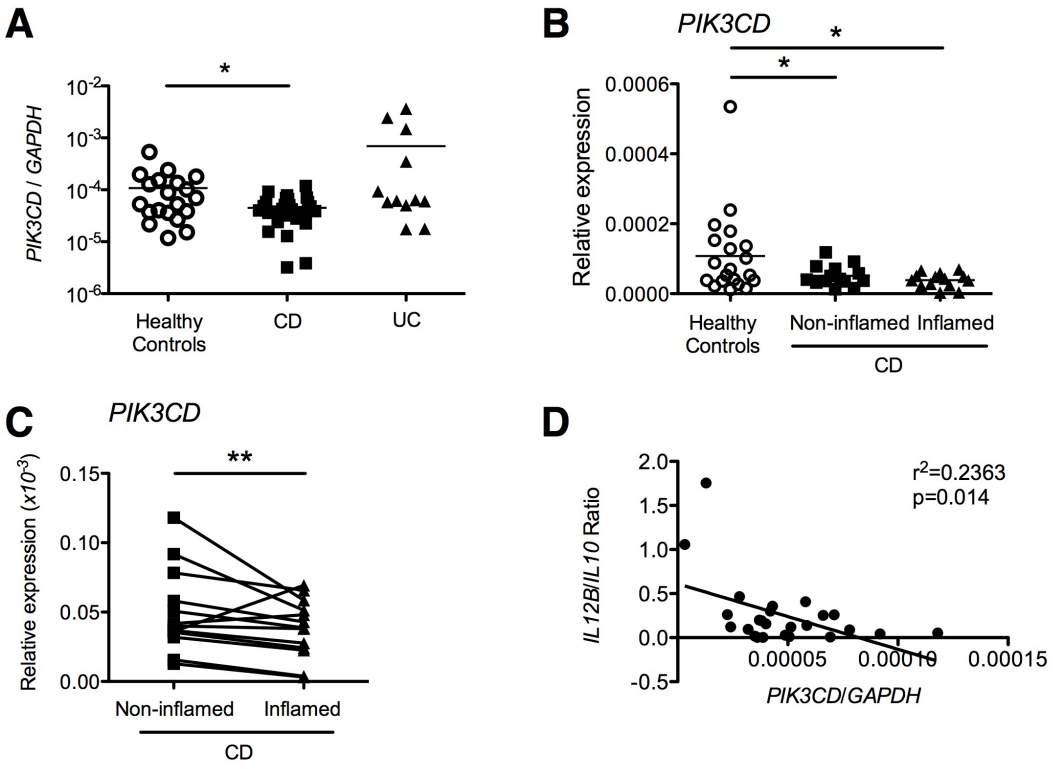
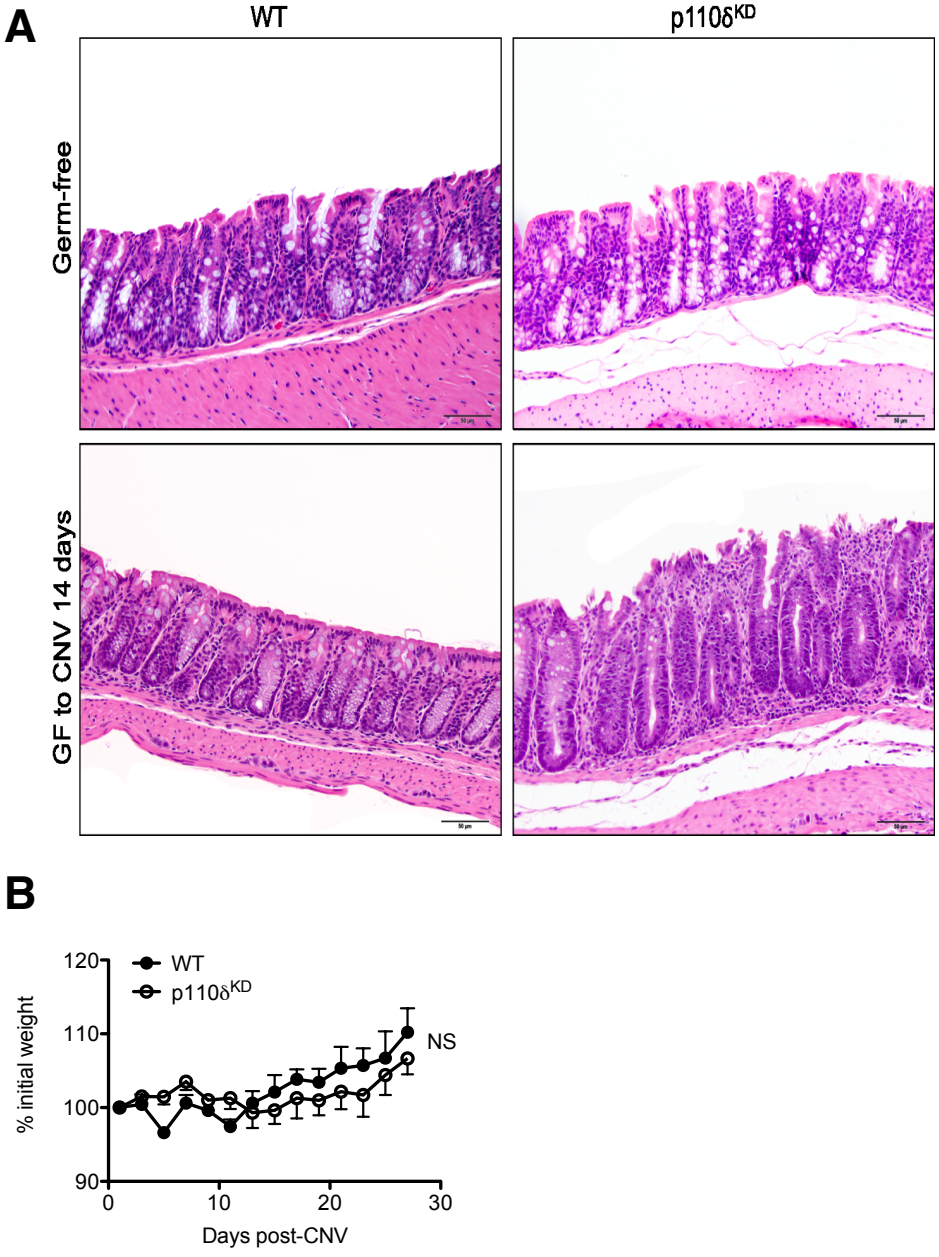
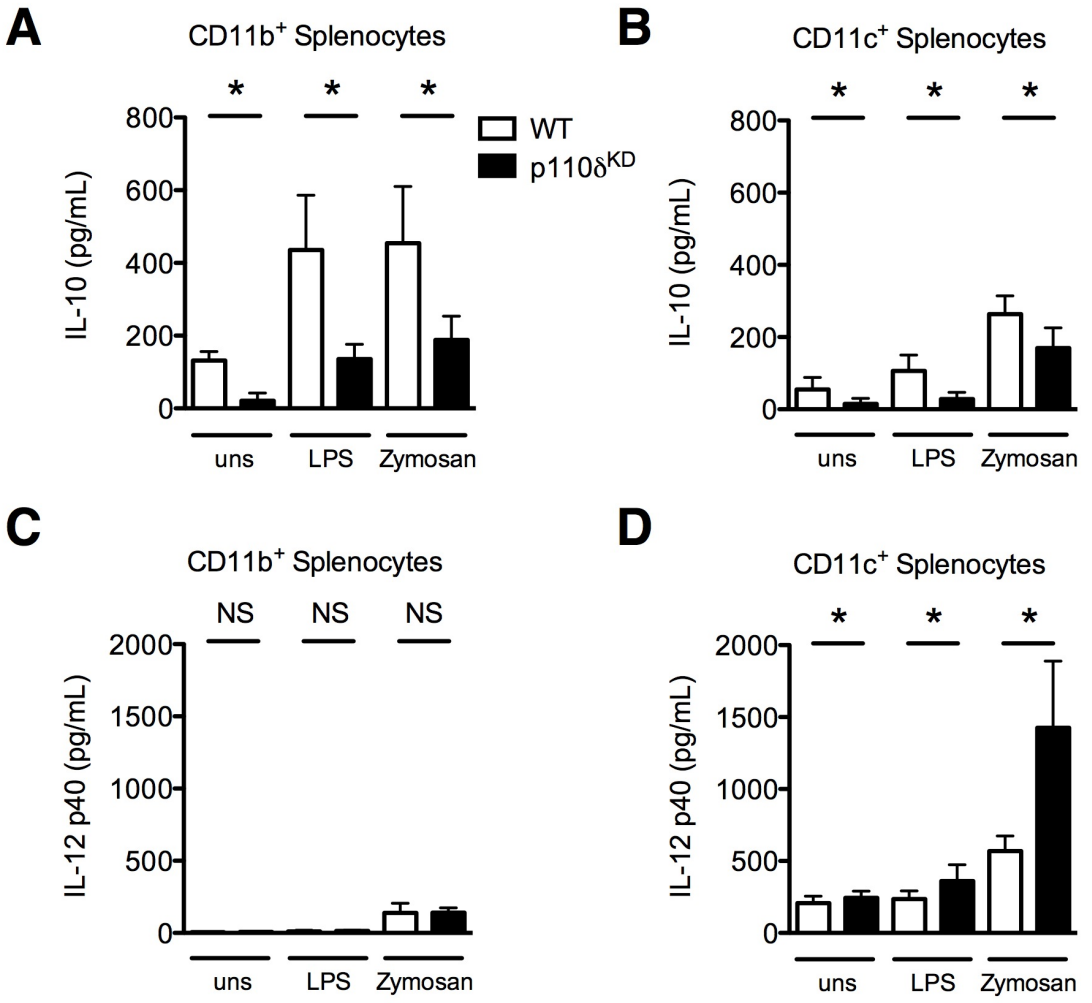


Figure 3.8. Human intestinal *PIK3CD* expression is decreased in patients with CD and inversely correlates with *IL12B*:*IL10* ratios. Macroscopically inflamed and non-inflamed colonic or ileal tissue was obtained from patients with CD *(n* = 14), UC (*n* = 6) or non-IBD control patients (non-inflamed tissue; *n* = 20) undergoing surgical resection. (A,B) Total RNA from samples was assessed for *PIK3CD* expression by quantitative real time RT-PCR in duplicate normalized to *GAPDH* expression. Error bars represent mean ± SEM (*, $p<0.05$). (C) Intestinal *PIK3CD* expression normalized to *GAPDH* expression from patients with CD was assessed at inflamed sites (n = 14) and compared to non-inflamed sites (n = 14) from the same patient. Lines connect samples from individual patients. Data was analyzed using a paired *t* test. (**, $p<0.005$). (D) Total RNA from samples was assessed for *IL12B* and *IL10* expression by quantitative real time RT-PCR in duplicate normalized to *GAPDH* expression. *IL12B*:*IL10* ratios in patients were correlated with *PIK3CD* expression ($r^2=0.2363$; $p=0.014$).

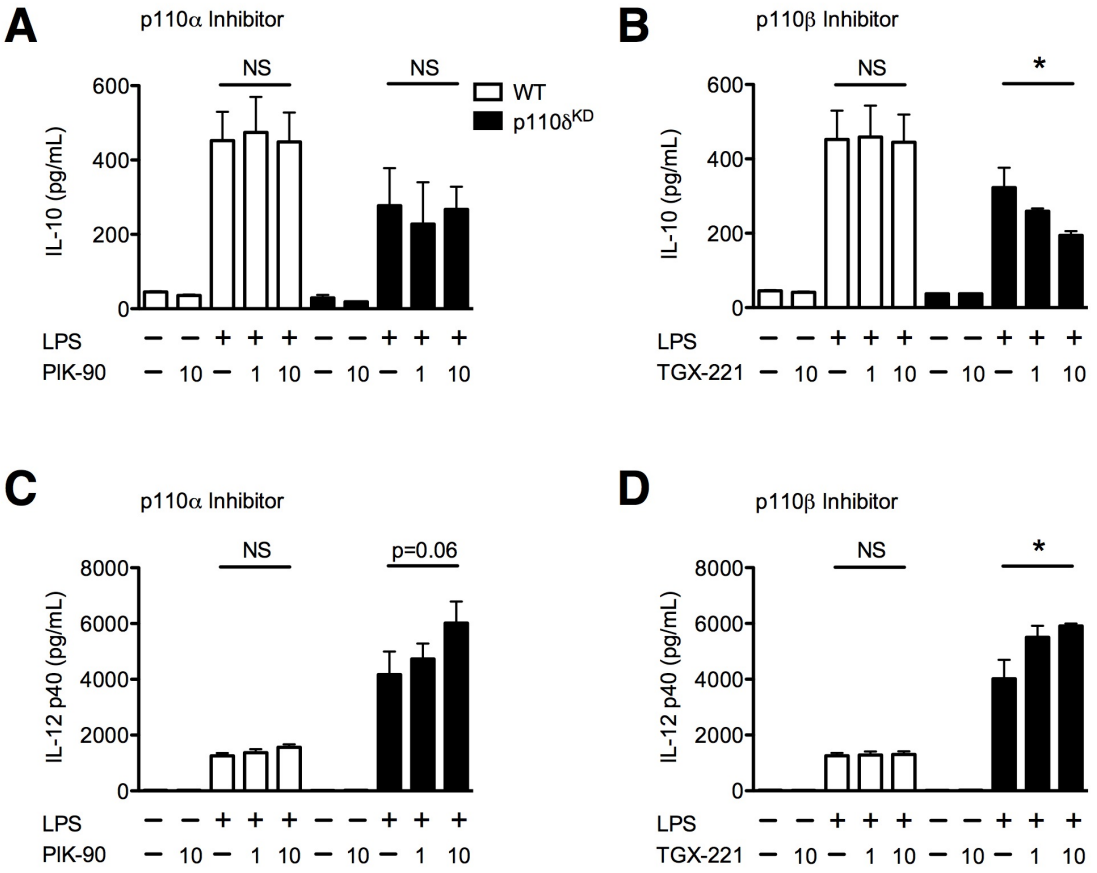
3.7 Supplemental Figures



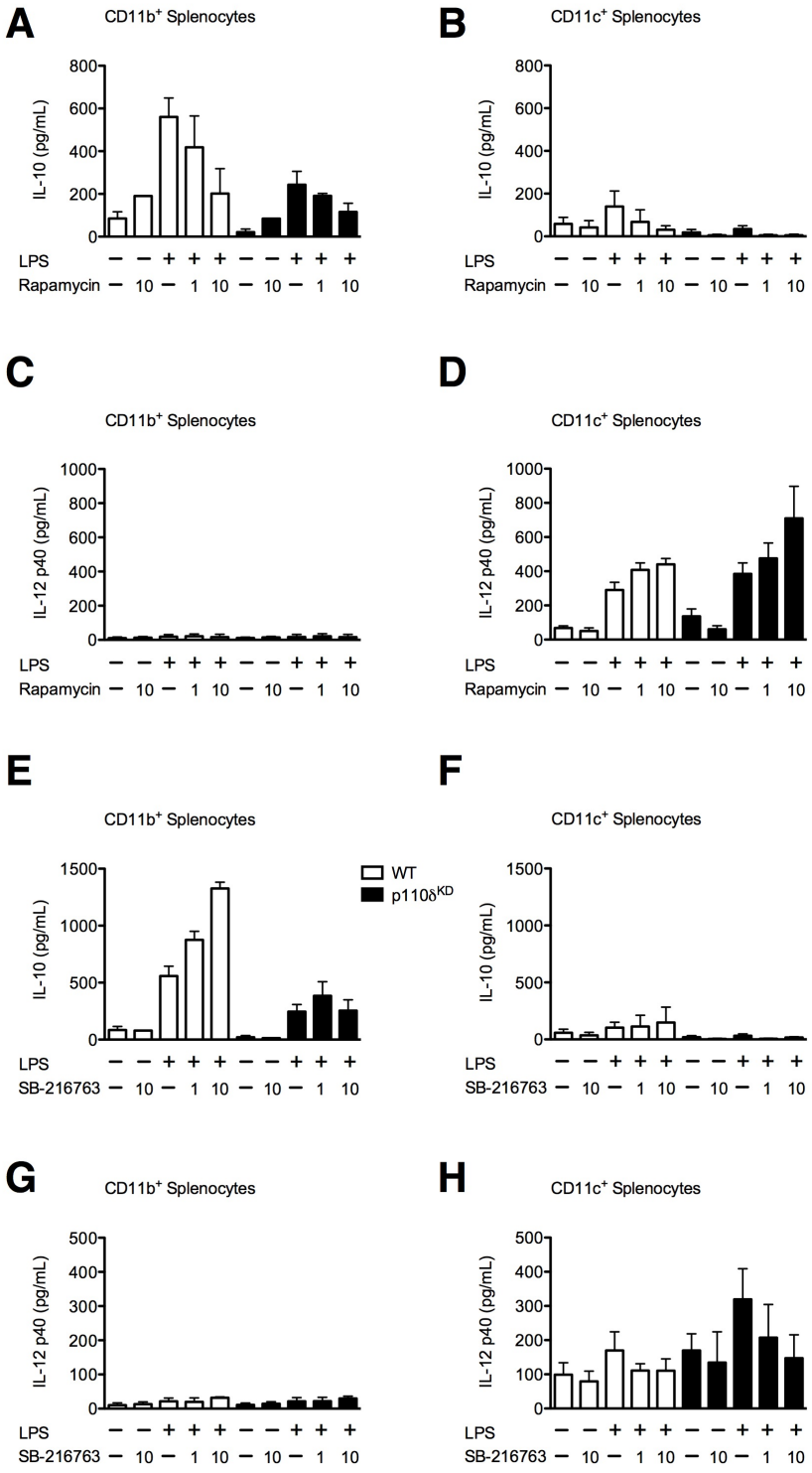
Supplemental Figure 3.1. GF to CNV p110δ^{KD} develop histologic colitis. GF WT (n = 6) and p110δ^{KD} (n = 12) mice were transitioned to conventional housing and monitored for colitis by weight and histology. (A) Representative H&E stained colonic tissue from GF and GF to CNV WT and p110δ^{KD} mice at 14 days after transfer. (B) Percentage of initial weight of GF to CNV WT and p110δ^{KD} mice after transfer. Error bars represent mean ± SEM (NS, not significant).



Supplemental Figure 3.2. Splenic macrophages and dendritic cells demonstrate increased Il-12p40 and impaired IL-10 production in response to TLR agonists. Splenic macrophages ($CD11b^+CD11c^-$) and DCs ($CD11b^-CD11c^+$) were stimulated with TLR agonists (LPS, 1 ng/ml; Zymosan A, 10 μg/ml) for 8 hours. Supernatants were collected and assessed for IL-10 (A and B, macrophages and DCs, respectively) and IL-12p40 (C and D, macrophages and DCs, respectively) production by ELISA. Error bars represent mean ± SEM from three independent experiments (NS, not significant; *, $p<0.05$).

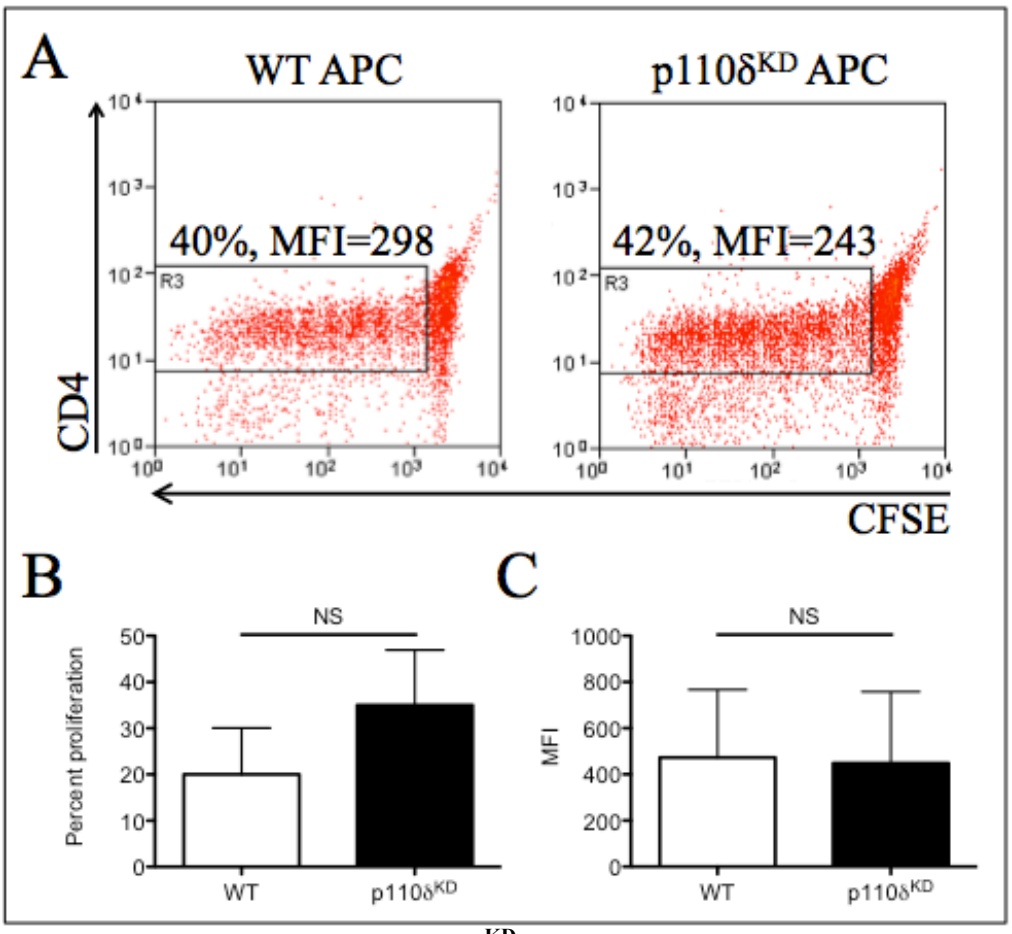


Supplemental Figure 3.3. Inhibition of the Class I_A PI3K isoforms p110α and p110β in WT macrophages has no effect on IL-10 and IL-12p40 production. WT and $p110\delta^{KD}$ BMDMs were stimulated with LPS (1 ng/ml) 1 hour after exposure to p110α- and p110β-specific inhibitors (PIK-90 and TGX-221, respectively) for 8 hours. Supernatants were collected and assessed for IL-10 (A,B) and IL-12p40 (C,D) by ELISA. Error bars represent mean ± SEM for three independent experiments (NS, not significant; *, $p<0.05$).

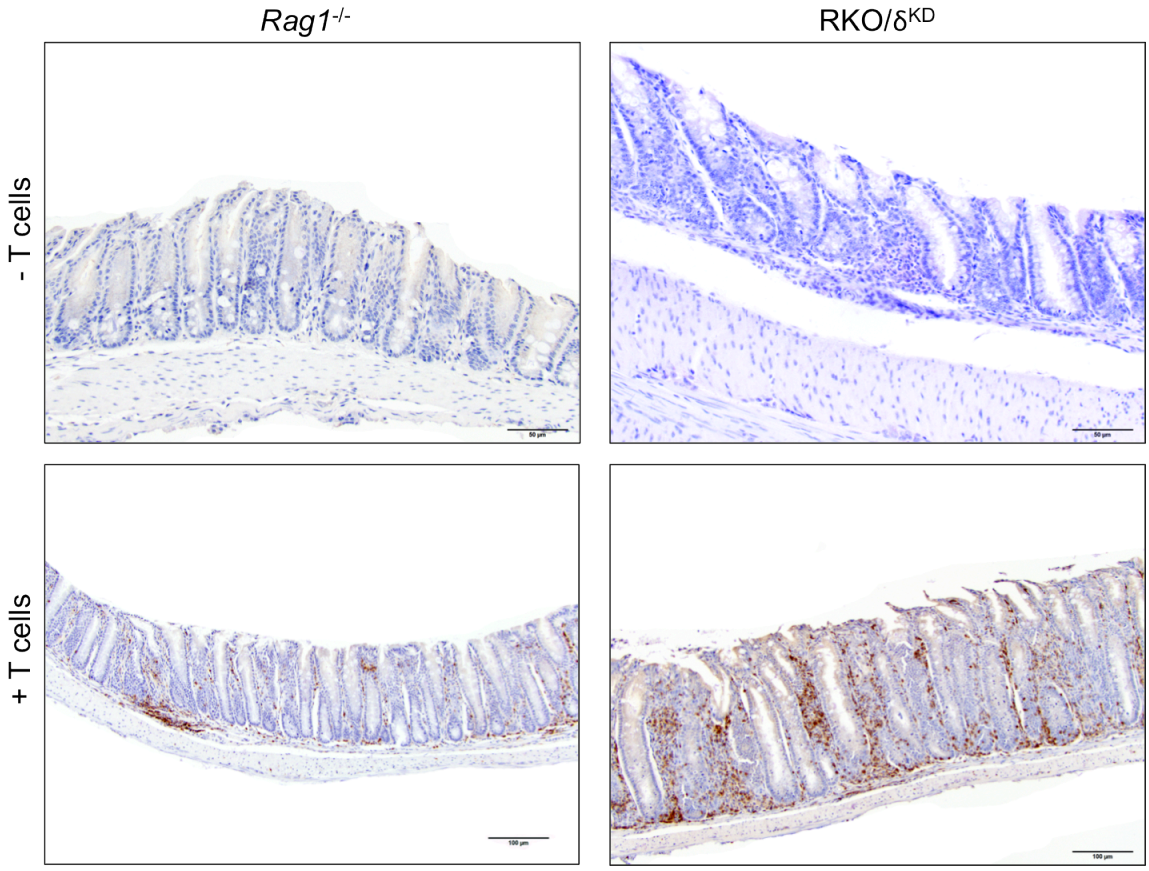


Supplemental Figure 3.4. LPS stimulated splenic macrophages and DCs modulate IL-10 and IL-12p40 expression in the presence of mTOR and GSK-3β inhibitors. (A-D) One hour after exposure to rapamycin (1 or 10 μM), splenic macrophages ($CD11c^-$/$CD11b^+$) and DCs ($CD11c^+$) were stimulated with LPS (10 ng/ml) for 24 hours. Supernatants were assessed for IL-10 (A and B, macrophage and DCs, respectively) and

IL-12p40 (C and D, macrophage and DCs, respectively) production by ELISA. Error bars represent mean ± SEM from three independent experiments. (E-H) One hour after exposure to SB216763 (1 or 10 μM), splenic macrophages ($CD11c^-/CD11b^+$) and DCs ($CD11c^+$) were stimulated with LPS (10 ng/ml) for 24 hours. Supernatants were collected and assessed for IL-10 (E and F, macrophage and DCs, respectively) and IL-12p40 (G and H, macrophage and DCs, respectively) production by ELISA. Error bars represent mean ± SEM from three independent experiments.



Supplemental Figure 3.5. WT and p110δ^{KD} APCs induce the same amount of antigen-specific CD4^{+} T cell proliferation. T cell-depleted splenocytes (APCs) from WT and p110 δ ^{KD} mice were activated with LPS (100 ng/ml) and pulsed overnight with OVA peptide (5 μ M). After washing extracellular antigen, APCs were co-cultured with CFSE-labeled OT-II (OVA-tg TCR) CD4^{+}CD62L^{+} T cells for 72 hours. CD4^{+} T cells were analyzed by flow cytometry. (A) Lymphocytes were gated on forward- and side-scatter and CD4. Figures shown are representative results from 3 independent experiments. (B) Percentage of proliferating CD4^{+} T cells was quantified from 3 independent experiments (NS, not significant). (C) Mean fluorescence intensity (MFI) of the CFSE signal was quantified from 3 independent experiments (NS, not significant).



Supplemental Figure 3.6. Adoptively transferred T cells localize to the colon of recipient mice. (20X, IHC) 8 week old *Rag1*$^{-/-}$ and RKO/δ^{KD} recipient mice were given 4x10^5 CD4$^+$CD45RB^{high} T cells admixed with 2x10^5 CD4$^+$CD45RB^{low} T cells by i.p. injection to induce colitis as described in the methods. Mice were assessed for colitis severity at 24 days after adoptive transfer. Paraffin-embedded sections of colon tissue were stained by immunohistochemistry for CD3.

CHAPTER 4

PI3K p110δ REGULATES BACTERICIDAL ACTIVITY OF MACROPHAGES[3]

4.1 Overview

GWAS studies identified IBD-associated SNPs supporting the hypothesis that altered phagosome function and subsequent impaired innate response to the enteric microbiota underlies the pathogenesis of IBDs. Therefore, as a model with relevance to the pathogenesis of IBD, we explored defects in phagosome formation and ROS production in macrophages from p110δ^{KD} mice. Additionally, we identified dysbiosis in the enteric microbiota of p110δ^{KD} mice. Functionally altered phagosomes kill microbes less effectively, resulting in prolonged immune activation and/or incomplete microbial clearance, contributing to the pathogenesis of IBDs.

4.2 Introduction

The human IBDs, CD and UC, result from an inappropriately directed immune response to enteric microbiota in a genetically susceptible host (Xavier and Podolsky, 2007). IBDs have a prevalence of 51-445 per 100,000 people in the United States and account for 100,000 hospitalizations per year (Loftus, 2004). However, the etiology of

[3] Erin C. Steinbach, Steven M. Russo, Taku Kobayashi, Shehzad Z. Sheikh, Nitsan Maharshak, Christopher D. Packey, R. Balfour Sartor, Scott E. Plevy. 2013. PI3K p110δ regulates bactericidal activity of macrophages. (Unpublished work).

IBDs remains elusive, and hospitalization rates have not decreased in recent years despite advancements in therapy (Loftus, 2004). Incomplete understanding of the pathways involved in IBD pathogenesis impedes the development of safer and more effective therapies.

The importance of the enteric microbiota in IBD pathogenesis is supported by studies in experimental models. Colitis is not observed when colitis-prone mouse strains are maintained germ free but emerges when they are reconstituted with normal enteric bacterial constituents (Sellon et al., 1998). Furthermore, it has been possible to induce colitis in a susceptible murine strain with a single species of non-pathogenic bacteria, for example, *Bacteroides vulgatus* in the *Il10*$^{-/-}$ mouse (Sellon et al., 1998). Macrophages are essential for the recognition, phagocytosis and clearance of commensal bacteria in the intestine that breach the epithelial barrier (Rioux et al., 2007). Recently, alterations in autophagy and phagosomal function have emerged as a central focus in the macrophage's ability to eradicate intracellular bacteria (Rioux et al., 2007). Indeed, the importance of microbicidal pathways in the pathogenesis of IBDs was highlighted by the discovery that a synonymous SNP in the auto-phagocytic gene *ATG16L1* and a SNP in the phagosomal gene *NCF4* are associated with enhanced risk for IBD (Rioux et al., 2007; Xavier and Podolsky, 2007). Therefore, a new paradigm in IBD pathogenesis research is a model of defective intracellular responses to commensal enteric bacteria.

Macrophages can kill or limit the replication of microorganisms through many possible mechanisms. Macrophages can limit the availability of essential nutrients, as well as produce antimicrobial peptides, lysosomal enzymes, and ROS/RNS (Ismail et al., 2002). Oxygen-dependent metabolites are perhaps the most efficient anti-microbial

effectors produced by macrophages. NADPH oxidases and associated accessory proteins are therefore essential components of cellular responses to microbial invasion. Hemoprotein complexes of the NADPH phagocyte membrane-bound gp91phox and p22phox subunits, along with the cytosolic p40phox, p47phox, p57phox and Rac proteins, can consume molecular oxygen (Rada et al., 2008; Robinson, 2008). The cytosolic components stabilize and activate phagocyte NADPH oxidase (Nox2). PI3K p110δ signaling can recruit p40phox and p47phox to the membrane signaling complex, implicating p110δ activation in ROS production (Kanai et al., 2001). Interestingly, pharmacologic inhibition of p110δ in neutrophils and other cell types decreases production of ROS and the respiratory burst (Yamamori et al., 2004). Compartmentalization of membrane-bound and cytosolic components of Nox2 ensures that the production of cytotoxic oxygen radicals is prevented in resting cells, thus avoiding "collateral damage". The discovery that chronic granulomatous disease is a consequence of genetic alteration in any one of the five essential subunits of Nox2 indicates the vital function of the enzyme (Segal et al., 2009). These patients are susceptible to bacterial infections but also develop chronic inflammatory bowel disease (IBD) with features similar to human CD (Segal et al., 2009). Furthermore, a SNP within the first intron of *NCF4* (p40phox) is associated with enhanced susceptibility to IBDs (Rioux et al., 2007).

Another important bactericidal pathway in macrophages following the phagocytosis of a microbe is phagosome maturation to the actively bactericidal vesicle, the phagolysosome. The nascent phagosome goes through three stages of maturation: early, late, and lysosome-interacting (Fairn and Grinstein, 2012). Rab-family GTPases

mediate the maturation process. Early phagosomes are marked by Rab5 decoration, which allows fusion with early endosomes via interactions with early endosome antigen 1 (EEA1) (Christoforidis et al., 1999; Kinchen et al., 2008). Recruitment of maturation factors such as EEA1 is mediated by the generation of membrane-bound PIP molecules by the Class III PI3K vacuolar protein sorting 34 (Vps34) (Kinchen et al., 2008; Scott et al., 2002). GTPase activity on late phagosomes transitions from Rab5 to Rab7, which mediates intracellular trafficking and fusion with lysosomes (Harrison et al., 2003; Johansson et al., 2007). The resulting phagolysosome drives a critical drop in intravesicular pH by pumping H^+ into the lumen via V-ATPase (Flannagan et al., 2009). The low pH of phagolysosomes directly impairs microbe function, activates host hydrolytic enzymes and assists in the generation of superoxide by NADPH oxidase. Furthermore, the H^+ gradient is used to pump essential microbial nutrients out of the phagolysosome.

The leukocyte-expressed PI3K p110δ protein regulates innate immune responses to bacteria (Fukao and Koyasu, 2003; Koyasu, 2003). In response to extracellular stimuli, phosphoinositides are phosphorylated on the 3-position of the inositol ring by PI3Ks. The products of PI3Ks govern fundamental cellular events such as cell growth and survival, and cytoskeletal remodeling. The PI3Ks are divided in four classes (I_A, I_B, II and III) on the basis of structural characteristics and substrate specificity. Class I_A enzymes have five different regulatory subunits, and three p110 catalytic subunits: p110α and p110β are expressed ubiquitously in many tissues whereas p110δ is expressed preferentially in leukocytes (Koyasu, 2003; Papakonstanti et al., 2008). Agents that activate macrophages and DCs to produce the inflammatory cytokine IL-12 also activate Class I_A PI3K (Fukao

et al., 2002). Activation of PI3K in turn blocks the expression of IL-12p40 mRNA (*Il12b*) (Fukao et al., 2002). Although inflammatory responses are essential for eradicating infectious microorganisms, excessive/prolonged activation of innate immunity is harmful to the host. Thus, PI3K-mediated negative feedback of IL-12 production is important to prevent excessive innate immune response. Therefore, the PI3K signal transduction pathway serves as an important molecular "brake" on inflammatory immune responses.

The clearest role of PI3K in chronic inflammation was described in a mouse with a knock-in kinase-dead point mutation in the p110δ subunit ($p110\delta^{KD}$) (Okkenhaug et al., 2002). The strategy of mutation rather than deletion was necessary as mice deficient in PI3K subunits had compensatory changes in expression and availability of regulatory subunits (Okkenhaug et al., 2002). In $p110\delta^{KD}$ mice, antigen receptor signaling in T and B cells is defective (Okkenhaug et al., 2002). Notably, these mice developed chronic segmental intestinal inflammation (Okkenhaug et al., 2002). The lesions were characterized by mucosal hyperplasia, crypt abscesses and mixed leukocyte infiltrates (Uno et al., 2010). We have demonstrated that these mice develop focal Th1/Th17-mediated colitis (Uno et al., 2010). Therefore, PI3K $p110\delta^{KD}$ mice are an invaluable reagent to understand the role of PI3K in the mucosal inflammatory response. PI3K p110δ is expressed predominantly in leukocytes (Koyasu, 2003), indicating that it plays a unique role in immune signaling.

Importantly, we recovered increased bacterial ribosomal DNA from the spleen and mesenteric lymph nodes (MLNs) of $p110\delta^{KD}$ mice (Uno et al., 2010), suggesting that in the absence of p110δ there is (1) an increase in bacterial translocation across the intestinal epithelial barrier, (2) an increase in bacterial persistence, or (3) a combination

of both increased translocation and persistence. Indeed, p110δ^{KD} macrophages demonstrate defective eradication of intracellular commensal, pathogenic and colitogenic bacteria, even when activated with IFN-γ or LPS (Uno et al., 2010). However, the specific way that p110δ contributes to enhancing bactericidal activity in macrophages is unknown.

SNPs identified by GWAS in humans support the hypothesis that altered phagosome function leading to a defective innate response to the enteric microbiota may underlie the pathogenesis of IBDs. Therefore, as a model with relevance to the pathogenesis of IBDs, we explored defects in phagosome formation and ROS production in macrophages from p110δ^{KD} mice. Additionally, we identified dysbiosis in the enteric microbiota of p110δ^{KD} mice. Functionally altered phagosomes kill microbes less effectively, resulting in prolonged immune activation and/or incomplete microbial clearance, contributing to the pathogenesis of IBDs.

4.3 Results

4.3.1 IFN-γ and E. coli-stimulated p110δ^{KD} macrophages demonstrate decreased lysosomal activation

Given that macrophages from p110δ^{KD} mice do not effectively eradicate intracellular bacteria (Uno et al., 2010), we sought to determine whether p110δ^{KD} macrophages demonstrated appropriately matured phagolysosomes. Previous results from our lab demonstrated greatly impaired induction of bactericidal activity in IFN-γ-activated p110δ^{KD} macrophages (Uno et al., 2010), and so we continued to activate macrophages with IFN-γ in our studies in order to maximize differences between those

from WT and p110δ^{KD} mice. WT and p110δ^{KD} BMDMs were stimulated with IFN-γ and cultured with GFP-expressing K12 *E. coli* for one hour. Cells were incubated with medium containing LysoTracker®, a weak base that permeates cell membranes and fluoresces upon protonation in low-pH environments. LysoTracker® detects phagolysosomal acidification by a fluorescence emission signal at 590 nm. Cells were washed, fixed, and visualized by fluorescent microscopy. Representative photographs were blinded, and the percentage of LysoTracker®-positive cells was calculated. PI3K p110δ^{KD} macrophages had significantly decreased percentages of LysoTracker®-positive cells (Fig. 4.1), suggesting that bacteria-laden phagosomes in p110δ^{KD} macrophages do not appropriately mature.

4.3.2 PI3K p110δ regulates reactive oxygen species generation in macrophages

PI(3)P molecule generation at cell and vesicular membranes regulates intracellular trafficking (Vanhaesebroeck et al., 2001). Generation of PI(3)P by PI3Ks is especially important in the maturation of nascent phagosomes and recruitment of Nox2 components to the phagosome membrane (Kinchen et al., 2008; Scott et al., 2002). While the Class III PI3K is the major generator of PI(3)P, Class I_A PI3Ks also make contribute to PI(3)P formation. Therefore we explored the production of ROS in IFN-γ-activated p110δ^{KD} macrophages during eradication of intracellular bacteria. IFN-γ-activated macrophages from p110δ^{KD} mice consistently demonstrated a lower peak of luminescence, representing the amount of ROS produced, when incubated with K12 *E. coli* or *S. typhimurium* compared to macrophages from WT mice (Fig. 4.2). Interestingly, there was no difference in the production of ROS between WT and p110δ^{KD}

macrophages when incubated with *E. faecalis*, a gram-positive enteric commensal organism.

To confirm these results, we measured ROS production in IFN-γ-activated WT macrophages pre-treated with the p110δ-specific inhibitor IC87114 (10 μM) and incubated with K12 *E. coli*, *E. faecalis* or *S. typhimurium*. In agreement with the data from p110δ^{KD} macrophages, inhibition of p110δ in WT macrophages decreased the production of ROS when incubated with K12 *E. coli* and *S. typhimurium*, but not *E. faecalis* (Fig. 4.3).

4.3.3 Phagosome maturation is regulated by p110δ

Given that p110δ appears to regulate lysosomes and the generation of ROS, we next sought to determine whether p110δ regulates phagosome maturation in macrophages during intracellular bacterial eradication. IFN-γ-activated WT and p110δ^{KD} macrophages were pulsed with FITC-labeled dextran followed by a short chase to allow the FITC-dextran to traffic to lysosomes. Macrophages were then incubated with mCherry-labeled K12 *E. coli* for 60 minutes, and confocal microscopy was performed to determine whether bacteria-laden phagosomes are able to fuse with lysosomes. While the observations were never quantified, we saw decreased co-localization of FITC-dextran with K12 *E. coli* in p110δ^{KD} macrophages compared to WT macrophages (Fig. 4.4).

EEA1 is a marker of nascent phagosomes, and its recruitment to the phagosome is dependent on the generation of PI(3)P at the vesicular membrane (Kinchen et al., 2008; Scott et al., 2002). Therefore we studied EEA1 recruitment to bacteria-laden phagosomes in IFN-γ-activated WT and p110δ^{KD} macrophages. We observed more co-localization

between GFP-expressing K12 *E. coli* and EEA1 in WT macrophages compared to p110δ^{KD} macrophages after 10 minutes of infection (Fig. 4.5). While this was not quantified, it represents a consistent observation. Furthermore, at 60 minutes after infection when EEA1 is no longer associated with the maturing phagosome, we observed more EEA1 and GFP K12 *E. coli* co-localization in p110δ^{KD} compared to WT macrophages (Fig. 4.6), suggesting that the kinetics of EEA1 association with the maturing phagosome is dysregulated in p110δ^{KD} macrophages.

4.3.4 Outcome of in vivo infection with Streptomycin-resistant Salmonella is no different between WT and p110δ^{KD} mice

To better understand how p110δ regulates bactericidal activity *in vivo*, we utilized the Streptomycin-*Salmonella* infection model (Barthel et al., 2003) in WT and p110δ^{KD} mice. Mice were given streptomycin (20 mg) 21 hours prior to oral gavage with 1x10^{8} CFUs of streptomycin-resistant *Salmonella typhimurium*. Weight loss and clinical signs of infection were followed until sacrifice at various times post-infection. Initially, p110δ^{KD} mice infected with streptomycin-resistant *S. typhimurium* lost more weight than matched WT mice (Fig. 4.7A). However, 48 hours post-infection and thereafter, weight loss in both *S. typhimurium*-infected WT and p110δ^{KD} mice precipitously dropped, and there was no significant difference between the two genotypes. At 48 hours post-infection, all mice exhibited clinical signs of dehydration, and, surprisingly, WT mice demonstrated decreased colon lengths compared to streptomycin-pretreated only controls and infected p110δ^{KD} mice (Fig. 4.7B). Colons from infected p110δ^{KD} mice were also significantly shorter than those from control mice. However, at 96 hours but not 72 hours post-infection, colons from p110δ^{KD} mice trended toward decreased length compared to

colons from WT mice (Fig. 4.7C,D). There was no significant difference in colonic production of IL-12p40 between infected WT and p110δ^{KD} mice at any time point (Fig. 4.7E).

We next studied the colony forming units (CFUs) recovered from various tissues in each infected WT and p110δ^{KD} mouse. We recovered significantly more *S. typhimurium* from feces collected from the ceca of WT mice compared to p110δ^{KD} mice at 72 hours post-infection, but not at the other time points (Fig. 4.8A). There was no difference in the amount of *S. tyhpimurium* recovered from colonic (distal) feces from WT or p110δ^{KD} mice at any time point (Fig. 4.8B). Interestingly, at tissues proximal to the gastrointestinal tract (cecal lamina propria, MLN), we recovered significantly more *S. typhimurium* from WT compared to p110δ^{KD} mice at 48 and 72 hours post-infection (Fig. 4.8C,D). At tissues distal to the gastrointestinal tract (spleen, liver), there was no difference in the numbers of *S. typhimurium* recovered from WT and p110δ^{KD} mice at any time point (Fig. 4.8E,F).

4.4.4 PI3K p110δ^{KD} mice demonstrate enteric microbiota dysbiosis

Although the functional relationship between altered microbiota and intestinal inflammation remains to be fully established, it is reasonable speculation that changes in the microbiome affect the host's ability to maintain homeostasis (Frank et al., 2011; Sartor, 2010). We conducted a microbial community composition analysis using terminal restriction fragment length polymorphisms (T-RFLP) on fecal samples from eight WT C57BL/6 mice and eight p110δ^{KD} mice. On a principle-components plot, enteric bacterial communities from p110δ^{KD} mice clustered independently of WT mice (Fig. 4.9A). Next,

fragment information for all enzymes was uploaded to Phylogenetic Assignment Tool and assigned to bacterial strains. We compared to the richness and evenness of both populations, demonstrating differences in diversity between the two populations (Fig. 4.9B).

Based on this preliminary analysis, we took a "candidate" approach to demonstrate differences in prevalent bacterial species between p110δ^{KD} and WT mice. Presence of bacterial populations from all four major phyla was analyzed by quantitative real time RT-PCR. There were notable differences in the assessment of individual bacterial species. Overall, more total bacterial DNA was isolated from the feces of p110δ^{KD} mice compared to WT mice (Fig. 4.9C). Furthermore, we detected significantly higher percentages of *Bacteroides fragilis*, *Bifidobacteria* and segmented filmentous bacteria, and a trend toward increased percentages of *Pseudomonas fluorescens* and *Clostridium coccoides*, in fecal samples from p110δ^{KD} mice compared to WT mice. There were no differences in the percentages of other bacteria (*Lactobacillus*, *Faecalibacterium prausnitzii*, *Enterococcus faecalis*, and *Escherichia coli*) isolated from feces of p110δ^{KD} compared to WT mice.

4.4 Discussion

We previously published that p110δ^{KD} macrophages demonstrate defective bactericidal activity against intracellular bacteria, and p110δ^{KD} mice harbor more systemic bacteria compared to WT mice (Uno et al., 2010). In the present studies we sought to mechanistically understand how p110δ regulates bactericidal activity. Studies of other Class I_A PI3K isoforms demonstrate participation in the regulation of bacterial

clearance. For instance, p110β positively regulates autophagy, an alternative bactericidal pathway in macrophages (Dou et al., 2010). While quantitative studies still need to be undertaken, we describe important observations that provide insights into where p110δ acts in pathways of intracellular eradication of bacteria *in vitro* and *in vivo*. Importantly, we have previously shown that IFN-γ-induced activation of $p110\delta^{KD}$ does not enhance microbicidal functions of macrophages (Uno et al., 2010), suggesting that p110δ regulates macrophage responses to IFN-γ. Indeed, p110δ is activated downstream of cytokine receptors, including IFN-γ (Hardy et al., 2009). It has been previously reported that p110δ activity is necessary for the production of IFN-γ in T cells and NK cells (Jarmin et al., 2008; Kim et al., 2007; Soond et al., 2010; Tassi et al., 2007), however, macrophage responses to IFN-γ in $p110\delta^{KD}$ have not been studied. There is mounting evidence suggesting Class I_A PI3Ks are necessary for proper macrophage activation and enhanced bactericidal activity, including autophagy, by IFN-γ (Hardy et al., 2009; Ling et al., 2006; Matsuzawa et al., 2012; Sakai et al., 2006), however p110δ specifically has not been shown to be involved. Isoform-specific studies carried out in the future will further delineate how p110δ signaling in macrophages induces IFN-γ-dependent activation.

We observed decreased Lysotracker®-positive $p110\delta^{KD}$ macrophages, compared to WT macrophages, during infection with K12 *E. coli*. Previously, PI3K p85α and ERK1/2 were shown to co-localize with vacuolar-type H^+-ATPase (V-ATPase), the proton pump responsible for acidifying vesicles, on endosomes and mediate endosomal acidification during infection with Influenza A (Marjuki et al., 2011). In contrast, p110γ negatively regulates endosomal acidification induced by CpG stimulation in macrophages (Hazeki et al., 2011). In general, the flux of PIP_2 and PIP_3 determines the maturation and

fate of phagosomes (Bohdanowicz et al., 2012), and the relative contributions of the PI3K subunits in the maturation and acidification of macrophage phagosomes are unknown. Indeed, the relevance of p110δ specifically in the regulation of phagosome maturation and acidification has not been shown. Furthermore, the method of phagocytosis (i.e., TLR-mediated, immunoglobulin- or complement-opsonized) affects the dynamics of phagosome maturation, and PI3Ks are differentially involved in these processes. For instance, TLR engagement during phagocytosis regulates the kinetics of bacterial uptake and phagosome maturation (Blander and Medzhitov, 2004), and PI3Ks are activated downstream of TLRs (Fukao et al., 2002). Phagocytosis of particles engaging Fcγ receptors (FcγR), which recognize the common chain of Ig molecules, initiates a single wave of PIP_3 production at the phagocytic cup. In contrast, engagement of complement receptors (CR) on the surface of macrophages produces two waves of PIP_3: one at the phagocytic cup that is necessary for the closure and formation of the nascent phagosome, and another that is involved in the propulsion of the phagosome away from the plasma membrane by generation of an actin tail (Thi and Reiner, 2012). It has been proposed that PIP_3 generated by Class I PI3Ks recruits Ca^{2+}-sensing proteins such as synaptotagmins (Syts) that prime SNARE proteins, which mediate cell membrane fusion, and Rab GTPases for the fusion of vesicles with the nascent phagosome (Thi and Reiner, 2012). Here we showed prolonged association of EEA1 at the phagosome in $p110\delta^{KD}$ macrophages, suggesting there is a loss in recruitment of the necessary vesicular membrane components to properly mature the phagosome. Thus, Class I PI3Ks have been implicated in phagosome maturation in several ways, and it will be important to understand which isoforms regulate this process.

We did not show differences in bacterial translocation and/or survival between WT and p110δ^{KD} mice infected with streptomycin-resistant *Salmonella enterica* serovar Typhimurium. However, at 48 and 72 hours post-infection we recovered significantly more CFUs from WT murine cecal lamina propria and MLNs compared to p110δ^{KD} murine tissues, and at 72 hours post-infection we recovered significantly more CFUs from WT cecal feces compared to p110δ^{KD} cecal feces. It is unclear whether this baseline difference in *S. typhimurium* cecal fecal growth affected our results. Indeed, baseline dysbiosis in p110δ^{KD} mice could affect the intraluminal growth of *S. typhimurium*. It has been shown that in B cells with non-functional p110δ, BCR-induced proliferation and function is impaired (Okkenhaug et al., 2002), and it is known that alterations in IgA production in the intestines affect the composition of the microbiota (Mantis et al., 2011). It is thus a short leap to hypothesize that p110δ^{KD} mice have altered intestinal IgA production and subsequent dysbiosis that affects the growth of *S. typhimurium*. However, IgA production in the intestines of p110δ^{KD} mice has not been explored.

Additionally, it is unknown whether translocation of *S. typhimurium* across the intestinal epithelium was different between WT and p110δ^{KD} mice. Indeed, it would be prudent to measure intestinal barrier permeability both at baseline and during infection in WT and p110δ^{KD} mice to understand whether p110δ regulates barrier function. The mere presence of inflammation in p110δ^{KD} mice, but not WT mice, suggests that barrier function is altered by p110δ, at least indirectly by the production of inflammatory mediators (Teshima et al., 2012). Furthermore, *S. typhimurium*-induced colitis in WT and p110δ^{KD} was maximally driven, as demonstrated by loss of 20% of starting body weight just 96 hours post-infection. Thus, while there was a trend for decreased colon length in

p110δ^{KD} compared to WT mice 96 hours post-infection, the experiment had to be terminated due to advanced clinical morbidity in both WT and p110δ^{KD} mice, precluding analysis of later time points where differences between the experimental groups may have emerged. Finally, *Salmonella* species are adept at escaping the macrophage phagosome to enhance their intracellular survival (Hashim et al., 2000). Thus, the differences between WT and p110δ^{KD} macrophage phagosome maturation observed *in vitro* with *E. coli*, which does not escape phagosomes, might not apply to infection with *S. typhimurium*. Indeed, it would be interesting to employ a peritoneal *Listeria monocytogenes* infection in p110δ^{KD} mice for several reasons: (1), intraperitoneal infection with *L. monocytogenes* would eliminate unclear results due to differences in barrier permeability between WT and p110δ^{KD} mice, and (2), eradication of *L. monocytogenes* requires IFN-γ-activated macrophages (Harty and Bevan, 1995; Huang et al., 1993) which has shown to be specifically defective in p110δ^{KD} macrophages (Uno et al., 2010).

PI3K p110δ^{KD} mice demonstrate enteric microbiota dysbiosis. Indeed, global immunologic defects are present in p110δ^{KD} mice (Jou et al., 2002; Okkenhaug et al., 2002; Okkenhaug et al., 2006; Patton et al., 2006) and likely shape the resident enteric microbial communities. However, we demonstrated increased percentages of *Bacteroides fragilis* and *Bifidobacteria* in the feces of p110δ^{KD} mice, an intriguing result given both *B. fragilis* and *Bifidobacteria* demonstrate homeostatic roles in other murine models of intestinal inflammation. *B. fragilis* protects mice from experimental colitis through a single microbial molecule, polysaccharide A (PSA). *B. fragilis* and purified PSA, but not PSA-deficient *B. fragilis*, suppress inflammation through homeostatic TLR2 signaling

and IL-10 production (Mazmanian et al., 2008; Round et al., 2011). *Bifidobacterium breve*, via TLR signaling on $CD103^+$ LPDCs, induces type 1 regulatory T (Tr1) cell production of IL-10 and maintenance of intestinal homeostasis (Jeon et al., 2012). It is possible that these bacteria, while protective in other colitis models, drive colonic inflammation in $p110\delta^{KD}$ mice due to the loss of homeostatic TLR signaling normally regulated by p110δ. Additionally, we observed an increase in percentage of fecal segmented filamentous bacteria (SFB) from $p110\delta^{KD}$ mice compared to WT mice. Indeed, mice lacking proper IgA production (activation-induced cytidine deaminase (AID)-deficient mice) demonstrate increased percentages of fecal SFB (Suzuki et al., 2004), and thus it follows that the defect in BCR signaling and Ig production in B cells lacking functional p110δ (Jou et al., 2002) leads to expansion of enteric SFB populations. Importantly, SFB has been shown to induce a wide range of protective immune responses, including the induction of Th17 cells and mucosal antimicrobial defenses, in the murine intestine (Ivanov et al., 2009; Klaasen et al., 1993). However, it is unknown whether SFB induces pathogenic Th17 immune responses, as seen in murine and human IBDs.

4.5 Materials and Methods

Mice. All mice were maintained on a C57BL/6 background in conventional housing. PI3K $p110\delta^{D910A/D910A}$ ($p110\delta^{KD}$) mice were previously obtained from Dr. Bart Vanhaesebroeck (Queen Mary University of London, London, England). All animal experiments were in compliance with protocols approved by the International Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Reagents. We obtained K12 *Escherichia coli* (MG1655) and *Salmonella enterica* serovar Typhimurium (700720D-5) from ATCC (Manassas, VA), and streptomycin-resistant *Salmonella enterica* serovar Typhimurium (SL1344), GFP-expressing K12 *E. coli* (MG1655) and *Enterococcus faecalis* (OG1RF) from Dr. R. Balfour Sartor (UNC-Chapel Hill, NC). Lysotracker® Red DND-99 was purchased from Life Technologies™ (Grand Island, NY). Recombinant IFN-γ was purchased from R&D Systems (Minneapolis, MN). Luminol, horseradish peroxidase and FITC-dextran were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Rabbit anti-EEA1 (ab2900) was purchased from Abcam (Cambridge, MA). K12 *E. coli* expressing mCherry red plasmid was graciously given to us by Miriam Braunstein (UNC, Chapel Hill, NC). IL-12p40 OptEIA™ ELISA kit was purchased from BD Biosciences (San Jose, CA).

Isolation and Culture of Bone Marrow-Derived Macrophages. Bone marrow-derived macrophages (BMDMs) were isolated and cultured as previously described (Uno et al., 2010).

Detection of Phagosomal Acidification. WT and p110δ^{KD} BMDMs were cultured overnight in antibiotic-free RPMI-1640 with IFN-γ (20 ng/ml) to induce activation. At the same time, an overnight culture of K12 *E. coli* was sub-cultured and grown to logarithmic phase and added to the BMDMs (multiplicity of infection, MOI, = 10). BMDMs were cultured with *E. coli* for one hour. Lysotracker® Red DND-99 (25 nM) was added when there were 30 minutes of culture time left. BMDMs were then rinsed

with cold PBS and fixed in 4% paraformaldehyde for 30 minutes. Nuclei were stained with DAPI and BMDMs were analyzed by fluorescence microscopy. At least 10 fields were randomly chosen and used to quantify the percentage of Lysotracker®-positive cells present.

Phagosomal Maturation Assays. WT and p110δ^{KD} BMDMs were cultured overnight in antibiotic-free RPMI-1640 with IFN-γ (20 ng/ml) to induce activation. At the same time, an overnight culture of K12 *E. coli* was sub-cultured and grown to logarithmic phase and added to the BMDMs (multiplicity of infection, MOI, = 10). BMDMs were cultured with FITC-dextran for several hours before infection with *E. coli* for various time points. BMDMs were then rinsed with cold PBS and fixed in 4% paraformaldehyde for 30 minutes. For immunofluorescence, BMDMs were permeabilized with 0.1% Triton X-100 for 5 minutes, washed and blocked with 10% normal rabbit serum for one hour. BMDMs were stained with anti-EEA1 (1/200) overnight at 4°C and with anti-Rabbit conjugated to Texas Red dye for one hour. Nuclei were stained with DAPI and BMDMs were analyzed by fluorescence confocal microscopy on a Zeiss CLSM 710 Spectral Confocal Laser Scanning Microscope (Thornwood, NY).

Detection of ROS. Detection of ROS by chemiluminescence was performed using luminol as previously described (Li et al., 1999). WT and p110δ^{KD} BMDMs were activated overnight with IFN-γ (20 ng/ml) and cultured with luminol with or without the p110δ-specific inhibitor IC87114 (10 μM) one hour prior to infection with K12 *E. coli*, *E. faecalis* or *S. typhimurium* (MOI = 100). Immediately after addition of bacteria to

BMDMs, chemiluminescence was measured using a Molecular Devices Lmax Luminometer Microplate Reader (Sunnyvale, CA).

***In vivo* Streptomycin-resistant *Salmonella typhimurium* Infection.** Infection with streptomycin-resistant *Salmonella typhimurium* was performed as previously described (Barthel et al., 2003). Six-eight-week-old WT and p110δ^{KD} mice were given streptomycin (20 mg) by oral gavage 24 hours prior to infection and gavaged with $1x10^8$ CFUs of streptomycin-resistant *S. typhimurium*. Total body weight was monitored over the course of the experiment. At 48-, 72- and 96-hours post-infection, mice were sacrificed and tissue collected for analysis of disease and for recovery of bacteria. For determination of CFUs *S. typhimurium* recovered, tissue was weighed and homogenized in PBS. Dilutions were plated on LB agar plates and incubated overnight at 37°C, after which colonies were enumerated and normalized to weight (g) of tissue isolated. Colonic tissue explant cultures were performed as described previously (Hegazi et al., 2005), and levels of IL-12p40 were determined by sandwich ELISA according to manufacturer's instructions.

Microbiota studies. We conducted a microbial community composition analysis using terminal restriction fragment length polymorphisms (T-RFLP) on fecal samples from 8 WT C57BL/6 mice and 8 p110δ^{KD} mice. Mice were randomly assigned to different cages at 6 weeks of age and feces collected at 12 weeks when p110δ^{KD} mice have documented colitis. Bacterial genomic DNA was isolated and amplified by universal primers specific for ribosomal 16S DNA, and amplified products from each mouse were digested by 3 T-

RFLP enzymes (RsaI, MspI and HhaI) and mixed with size standard (Bioventures Map Marker 1000). A principal-coordinates graph representing the relationship between samples in multidimensional space was generated using Qiime. Next, fragment information for all enzymes was uploaded to Phylogenetic Assignment Tool and assigned to bacterial strains. Using diversity functions (Shannon and Simpson), we compared to the richness and evenness of both populations. Presence of bacterial populations from all four major phyla was analyzed by quantitative RT-PCR using bacterial species-specific 16S rRNA primers normalized to total bacterial DNA.

4.6 Figures

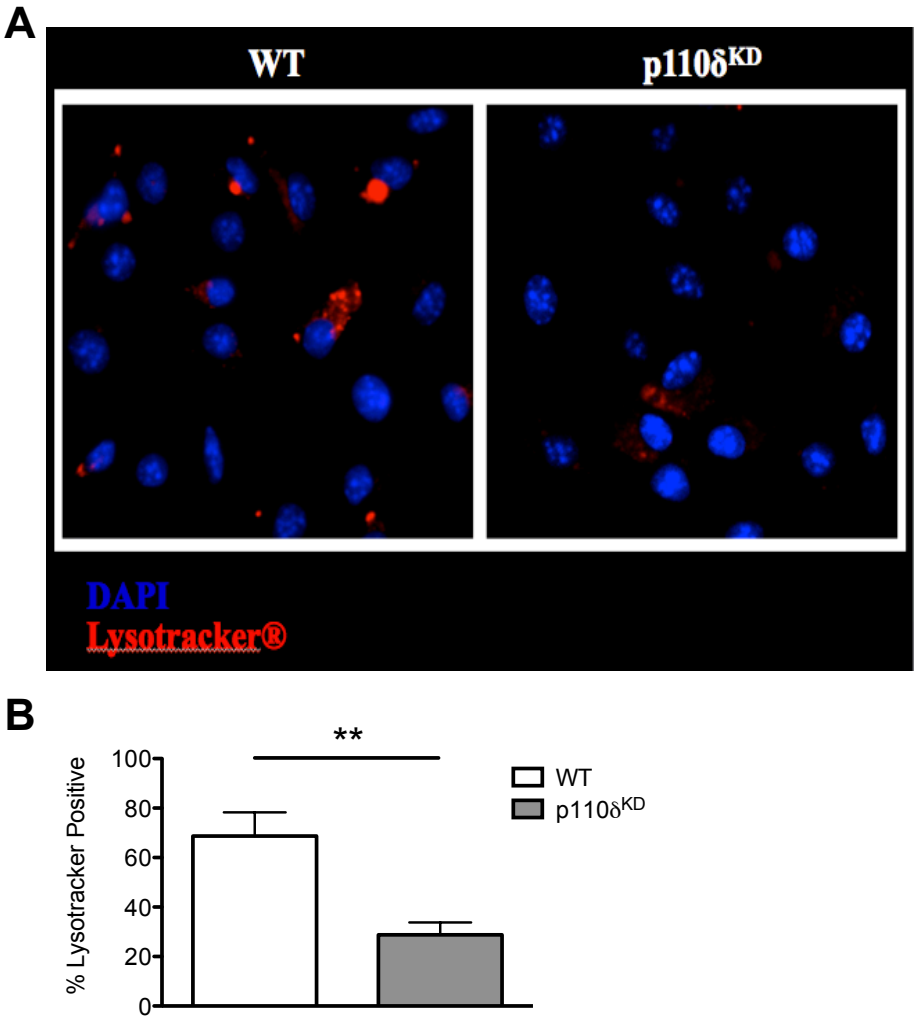


Figure 4.1. IFN-γ-activated, *E. coli*-infected p110δ^{KD} BMDMs demonstrate decreased phagosome acidification. WT and p110δ^{KD} bone marrow-derived macrophages (BMDMs) were activated overnight with IFN-γ (20 ng/ml) and cultured with K12 *E. coli* for one hour. Lysotracker® Red DND-99 (25 nM) was added for the final 30 minutes of culture. Three independent experiments were performed. (A) Pictures shown are representative of results from IFN-γ-activated, *E. coli*-infected BMDMs. (B) Percentage of Lysotracker®-positive cells was quantified from at least 10 random fields for each experimental group by a colleague (SMR) blinded to the experimental groups. Error bars represent mean ± SEM for three independent experiments (**, $p<0.005$ versus WT BMDMs).

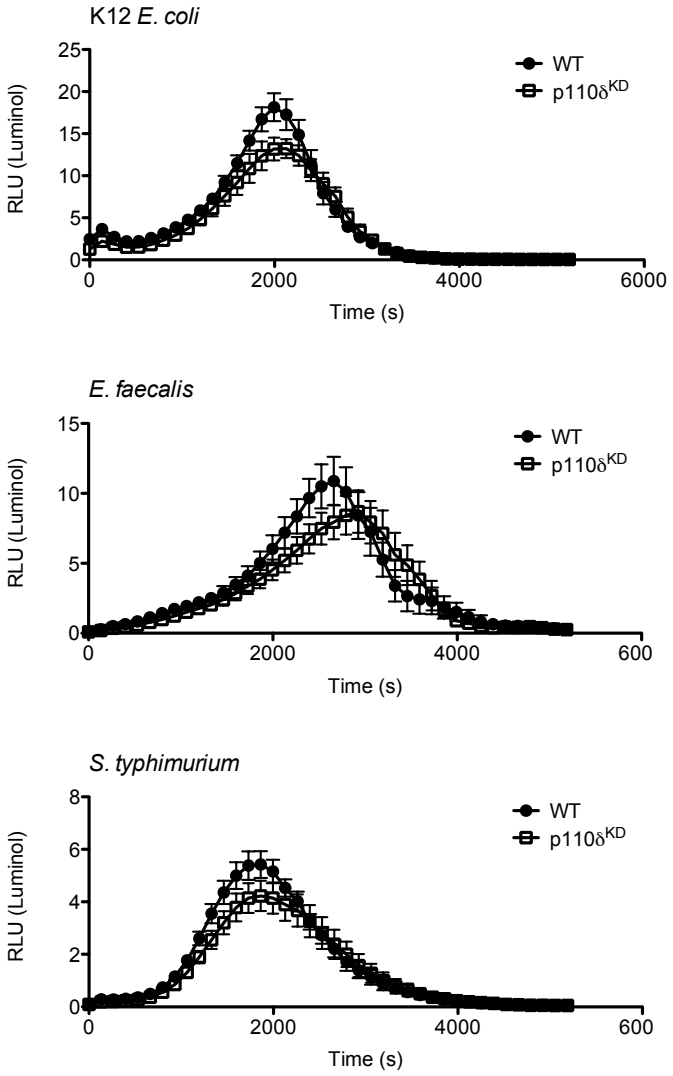


Figure 4.2. Reactive oxygen species production is diminished in p110δ^{KD} BMDMs. WT and p110δ^{KD} BMDMs were cultured with either K12 *E. coli*, *E. faecalis* or *S. typhimurium* in the presence of luminol and chemiluminescence was measured over 90 minutes. Graphs representative of typical results from three independent experiments are shown. Error bars represent mean ± SEM from triplicates per experiment.

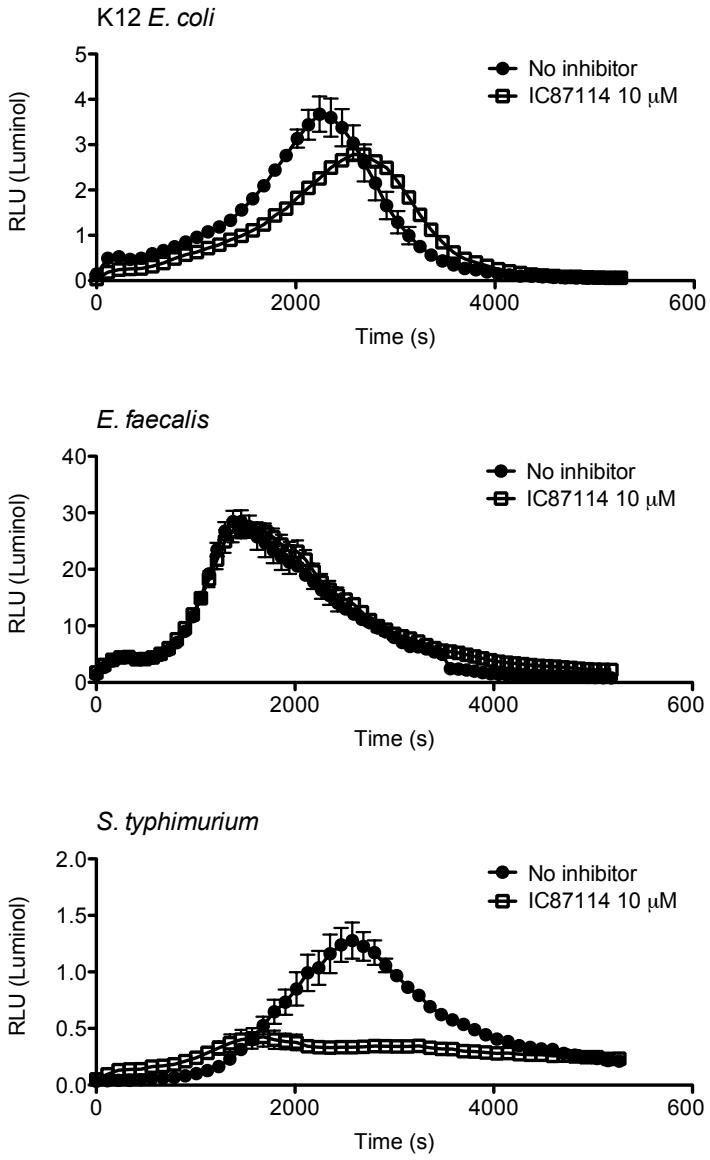


Figure 4.3. Inhibition of p110δ impairs reactive oxygen species production in WT BMDMs. IFN-γ-activated WT BMDMs were cultured in the presence or absence of the p110δ-specific inhibitor IC87114 (10 μM) and various microbes (K12 *E. coli*, *E. faecalis* or *S. typhimurium*), and chemiluminescence was measured over 90 minutes. Graphs representative of typical results from three independent experiments are shown. Error bars represent mean ± SEM from triplicates per experiment.

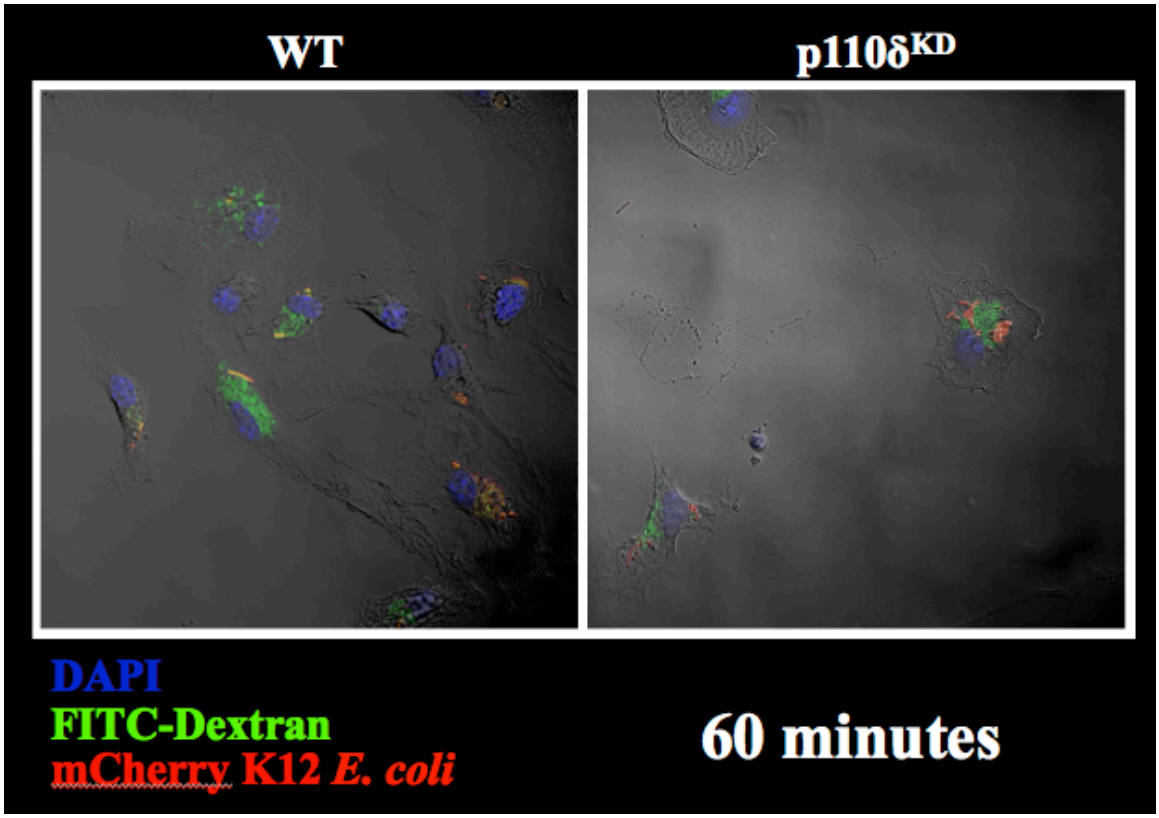


Figure 4.4. K12 *E. coli*-laden phagosome colocalization with FITC-dextran is decreased in IFN-γ-activated p110δ^{KD} BMDMs. WT and p110δ^{KD} BMDMs were activated overnight with IFN-γ and loaded with FITC-dextran. After 60 minutes of culture with K12 *E. coli*, cells were fixed and analyzed by immunofluorescence confocal microscopy. Pictures representative of typical results from three independent experiments are shown.

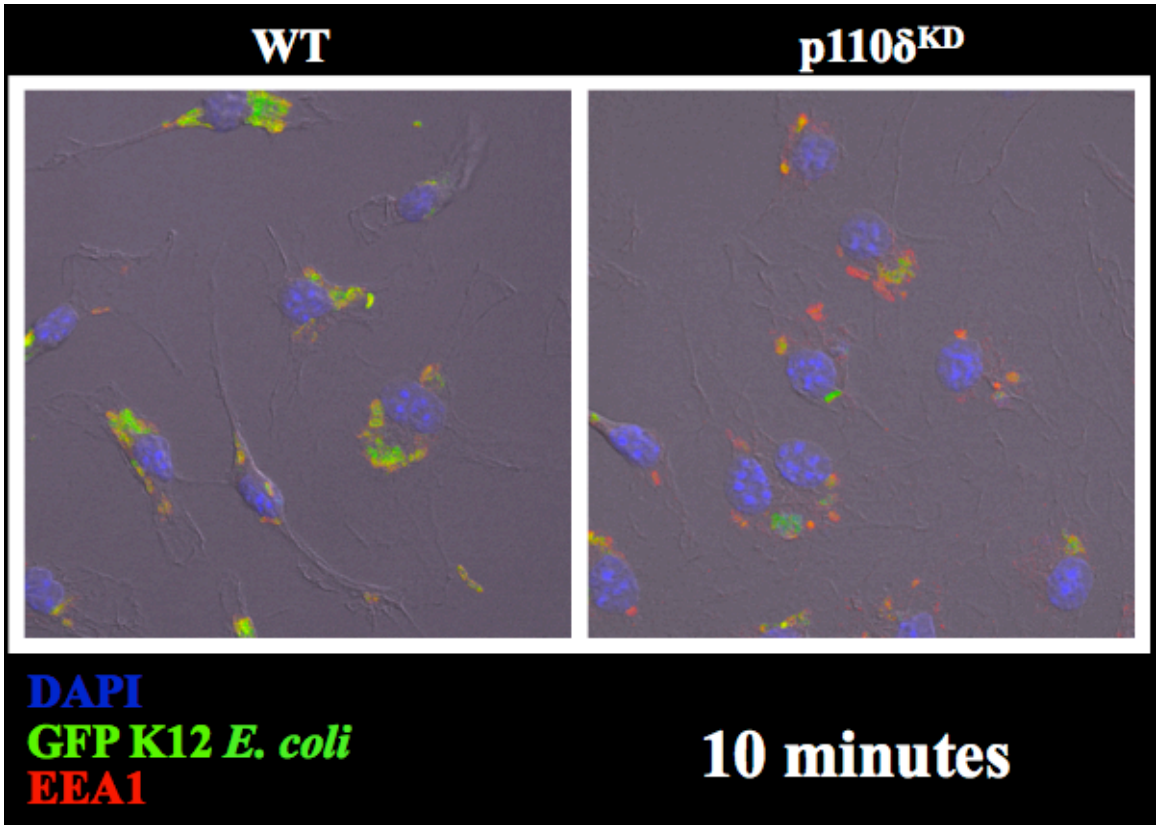


Figure 4.5. K12 *E. coli*-laden phagosome colocalization with EEA1 is decreased in IFN-γ-activated p110δ^{KD} BMDMs after 10 minutes. WT and p110δ^{KD} BMDMs were activated overnight with IFN-γ and cultured with K12 *E. coli* for 10 minutes. Cells were then fixed and stained for EEA1 and analyzed by immunofluorescence confocal microscopy. Pictures representative of typical results from three independent experiments are shown.

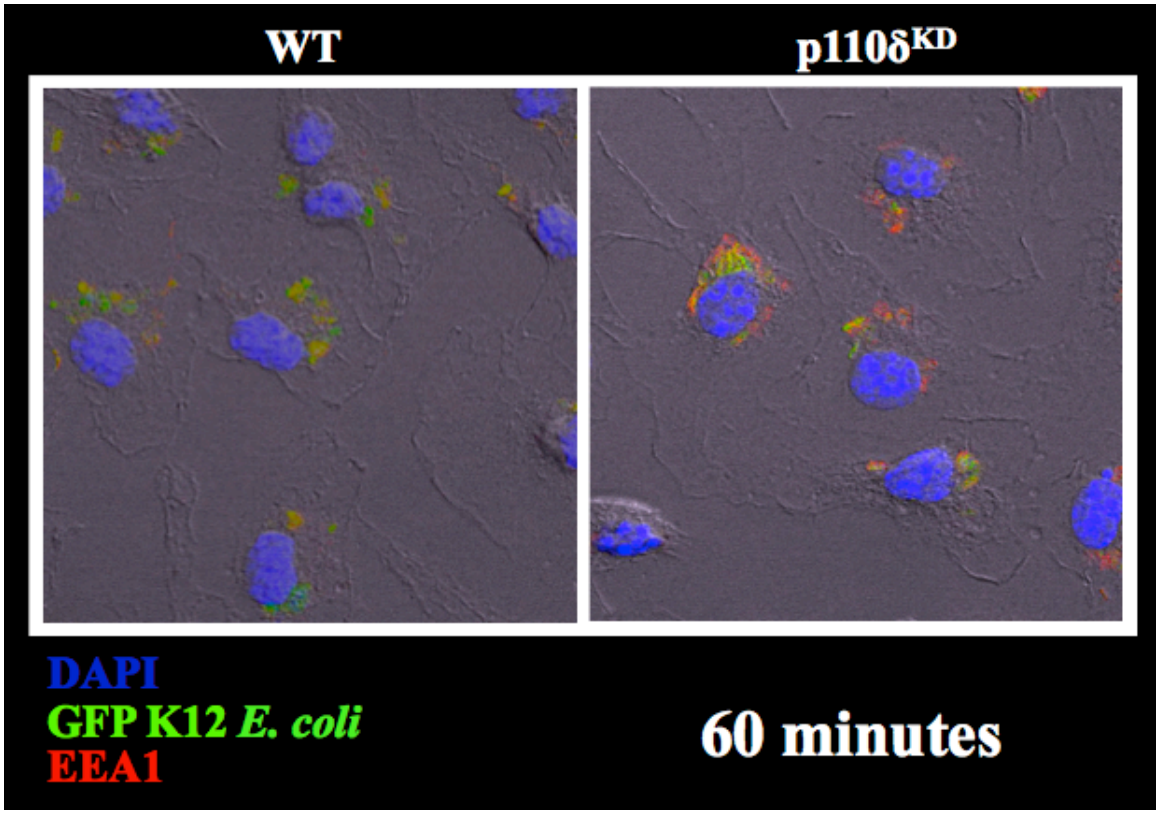


Figure 4.6. K12 *E. coli*-laden phagosome colocalization with EEA1 is decreased in IFN-γ-activated p110δ^{KD} BMDMs after 60 minutes. WT and p110δ^{KD} BMDMs were activated overnight with IFN-γ and cultured with K12 *E. coli* for 60 minutes. Cells were then fixed and stained for EEA1 and analyzed by immunofluorescence confocal microscopy. Pictures representative of typical results from three independent experiments are shown.

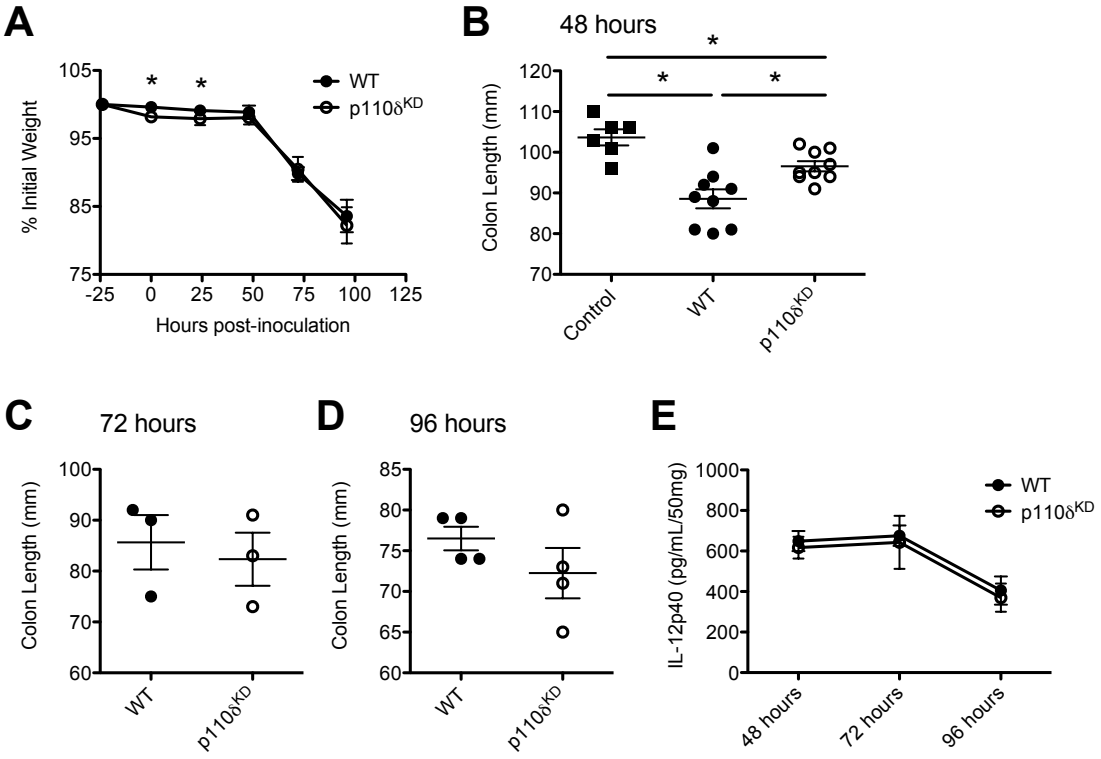


Figure 4.7. Streptomycin-resistant *Salmonella typhimurium* colitis is not worsened in p110δ^{KD} mice. WT (48 hours, *n* = 8; 72 hours, *n* = 3; 96 hours, *n* = 4) and p110δ^{KD} (48 hours, *n* = 9; 72 hours, *n* = 3; 96 hours, *n* = 4) mice were orally gavaged with streptomycin (20 mg) 24 hours prior to oral infection with streptomycin-resistant *Salmonella typhimurium*. Body weight loss was monitored over the course of the experiment, and mice were sacrificed at 48-, 72- and 96-hours post-infection. (A) Percent initial weight of *S. typhimurium*-infected WT and p110δ^{KD} mice was monitored up to 96 hours post-infection. Error bars represent mean ± SEM (*, $p<0.05$ versus WT). (B,C,D) Colon lengths were measured at 48 hours (B), 72 hours (C) and 96 hours (D) post-infection. Error bars represent mean ± SEM of two independent experiments (*, $p<0.05$ versus WT). (E) IL-12p40 production was measured by ELISA from colonic tissue explant cultures.

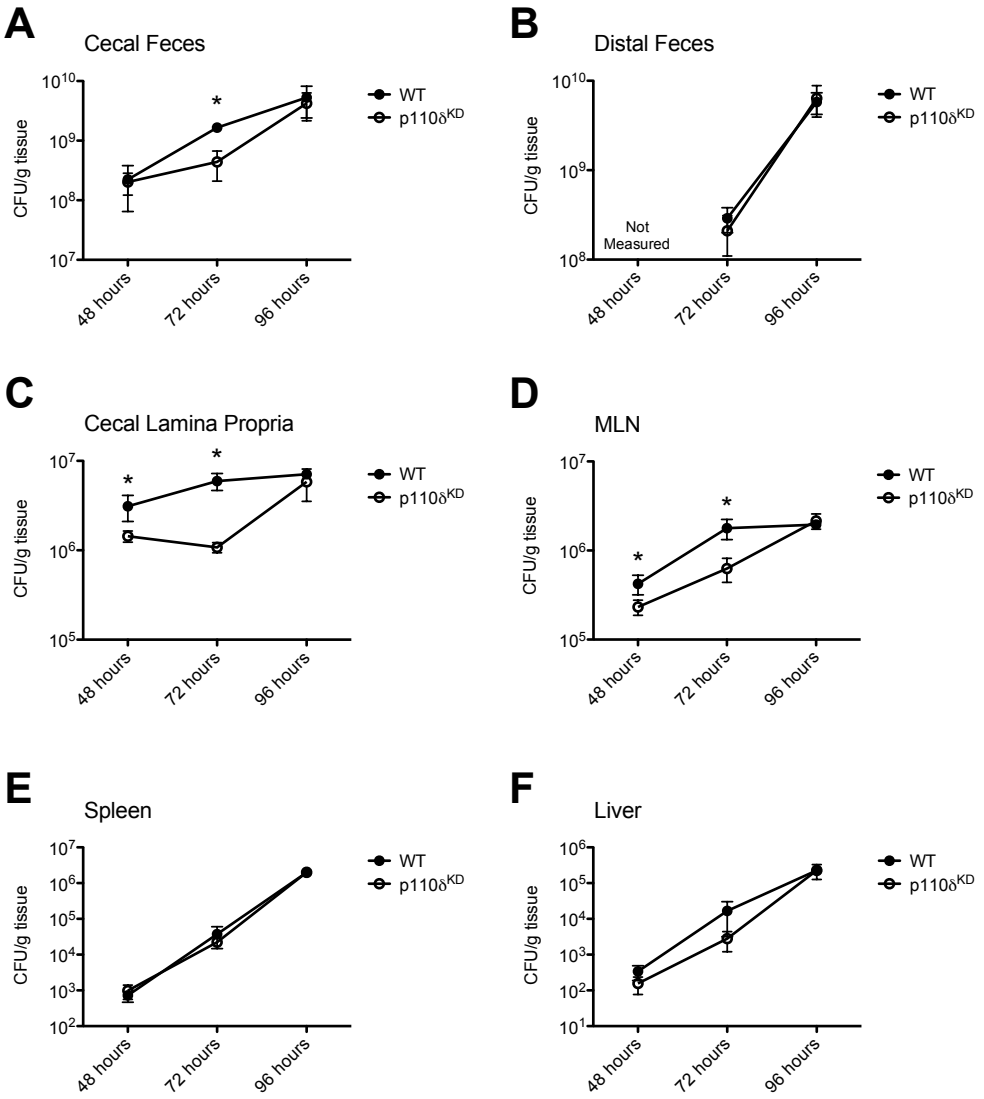
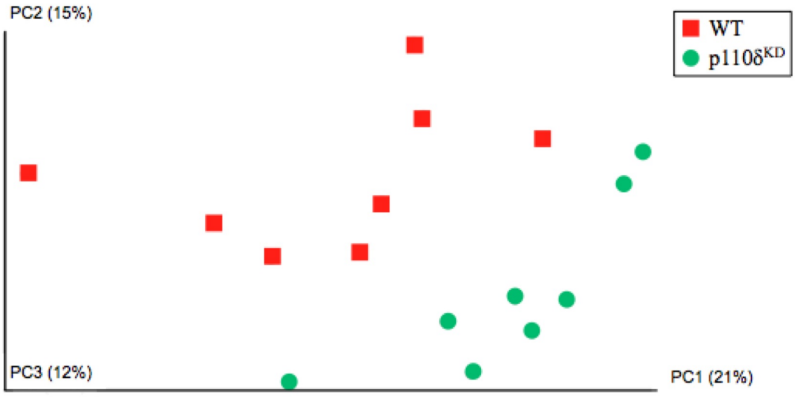


Figure 4.8. *Salmonella* CFUs recovered from cecal feces, cecal lamina propria and mesenteric lymph nodes of p110δ^{KD} mice was decreased compared to that from WT mice. WT (48 hours, *n* = 8; 72 hours, *n* = 3; 96 hours, *n* = 4) and p110δ^{KD} (48 hours, *n* = 9; 72 hours, *n* = 3; 96 hours, *n* = 4) mice were orally gavaged with streptomycin (20 mg) 24 hours prior to oral infection with streptomycin-resistant *Salmonella typhimurium*. Mice were sacrificed at 48-, 72- and 96-hours post-infection, and various tissues were collected for determination of CFUs. Results are normalized to weight (g) of tissue. (A-F) Streptomycin-resistant *Salmonella typhimurium* was recovered from WT and p110δ^{KD} cecal feces (A), distal colonic feces (B), cecal lamina propria (C), MLN (D), spleen (E) and liver (F) at 48-, 72- and 96-hours post-infection and normalized to weight (g) of tissue. Error bars represent mean ± SEM from two independent experiments (*, $p<0.05$ versus WT).

A



B

	Number	Shannon	Shannon Evenness	Simpson
WT	18.1	2.3	0.82	0.86
p110δ^{KD}	21	2.63	0.87	0.91
p value	0.41	0.12	0.07	0.07

C

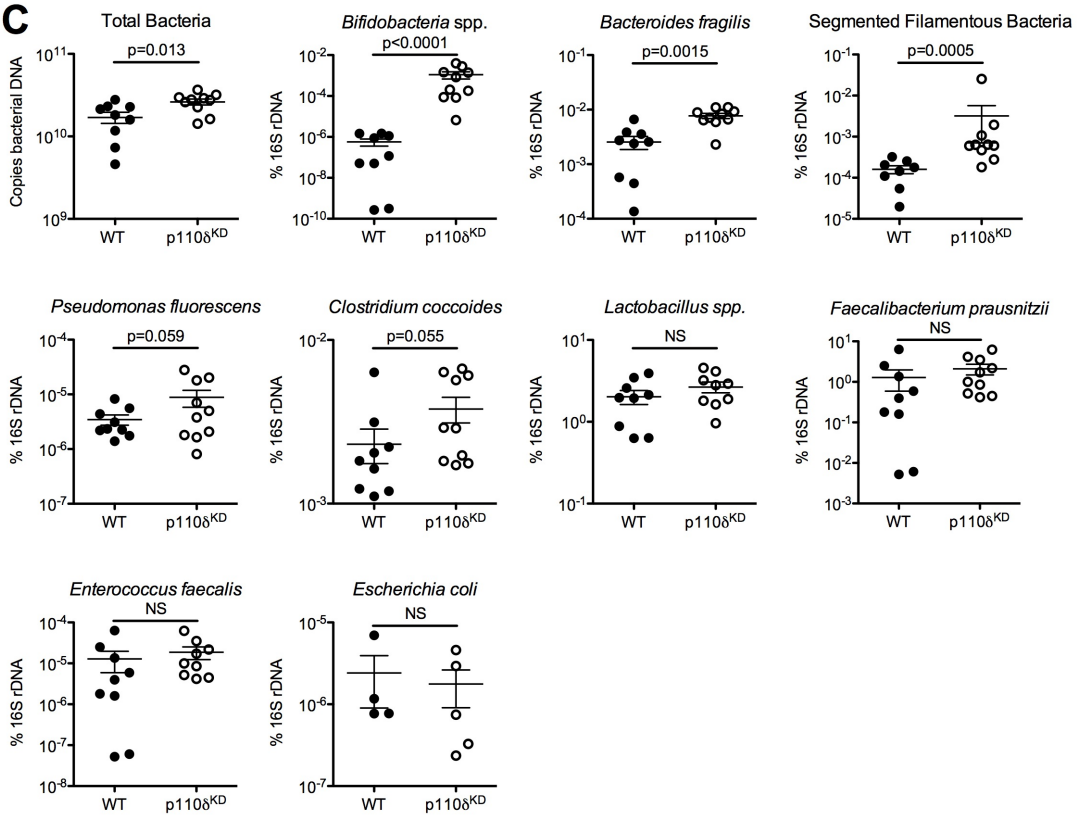


Figure 4.9. PI3K p110δ^{KD} mice demonstrate unique microbial community composition. Bacterial DNA was recovered from fecal samples from WT (n = 9) and p110δ^{KD} (n = 10) mice for analysis of microbial community composition by T-RFLP and

quantitative RT-PCR. (A) Principle components plots were made from T-RFLP analysis using Qiime. (B) Diversity and evenness of microbial communities were analyzed using Shannon and Simpson diversity functions. (C) Total bacterial numbers and percentages of various bacterial species were determined using quantitative RT-PCR. Error bars represent mean ± SEM of two independent experiments (NS, not significant).

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Overview

The human IBDs, CD and UC, result from an incompletely defined and complex interaction between host immune responses, genetic susceptibility, environmental factors and the enteric luminal contents (Xavier and Podolsky, 2007). IBD-associated SNPs have identified macrophages and DCs as key players in IBD pathogenesis (Jostins et al., 2012). IBDs represent an increasing burden on global and United States health care systems (Park and Bass, 2011). Thus, there is a pressing need for understanding IBD pathogenesis and for the development of specific, novel therapeutic targets.

We identified innate immune cell p110δ as a critical regulator of intestinal homeostasis through modulating innate immune cell responses to microbes and promoting subsequent homeostatic adaptive immune responses, and enhancing intracellular clearance of microbes in the gastrointestinal tract. In the absence of functional p110δ ($p110\delta^{KD}$), mice develop chronic colonic inflammation characterized by patchy inflammatory infiltrates, epithelial hyperplasia, intraepithelial lymphocytosis and hypersecretion of Th1/Th17 cytokines (Uno et al., 2010). Furthermore, colonic inflammation in $p110\delta^{KD}$ mice is microbiota-dependent. Indeed, p110δ activity in colonic $CD11b^{+}$ LPMCs provides a functional "brake" on TLR-induced responses, allowing for

the maintenance of intestinal homeostasis. Colonic $CD11b^+$ LPMCs from $p110\delta^{KD}$ mice also produced significantly more IL-12p40 and less IL-10 in response to heat-killed *E. coli*. While $p110\delta^{KD}$ mice without mature T and B cells ($Rag1^{-/-}/p110\delta^{KD}$) did develop mild histopathologic colitis, reconstitution of $Rag1^{-/-}/p110\delta^{KD}$ mice with WT naïve T cells induced severe colitis. We propose that chronic colitis in $p110\delta^{KD}$ mice is driven by LPMCs producing inappropriate inflammatory mediators in response to the enteric microbiota and subsequently promoting the development of pathogenic T cell responses. Furthermore, enhanced survival of intracellular microbes provides chronic stimulation of already hyper-inflammatory innate immune cells, perpetuating inflammation in the intestines.

Understanding how p110δ regulates innate immune cell responses to microbes in the GALT has significant implications for human IBDs. We show that *PIK3CD* expression from intestinal tissue biopsies, both inflamed and non-inflamed, from patients with CD inversely correlated with the ratio of *IL12* to *IL10*, suggesting that human p110δ regulates the balance of homeostatic cytokines in the intestines. Furthermore, *PIK3CD* expression in tissue from patients with CD was significantly decreased compared to tissue from normal, non-inflamed control patients. This is surprising given that expression of *PIK3CD* is highly enriched in leukocytes, and intestines from patients with CD demonstrate significant numbers of infiltrating inflammatory cells. Interestingly, we did not demonstrate a similar decrease in *PIK3CD* expression in intestinal biopsy specimens from patients with UC. However we need a larger pool of samples from patients with UC to confirm these findings, as the cohort of UC patients was small. The

implications for CD-specific downregulation of *PIK3CD* expression are exciting and may lead to development of CD-specific therapies.

Most studies of *PIK3CD* expression focus on posttranslational regulation, as it was shown that changes in p110δ levels do not change acutely in response to various agonists (Kok et al., 2009). We have shown that p110δ expression is induced in BMDMs stimulated with TLR agonists and in the intestines upon colonization with enteric microbiota (Fig. 2.6) (Uno et al., 2010). This may represent a novel concept in p110δ regulation that needs further study. Indeed, *PIK3CD* lies within an IBD susceptibility locus (1p36) (Cho et al., 1998), and we speculate that an IBD-associated SNP lies within a regulatory region of *PIK3CD* leading to decreased expression in IBD susceptible individuals. Thus the full impact that p110δ has on IBD pathogenesis remains to be elucidated.

Finally, p110δ inhibitors are being developed in the clinic for the treatment of inflammatory disorders (Durand et al., 2013; Haylock-Jacobs et al., 2011; Matteoli et al., 2010; Ying et al., 2012). Both human and murine studies strongly implicate innate p110δ signaling in the maintenance of intestinal homeostasis. While p110δ inhibition has the desired effects of inhibiting adaptive immunity, blockade of p110δ in diseases where innate immune processes are central drivers of pathogenesis, such as IBDs, may actually be harmful.

5.2 IBD Heterogeneity and p110δ

Differences between CD and UC, the two major subtypes of IBDs, suggest that there are diverse etiologies driving different forms of IBDs. This presents a barrier to

therapeutic development, as human IBDs remain poorly defined clinically, and there is significant overlap in the way CD and UC present clinically, endoscopically and histopathologically. Furthermore, even less is understood about the more rare IBDs, including lymphocytic colitis and collagenous colitis (both types of "microscopic colitis") and eosinophilic colitis. These inflammatory conditions are marked by chronic, watery diarrhea, and the incidence is increasing worldwide (Bohr et al., 1995; Fernandez-Banares et al., 1999; Olesen et al., 2004; Pardi et al., 2007; Stewart et al., 2011; Williams et al., 2008). Interestingly, intraepithelial lymphocytosis is common in these human disorders (Yen and Pardi, 2012), and we described this distinct finding in the colons of p110δ^{KD} mice (Uno et al., 2010). This mouse model presents a unique opportunity to understand how intraepithelial lymphocytosis develops and affects IBD pathogenesis.

Colitis in p110δ^{KD} mice is 100% penetrant but relatively mild compared to other well-characterized murine models of IBDs. Genetic background greatly affects disease outcome in rodent models of IBDs, as unknown modifier genes contribute to disease susceptibility and phenotype (Buchler et al., 2012; Valatas et al., 2013). Indeed, host genotype is but one reason human IBDs are so heterogeneous. Although we found low expression of *PIK3CD* in intestinal tissue biopsies from patients with CD, the patient pool was small. Thus, there could be differences in *PIK3CD* expression between subtypes of CD, between locations of biopsies (e.g., ileum versus distal colon) or depending on the disease status of the patient (e.g., remission versus flare). To further characterize *PIK3CD* expression in human IBDs, it will be important to expand our patient population and correlate clinical parameters with *PIK3CD* expression in specific cell populations from specific sites along the gastrointestinal tract.

The p110δ^{KD} mouse is currently maintained on a C57BL/6 background, a strain that is relatively resistant to autoimmune pathologies. To further understand how host genetics alters IBDs, it would be interesting to backcross p110δ^{KD} mice onto other more susceptible genetic backgrounds (e.g., C3H/HeJBir, BALB/c) and identify quantitative trait loci (QTL) contributing to disease variability. This approach may also uncover novel functions of p110δ.

5.3 Development of Novel Tools to Study p110δ Function in Innate Immunity

While the T cell adoptive transfer model of colitis allowed us to study the effects of defective p110δ activity in innate immune cells on T cells, ideally we would use a cell-specific p110δ^{KD} knock-in mouse to determine whether intestinal inflammation develops spontaneously as in the p110δ^{KD} mouse. We could create a macrophage-specific p110δ^{KD} knock-in mouse using a previously described genetic engineering strategy (Skvorak et al., 2006). This strategy utilizes a construct with both the WT and mutant alleles present, but only the WT allele is expressed at baseline. Expression of the mutant allele is induced only when Cre recombinase is expressed and removes the WT allele from the construct. We will be able to restrict the expression of the mutant p110δ allele to macrophages by using Cre recombinase expressed under the control of the macrophage-specific lysozyme-2 (LysM) promoter (LysM^{Cre}). The development of this mouse will greatly enhance our ability to understand the role of macrophage p110δ in IBD pathogenesis.

One of the obvious criticisms of our studies is the use of BMDMs as a surrogate cell type for colonic macrophages. While we always tried to confirm results from BMDMs in colonic macrophages when technically feasible, these two cells types are

distinctly different. Ideally, colonic macrophages would be used in all studies; however, realistically this is difficult for some molecular experiments due to the low numbers of $CD11b^+$ cells in the colon as well as potential phenotypic changes during the isolation process. Furthermore, an intestinal macrophage-specific knock-in inactive $p110\delta^{KD}$ mouse would be an indispensable tool for the study of p110δ in intestinal homeostasis, but there are as yet no known intestinal macrophage-specific markers.

5.4 PI3K p110δ Expression and Regulation in IBDs

PIK3CD is mainly regulated at the transcriptional level in both mice and humans, and transcript levels correlate well with protein levels (Kok et al., 2009). We previously showed that p110δ mRNA and protein levels are increased in macrophages following TLR stimulation, and that colonization of the gastrointestinal tract with commensal microbiota induces intestinal p110δ expression (Fig. 2.6) (Uno et al., 2010). To continue these studies, we would characterize chromatin modifications and transcription factor recruitment at the *Pik3cd* locus in intestinal macrophages from GF mice exposed to bacteria.

While *PIK3CD* mRNA levels correlate well with p110δ levels, it is unknown whether this holds true for tissues from patients with IBDs. It would be informative to measure protein levels and identify subcellular localization of p110δ in tissues obtained from patients with IBD. We could obtain whole tissue for use in western blot and immunofluorescence analysis, as human p110δ antibodies are widely available and well characterized (Vanhaesebroeck et al., 1997). Additionally, we would look for changes in chromatin modifications at the *PIK3CD* locus to identify potential epigenetic

mechanisms that could account for the decrease in *PIK3CD* expression in tissues from patients with CD. These studies may reveal novel mechanisms of regulating p110δ expression during health and disease that could be exploited for the development of IBD therapeutics.

5.5 The Enteric Microbiota, p110δ and IBDs

We showed that the composition of enteric microbial communities from $p110\delta^{KD}$ mice is different from that of WT mice. Although the functional relationship between altered microbiota and intestinal inflammation remains to be fully established, it is reasonable speculation that changes in the microbiome affect the host's ability to maintain homeostasis (Frank et al., 2011; Sartor, 2010). A microbiota "knockout" of specific types of bacteria does not yet exist but would be enormously helpful in delineating the effects of certain microbes on IBD pathogenesis. A major barrier to understanding IBD pathogenesis is a veritable lack of tools for studying the effect of the enteric microbiota on disease development and severity. It has already been shown in numerous rodent models of intestinal inflammation that in the absence of the enteric microbiota, IBD does not develop (Nell et al., 2010). However, human IBDs are not cured with antibiotics, and the efficacy of antibiotics as primary or adjuvant therapy in IBD is controversial (Sartor, 2004; Wang et al., 2012).

Exciting technological advances in bioinformatics have made collection and analysis of massive data sets manageable. It is now possible to employ deep sequencing on microbial communities to identify microbes down to the species level for some taxa. Furthermore, the depth of reads generated by deep sequencing allows for identification of

less abundant taxa. However, despite showing considerable variability between individual's enteric microbiota compositions, studies of microbial function demonstrate widespread functional conservation between different individuals (Consortium, 2012; Qin et al., 2012). Thus, the functional characterization of microbial communities is necessary to understand the potential metabolic impact changes in the community may have on an individual in health and disease. This next step will employ whole-population integrated "-omics" studies, including metagenomics, metatranscriptomics, metabolomics and metaproteomics, to functionally characterize microbial communities. Additionally, studies on enteric resident archaea, viruses, fungi and protists are necessary.

A complete study to understand the role of the microbiota in colitis pathogenesis in the p110δ^{KD} mouse must contain many elements. First, a robust time course of sample collection is necessary to follow compositional changes before and during when inflammation develops in the p110δ^{KD} mouse. Second, the location of sample collection importantly allows for characterization of specific, ecologically distinct niches. We would collect luminal and mucosal-associated samples from different areas of the gastrointestinal tract (e.g., cecum, proximal colon, distal colon). Third, we would combine "-omics" studies on bacterial samples with complete characterization of host colitis. Thus, we would be able to combine robust techniques for the community and functional characterization of the microbiota with important host parameters of disease progression.

5.6 Macrophage Intracellular Microbe Eradication and p110δ

Macrophages with impaired p110δ function demonstrate impaired intracellular eradication of several species of gram-negative enteric bacteria (Fig. 2.5) (Uno et al., 2010). To determine whether this defect extends to gram-positive enteric bacteria, we will perform gentamicin protection assays with WT and p110δ^{KD} macrophages using *Enterococcus faecalis*, *Staphylococcus aureus*, *Clostridium leptum*, and *Listeria monocytogenes*. Macrophage proteins that specifically regulate intracellular bactericidal activity of gram-negative bacteria exist (Berger et al., 2010). It would be interesting to know whether regulation of bacterial eradication by p110δ is restricted to certain types of bacteria. Additionally, macrophage eradication of fungi (i.e., *Histoplasma capsulatum*) may also be affected by p110δ activity.

To continue characterizing how p110δ regulates intracellular eradication of bacteria in macrophages, we would first need to supplement our documented observations with quantitative colocalization analyses (Fig. 4.4-4.6). Next, we would further follow GFP *E. coli*-laden phagosome maturation using immunofluorescence for markers of late phagosomes (LAMP-2, Rab7) and phagolysosomes (LAMP-1). We would measure phagolysosomal pH in WT and p110δ^{KD} macrophages infected with *E. coli*. To determine whether p110δ regulates the recruitment of NADPH oxidase to the phagosome, we would localize NADPH oxidase subunits to GFP *E. coli*-laden phagosomes in WT and p110δ^{KD} macrophages. Finally, we can measure the activity of proteases within phagosomes, as previously described (Yates et al., 2007), from WT and p110δ^{KD} macrophages infected with *E. coli*. Ideally these studies would be conducted in

intestinal macrophages. The conclusion of these studies will fully characterize the role of p110δ in regulating macrophage intracellular eradication of bacteria.

5.7 PI3K p110δ as a Therapeutic Target in IBDs

The Class I_A PI3Ks and their downstream signaling molecules are popular targets for anti-cancer therapy; inhibition of p110 isoforms, Akt or mTOR has shown varying effects on tumor growth and progression (Rodon et al., 2013). While there have been no reported side effects of intestinal inflammation with the use of p110δ-specific inhibitors, it is possible, given our studies, that p110δ inhibition in susceptible individuals may induce IBDs. Because PI3Ks regulate diverse pathways involved in cell growth, proliferation, differentiation and survival, it will be prudent to target specific pathways downstream of PI3K that regulate cytokine production and intracellular bacterial eradication for the therapeutic use in IBDs.

One promising candidate is GSK-3β. Inhibition of GSK-3β ameliorates murine colitis caused by chronic administration of DSS (Hofmann et al., 2010). While DSS as an IBD model has many weaknesses, this study suggests that inhibition of GSK-3β, normally a function of PI3Ks, can reverse exaggerated immune responses to the enteric microbiota during intestinal inflammation. We showed that GSK-3β inhibition in TLR-stimulated $p110\delta^{KD}$ BMDMs enhanced the production of IL-10 and decreased the production of IL-12p40 (Fig. 3.4E-H). The next step is *in vivo* administration of the GSK-3β inhibitor SB216763 to $p110\delta^{KD}$ mice to determine whether this can prevent colitis development or reverse damages from chronic intestinal inflammation. A powerful tool available is the GF $p110\delta^{KD}$ mouse colony at the UNC Center for Gastrointestinal

Biology and Disease (CGIBD) Gnotobiotic Facility. To remove some of the variability of disease onset seen in p110δ^{KD} mice, we can coordinately initiate intestinal inflammation in a cohort of GF p110δ^{KD} mice by colonizing them with a predefined commensal enteric microbiota. Treatment with SB216763 would occur either at the onset of microbiota colonization or several weeks after colonization to address the effects of GSK-3β inhibition on IBD development and on therapy during active inflammation.

5.8 Conclusion

Defects in innate immune cell functions are central events in the pathogenesis of IBDs (Jostins et al., 2012). We provide compelling evidence for the involvement of the PI3K catalytic subunit p110δ in maintaining intestinal homeostasis in mice and humans through regulating innate immune cell responsiveness to microbial stimuli. Additionally, p110δ regulates intracellular eradication of bacteria in macrophages. In the absence of functional p110δ, chronic stimulation of macrophages from persistent bacteria induces dysregulated cytokine production, promoting pathogenic T cell differentiation and the development of chronic intestinal inflammation. Understanding the role of p110δ in these processes in rodents and humans will elucidate novel pathogenesis-based therapeutic strategies to treat human IBDs.

REFERENCES

Abe, K., K.P. Nguyen, S.D. Fine, J.H. Mo, C. Shen, S. Shenouda, M. Corr, S. Jung, J. Lee, L. Eckmann, and E. Raz. 2007. Conventional dendritic cells regulate the outcome of colonic inflammation independently of T cells. *Proc Natl Acad Sci U S A* 104:17022-17027.

Abreu, M.T. 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol* 10:131-144.

Agrawal, A., and B. Pulendran. 2004. Anthrax lethal toxin: a weapon of multisystem destruction. *Cell Mol Life Sci* 61:2859-2865.

Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2:675-680.

Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124:783-801.

Aksoy, E., S. Taboubi, D. Torres, S. Delbauve, A. Hachani, M.A. Whitehead, W.P. Pearce, I. Berenjeno-Martin, G. Nock, A. Filloux, R. Beyaert, V. Flamand, and B. Vanhaesebroeck. 2012. The p110δ isoform of the kinase PI(3)K controls the subcellular compartmentalization of TLR4 signaling and protects from endotoxic shock. *Nat Immunol* 13:1045-1054.

Aksoy, E., W. Vanden Berghe, S. Detienne, Z. Amraoui, K.A. Fitzgerald, G. Haegeman, M. Goldman, and F. Willems. 2005. Inhibition of phosphoinositide 3-kinase enhances TRIF-dependent NF-κB activation and IFN-β synthesis downstream of Toll-like receptor 3 and 4. *Eur J Immunol* 35:2200-2209.

Al-Alwan, M.M., K. Okkenhaug, B. Vanhaesebroeck, J.S. Hayflick, and A.J. Marshall. 2007. Requirement for phosphoinositide 3-kinase p110δ signaling in B cell antigen receptor-mediated antigen presentation. *J Immunol* 178:2328-2335.

Andrade, R.M., M. Wessendarp, M.J. Gubbels, B. Striepen, and C.S. Subauste. 2006. CD40 induces macrophage anti-*Toxoplasma gondii* activity by triggering autophagy-dependent fusion of pathogen-containing vacuoles and lysosomes. *J Clin Invest* 116:2366-2377.

Antoniv, T.T., and L.B. Ivashkiv. 2011. Interleukin-10-induced gene expression and suppressive function are selectively modulated by the PI3K-Akt-GSK3 pathway. *Immunology* 132:567-577.

Arita, M., M. Yoshida, S. Hong, E. Tjonahen, J.N. Glickman, N.A. Petasis, R.S. Blumberg, and C.N. Serhan. 2005. Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. *Proc Natl Acad Sci U S A* 102:7671-7676.

Arranz, A., C. Doxaki, E. Vergadi, Y. Martinez de la Torre, K. Vaporidi, E.D. Lagoudaki, E. Ieronymaki, A. Androulidaki, M. Venihaki, A.N. Margioris, E.N. Stathopoulos, P.N. Tsichlis, and C. Tsatsanis. 2012. Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization. *Proc Natl Acad Sci U S A* 109:9517-9522.

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

Backer, J.M. 2008. The regulation and function of Class III PI3Ks: novel roles for Vps34. *Biochem J* 410:1-17.

Baetselier, P.D., B. Namangala, W. Noel, L. Brys, E. Pays, and A. Beschin. 2001. Alternative versus classical macrophage activation during experimental African trypanosomosis. *Int J Parasitol* 31:575-587.

Barthel, M., S. Hapfelmeier, L. Quintanilla-Martinez, M. Kremer, M. Rohde, M. Hogardt, K. Pfeffer, H. Russmann, and W.D. Hardt. 2003. Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infect Immun* 71:2839-2858.

Baumgart, D.C., D. Metzke, O. Guckelberger, A. Pascher, C. Grotzinger, I. Przesdzing, Y. Dorffel, J. Schmitz, and S. Thomas. 2011. Aberrant plasmacytoid dendritic cell distribution and function in patients with Crohn's disease and ulcerative colitis. *Clin Exp Immunol* 166:46-54.

Baumgart, D.C., D. Metzke, J. Schmitz, A. Scheffold, A. Sturm, B. Wiedenmann, and A.U. Dignass. 2005. Patients with active inflammatory bowel disease lack

immature peripheral blood plasmacytoid and myeloid dendritic cells. *Gut* 54:228-236.

Baumgart, D.C., S. Thomas, I. Przesdzing, D. Metzke, C. Bielecki, S.M. Lehmann, S. Lehnardt, Y. Dorffel, A. Sturm, A. Scheffold, J. Schmitz, and A. Radbruch. 2009. Exaggerated inflammatory response of primary human myeloid dendritic cells to lipopolysaccharide in patients with inflammatory bowel disease. *Clin Exp Immunol* 157:423-436.

Bemark, M., P. Boysen, and N.Y. Lycke. 2012. Induction of gut IgA production through T cell-dependent and T cell-independent pathways. *Ann N Y Acad Sci* 1247:97-116.

Benoit, M., B. Barbarat, A. Bernard, D. Olive, and J.L. Mege. 2008. *Coxiella burnetii*, the agent of Q fever, stimulates an atypical M2 activation program in human macrophages. *Eur J Immunol* 38:1065-1070.

Berger, S.B., X. Romero, C. Ma, G. Wang, W.A. Faubion, G. Liao, E. Compeer, M. Keszei, L. Rameh, N. Wang, M. Boes, J.R. Regueiro, H.C. Reinecker, and C. Terhorst. 2010. SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages. *Nat Immunol* 11:920-927.

Bergsbaken, T., S.L. Fink, and B.T. Cookson. 2009. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* 7:99-109.

Berndt, B.E., M. Zhang, G.H. Chen, G.B. Huffnagle, and J.Y. Kao. 2007. The role of dendritic cells in the development of acute dextran sulfate sodium colitis. *J Immunol* 179:6255-6262.

Bettelli, E., M. Oukka, and V.K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8:345-350.

Bewtra, M., L.M. Kaiser, T. TenHave, and J.D. Lewis. 2013. Crohn's disease and ulcerative colitis are associated with elevated standardized mortality ratios: a meta-analysis. *Inflamm Bowel Dis* 19:599-613.

Bhoj, V.G., and Z.J. Chen. 2009. Ubiquitylation in innate and adaptive immunity. *Nature* 458:430-437.

Bilsborough, J., T.C. George, A. Norment, and J.L. Viney. 2003. Mucosal CD8α+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. *Immunology* 108:481-492.

Birmingham, C.L., A.C. Smith, M.A. Bakowski, T. Yoshimori, and J.H. Brumell. 2006. Autophagy controls *Salmonella* infection in response to damage to the *Salmonella*-containing vacuole. *J Biol Chem* 281:11374-11383.

Bishop, J.L., L.M. Sly, G. Krystal, and B.B. Finlay. 2008. The inositol phosphatase SHIP controls *Salmonella enterica* serovar Typhimurium infection *in vivo*. *Infect Immun* 76:2913-2922.

Biswas, S.K., L. Gangi, S. Paul, T. Schioppa, A. Saccani, M. Sironi, B. Bottazzi, A. Doni, B. Vincenzo, F. Pasqualini, L. Vago, M. Nebuloni, A. Mantovani, and A. Sica. 2006. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). *Blood* 107:2112-2122.

Blander, J.M., and R. Medzhitov. 2004. Regulation of phagosome maturation by signals from toll-like receptors. *Science* 304:1014-1018.

Bode, J.G., C. Ehlting, and D. Haussinger. 2012. The macrophage response towards LPS and its control through the p38(MAPK)-STAT3 axis. *Cell Signal* 24:1185-1194.

Bohdanowicz, M., D.M. Balkin, P. De Camilli, and S. Grinstein. 2012. Recruitment of OCRL and Inpp5B to phagosomes by Rab5 and APPL1 depletes phosphoinositides and attenuates Akt signaling. *Mol Biol Cell* 23:176-187.

Bohr, J., C. Tysk, S. Eriksson, and G. Jarnerot. 1995. Collagenous colitis in Orebro, Sweden, an epidemiological study 1984-1993. *Gut* 37:394-397.

Boonstra, A., R. Rajsbaum, M. Holman, R. Marques, C. Asselin-Paturel, J.P. Pereira, E.E. Bates, S. Akira, P. Vieira, Y.J. Liu, G. Trinchieri, and A. O'Garra. 2006. Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J Immunol* 177:7551-7558.

Booth, J.W., D. Telio, E.H. Liao, S.E. McCaw, T. Matsuo, S. Grinstein, and S.D. Gray-Owen. 2003. Phosphatidylinositol 3-kinases in carcinoembryonic antigen-related

cellular adhesion molecule-mediated internalization of *Neisseria gonorrhoeae*. *J Biol Chem* 278:14037-14045.

Bouchon, A., J. Dietrich, and M. Colonna. 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 164:4991-4995.

Boudeau, J., A.L. Glasser, E. Masseret, B. Joly, and A. Darfeuille-Michaud. 1999. Invasive ability of an *Escherichia coli* strain isolated from the ileal mucosa of a patient with Crohn's disease. *Infect Immun* 67:4499-4509.

Buchler, G., M.L. Wos-Oxley, A. Smoczek, N.H. Zschemisch, D. Neumann, D.H. Pieper, H.J. Hedrich, and A. Bleich. 2012. Strain-specific colitis susceptibility in IL10-deficient mice depends on complex gut microbiota-host interactions. *Inflamm Bowel Dis* 18:943-954.

Burisch, J., and P. Munkholm. 2013. Inflammatory bowel disease epidemiology. *Curr Opin Gastroenterol* 29:357-362.

Bystrom, J., I. Evans, J. Newson, M. Stables, I. Toor, N. van Rooijen, M. Crawford, P. Colville-Nash, S. Farrow, and D.W. Gilroy. 2008. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. *Blood* 112:4117-4127.

Campos, N., F. Magro, A.R. Castro, J. Cabral, P. Rodrigues, R. Silva, R. Appelberg, S. Rodrigues, S. Lopes, G. Macedo, and A. Sarmento. 2011. Macrophages from IBD patients exhibit defective tumour necrosis factor-α secretion but otherwise normal or augmented pro-inflammatory responses to infection. *Immunobiology* 216:961-970.

Caradonna, L., L. Amati, P. Lella, E. Jirillo, and D. Caccavo. 2000. Phagocytosis, killing, lymphocyte-mediated antibacterial activity, serum autoantibodies, and plasma endotoxins in inflammatory bowel disease. *Am J Gastroenterol* 95:1495-1502.

Cavaglieri, C.R., A. Nishiyama, L.C. Fernandes, R. Curi, E.A. Miles, and P.C. Calder. 2003. Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes. *Life Sci* 73:1683-1690.

Checroun, C., T.D. Wehrly, E.R. Fischer, S.F. Hayes, and J. Celli. 2006. Autophagy-mediated reentry of *Francisella tularensis* into the endocytic compartment after cytoplasmic replication. *Proc Natl Acad Sci U S A* 103:14578-14583.

Chirdo, F.G., O.R. Millington, H. Beacock-Sharp, and A.M. Mowat. 2005. Immunomodulatory dendritic cells in intestinal lamina propria. *Eur J Immunol* 35:1831-1840.

Cho, J.H., and S.R. Brant. 2011. Recent insights into the genetics of inflammatory bowel disease. *Gastroenterology* 140:1704-1712.

Cho, J.H., D.L. Nicolae, L.H. Gold, C.T. Fields, M.C. LaBuda, P.M. Rohal, M.R. Pickles, L. Qin, Y. Fu, J.S. Mann, B.S. Kirschner, E.W. Jabs, J. Weber, S.B. Hanauer, T.M. Bayless, and S.R. Brant. 1998. Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1p, 3q, and 4q: evidence for epistasis between 1p and IBD1. *Proc Natl Acad Sci U S A* 95:7502-7507.

Christoforidis, S., H.M. McBride, R.D. Burgoyne, and M. Zerial. 1999. The Rab5 effector EEA1 is a core component of endosome docking. *Nature* 397:621-625.

Collins, C.B., C.M. Aherne, E.N. McNamee, M.D. Lebsack, H. Eltzschig, P. Jedlicka, and J. Rivera-Nieves. 2012. Flt3 ligand expands CD103(+) dendritic cells and FoxP3(+) T regulatory cells, and attenuates Crohn's-like murine ileitis. *Gut* 61:1154-1162.

Cong, Y., C.T. Weaver, A. Lazenby, and C.O. Elson. 2002. Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. *J Immunol* 169:6112-6119.

Conley, M.E., A.K. Dobbs, A.M. Quintana, A. Bosompem, Y.D. Wang, E. Coustan-Smith, A.M. Smith, E.E. Perez, and P.J. Murray. 2012. Agammaglobulinemia and absent B lineage cells in a patient lacking the p85α subunit of PI3K. *J Exp Med* 209:463-470.

Consortium, H.M.P. 2012. Structure, function and diversity of the healthy human microbiome. *Nature* 486:207-214.

Coombes, J.L., K.R. Siddiqui, C.V. Arancibia-Carcamo, J. Hall, C.M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+

DCs induces Foxp3+ regulatory T cells via a TGF-β and retinoic acid-dependent mechanism. *J Exp Med* 204:1757-1764.

Dale, D.C., L. Boxer, and W.C. Liles. 2008. The phagocytes: neutrophils and monocytes. *Blood* 112:935-945.

Danese, S. 2008. Nonimmune cells in inflammatory bowel disease: from victim to villain. *Trends Immunol* 29:555-564.

Darfeuille-Michaud, A., J. Boudeau, P. Bulois, C. Neut, A.L. Glasser, N. Barnich, M.A. Bringer, A. Swidsinski, L. Beaugerie, and J.F. Colombel. 2004. High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. *Gastroenterology* 127:412-421.

Denning, T.L., Y.C. Wang, S.R. Patel, I.R. Williams, and B. Pulendran. 2007. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol* 8:1086-1094.

Diehl, G.E., R.S. Longman, J.X. Zhang, B. Breart, C. Galan, A. Cuesta, S.R. Schwab, and D.R. Littman. 2013. Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells. *Nature* 494:116-120.

Dieleman, L.A., B.U. Ridwan, G.S. Tennyson, K.W. Beagley, R.P. Bucy, and C.O. Elson. 1994. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* 107:1643-1652.

Diez, R., M.J. Garcia, S. Vivas, L. Arias, G. Rascarachi, E. Pozo, L.M. Vaquero, A. Miguel, M. Sierra, S. Calleja, and J.M. Ruiz De Morales. 2010. [Gastrointestinal manifestations in patients with primary immunodeficiencies causing antibody deficiency]. *Gastroenterol Hepatol* 33:347-351.

Dil, N., and A.J. Marshall. 2009. Role of phosphoinositide 3-kinase p110δ in TLR4- and TLR9-mediated B cell cytokine production and differentiation. *Mol Immunol* 46:1970-1978.

Dou, Z., M. Chattopadhyay, J.A. Pan, J.L. Guerriero, Y.P. Jiang, L.M. Ballou, Z. Yue, R.Z. Lin, and W.X. Zong. 2010. The class IA phosphatidylinositol 3-kinase p110β subunit is a positive regulator of autophagy. *J Cell Biol* 191:827-843.

Durand, C.A., M.J. Richer, K. Brenker, M. Graves, I. Shanina, K. Choi, M.S. Horwitz, K.D. Puri, and M.R. Gold. 2013. Selective pharmacological inhibition of phosphoinositide 3-kinase p110δ opposes the progression of autoimmune diabetes in non-obese diabetic (NOD) mice. *Autoimmunity* 46:62-73.

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

El Kasmi, K.C., A.M. Smith, L. Williams, G. Neale, A.D. Panopoulos, S.S. Watowich, H. Hacker, B.M. Foxwell, and P.J. Murray. 2007. Cutting edge: A transcriptional repressor and corepressor induced by the STAT3-regulated anti-inflammatory signaling pathway. *J Immunol* 179:7215-7219.

Fairn, G.D., and S. Grinstein. 2012. How nascent phagosomes mature to become phagolysosomes. *Trends Immunol* 33:397-405.

Fairweather, D., and D. Cihakova. 2009. Alternatively activated macrophages in infection and autoimmunity. *J Autoimmun* 33:222-230.

Falasca, M., and T. Maffucci. 2012. Regulation and cellular functions of class II phosphoinositide 3-kinases. *Biochem J* 443:587-601.

Feng, G.J., H.S. Goodridge, M.M. Harnett, X.Q. Wei, A.V. Nikolaev, A.P. Higson, and F.Y. Liew. 1999. Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: *Leishmania* phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *J Immunol* 163:6403-6412.

Fernandez-Banares, F., A. Salas, M. Forne, M. Esteve, J. Espinos, and J.M. Viver. 1999. Incidence of collagenous and lymphocytic colitis: a 5-year population-based study. *Am J Gastroenterol* 94:418-423.

Flannagan, R.S., G. Cosio, and S. Grinstein. 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol* 7:355-366.

Fortin, C.F., A. Cloutier, T. Ear, S. Sylvain-Prevost, T.Z. Mayer, R. Bouchelaghem, and P.P. McDonald. 2011. A class IA PI3K controls inflammatory cytokine production in human neutrophils. *Eur J Immunol* 41:1709-1719.

Frank, D.N., C.E. Robertson, C.M. Hamm, Z. Kpadeh, T. Zhang, H. Chen, W. Zhu, R.B. Sartor, E.C. Boedeker, N. Harpaz, N.R. Pace, and E. Li. 2011. Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. *Inflamm Bowel Dis* 17:179-184.

Frank, D.N., A.L. St Amand, R.A. Feldman, E.C. Boedeker, N. Harpaz, and N.R. Pace. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* 104:13780-13785.

Friedman, D.J., B.M. Kunzli, A.R. YI, J. Sevigny, P.O. Berberat, K. Enjyoji, E. Csizmadia, H. Friess, and S.C. Robson. 2009. From the Cover: CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. *Proc Natl Acad Sci U S A* 106:16788-16793.

Fukao, T., and S. Koyasu. 2003. PI3K and negative regulation of TLR signaling. *Trends Immunol* 24:358-363.

Fukao, T., M. Tanabe, Y. Terauchi, T. Ota, S. Matsuda, T. Asano, T. Kadowaki, T. Takeuchi, and S. Koyasu. 2002. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat Immunol* 3:875-881.

Garcon, F., D.T. Patton, J.L. Emery, E. Hirsch, R. Rottapel, T. Sasaki, and K. Okkenhaug. 2008. CD28 provides T-cell costimulation and enhances PI3K activity at the immune synapse independently of its capacity to interact with the p85/p110 heterodimer. *Blood* 111:1464-1471.

Gisbert, J.P., and J. Panes. 2009. Loss of response and requirement of infliximab dose intensification in Crohn's disease: a review. *Am J Gastroenterol* 104:760-767.

Goodridge, H.S., R.M. Simmons, and D.M. Underhill. 2007. Dectin-1 stimulation by *Candida albicans* yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. *J Immunol* 178:3107-3115.

Green, P.H., and C. Cellier. 2007. Celiac disease. *N Engl J Med* 357:1731-1743.

Gringhuis, S.I., J. den Dunnen, M. Litjens, B. van Het Hof, Y. van Kooyk, and T.B. Geijtenbeek. 2007. C-type lectin DC-SIGN modulates Toll-like receptor signaling

via Raf-1 kinase-dependent acetylation of transcription factor NF-κB. *Immunity* 26:605-616.

Gross, O., A. Gewies, K. Finger, M. Schafer, T. Sparwasser, C. Peschel, I. Forster, and J. Ruland. 2006. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* 442:651-656.

Guarner, F. 2008. What is the role of the enteric commensal flora in IBD? *Inflamm Bowel Dis* 14 Suppl 2:S83-84.

Gutierrez, M.G., S.S. Master, S.B. Singh, G.A. Taylor, M.I. Colombo, and V. Deretic. 2004. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* 119:753-766.

Hadis, U., B. Wahl, O. Schulz, M. Hardtke-Wolenski, A. Schippers, N. Wagner, W. Muller, T. Sparwasser, R. Forster, and O. Pabst. 2011. Intestinal tolerance requires gut homing and expansion of Foxp3+ regulatory T cells in the lamina propria. *Immunity* 34:237-246.

Hardison, S.E., and G.D. Brown. 2012. C-type lectin receptors orchestrate antifungal immunity. *Nat Immunol* 13:817-822.

Hardy, P.O., T.O. Diallo, C. Matte, and A. Descoteaux. 2009. Roles of phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase in the regulation of protein kinase C-α activation in interferon-γ-stimulated macrophages. *Immunology* 128:e652-660.

Harris, J., S.A. De Haro, S.S. Master, J. Keane, E.A. Roberts, M. Delgado, and V. Deretic. 2007. T helper 2 cytokines inhibit autophagic control of intracellular *Mycobacterium tuberculosis*. *Immunity* 27:505-517.

Harrison, R.E., C. Bucci, O.V. Vieira, T.A. Schroer, and S. Grinstein. 2003. Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP. *Mol Cell Biol* 23:6494-6506.

Hart, A.L., H.O. Al-Hassi, R.J. Rigby, S.J. Bell, A.V. Emmanuel, S.C. Knight, M.A. Kamm, and A.J. Stagg. 2005. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology* 129:50-65.

Harty, J.T., and M.J. Bevan. 1995. Specific immunity to *Listeria monocytogenes* in the absence of IFN-γ. *Immunity* 3:109-117.

Hashim, S., K. Mukherjee, M. Raje, S.K. Basu, and A. Mukhopadhyay. 2000. Live *Salmonella* modulate expression of Rab proteins to persist in a specialized compartment and escape transport to lysosomes. *J Biol Chem* 275:16281-16288.

Hasko, G., B. Csoka, Z.H. Nemeth, E.S. Vizi, and P. Pacher. 2009. A(2B) adenosine receptors in immunity and inflammation. *Trends Immunol* 30:263-270.

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

Haylock-Jacobs, S., I. Comerford, M. Bunting, E. Kara, S. Townley, M. Klingler-Hoffmann, B. Vanhaesebroeck, K.D. Puri, and S.R. McColl. 2011. PI3Kδ drives the pathogenesis of experimental autoimmune encephalomyelitis by inhibiting effector T cell apoptosis and promoting Th17 differentiation. *J Autoimmun* 36:278-287.

Hazeki, K., Y. Kametani, H. Murakami, M. Uehara, Y. Ishikawa, K. Nigorikawa, S. Takasuga, T. Sasaki, T. Seya, M. Matsumoto, and O. Hazeki. 2011. PI3Kγ controls the intracellular localization of CpG to limit DNA-PKcs-dependent IL-10 production in macrophages. *PLoS One* 6:e26836.

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

Hemmings, B.A., and D.F. Restuccia. 2012. PI3K-PKB/Akt pathway. *Cold Spring Harb Perspect Biol* 4:a011189.

Hirschfeld, M., Y. Ma, J.H. Weis, S.N. Vogel, and J.J. Weis. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol* 165:618-622.

Hochdorfer, T., M. Kuhny, C.N. Zorn, R.W. Hendriks, B. Vanhaesebroeck, T. Bohnacker, G. Krystal, and M. Huber. 2011. Activation of the PI3K pathway

increases TLR-induced TNF-α and IL-6 but reduces IL-1β production in mast cells. *Cell Signal* 23:866-875.

Hofmann, C., N. Dunger, J. Scholmerich, W. Falk, and F. Obermeier. 2010. Glycogen synthase kinase 3-β: a master regulator of toll-like receptor-mediated chronic intestinal inflammation. *Inflamm Bowel Dis* 16:1850-1858.

Horz, H.P., M.E. Vianna, B.P. Gomes, and G. Conrads. 2005. Evaluation of universal probes and primer sets for assessing total bacterial load in clinical samples: general implications and practical use in endodontic antimicrobial therapy. *J Clin Microbiol* 43:5332-5337.

Huang, F.P., N. Platt, M. Wykes, J.R. Major, T.J. Powell, C.D. Jenkins, and G.G. MacPherson. 2000. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med* 191:435-444.

Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R.M. Zinkernagel, and M. Aguet. 1993. Immune response in mice that lack the interferon-γ receptor. *Science* 259:1742-1745.

Hunter, M.M., A. Wang, K.S. Parhar, M.J. Johnston, N. Van Rooijen, P.L. Beck, and D.M. McKay. 2010. *In vitro*-derived alternatively activated macrophages reduce colonic inflammation in mice. *Gastroenterology* 138:1395-1405.

Iliev, I.D., V.A. Funari, K.D. Taylor, Q. Nguyen, C.N. Reyes, S.P. Strom, J. Brown, C.A. Becker, P.R. Fleshner, M. Dubinsky, J.I. Rotter, H.L. Wang, D.P. McGovern, G.D. Brown, and D.M. Underhill. 2012. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science* 336:1314-1317.

Iliev, I.D., E. Mileti, G. Matteoli, M. Chieppa, and M. Rescigno. 2009. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol* 2:340-350.

Ishida, T., M. Yoshida, M. Arita, Y. Nishitani, S. Nishiumi, A. Masuda, S. Mizuno, T. Takagawa, Y. Morita, H. Kutsumi, H. Inokuchi, C.N. Serhan, R.S. Blumberg, and T. Azuma. 2010. Resolvin E1, an endogenous lipid mediator derived from eicosapentaenoic acid, prevents dextran sulfate sodium-induced colitis. *Inflamm Bowel Dis* 16:87-95.

Ishii, E., T. Matui, M. Iida, T. Inamitu, and K. Ueda. 1987. Chediak-Higashi syndrome with intestinal complication. Report of a case. *J Clin Gastroenterol* 9:556-558.

Ismail, N., J.P. Olano, H.M. Feng, and D.H. Walker. 2002. Current status of immune mechanisms of killing of intracellular microorganisms. *FEMS Microbiol Lett* 207:111-120.

Ivanov, I.I., K. Atarashi, N. Manel, E.L. Brodie, T. Shima, U. Karaoz, D. Wei, K.C. Goldfarb, C.A. Santee, S.V. Lynch, T. Tanoue, A. Imaoka, K. Itoh, K. Takeda, Y. Umesaki, K. Honda, and D.R. Littman. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485-498.

Jaensson-Gyllenback, E., K. Kotarsky, F. Zapata, E.K. Persson, T.E. Gundersen, R. Blomhoff, and W.W. Agace. 2011. Bile retinoids imprint intestinal CD103+ dendritic cells with the ability to generate gut-tropic T cells. *Mucosal Immunol* 4:438-447.

Janeway, C.A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu Rev Immunol* 20:197-216.

Jarmin, S.J., R. David, L. Ma, J.G. Chai, H. Dewchand, A. Takesono, A.J. Ridley, K. Okkenhaug, and F.M. Marelli-Berg. 2008. T cell receptor-induced phosphoinositide-3-kinase p110δ activity is required for T cell localization to antigenic tissue in mice. *J Clin Invest* 118:1154-1164.

Jeon, S.G., H. Kayama, Y. Ueda, T. Takahashi, T. Asahara, H. Tsuji, N.M. Tsuji, H. Kiyono, J.S. Ma, T. Kusu, R. Okumura, H. Hara, H. Yoshida, M. Yamamoto, K. Nomoto, and K. Takeda. 2012. Probiotic *Bifidobacterium breve* induces IL-10-producing Tr1 cells in the colon. *PLoS Pathog* 8:e1002714.

Johansson, M., N. Rocha, W. Zwart, I. Jordens, L. Janssen, C. Kuijl, V.M. Olkkonen, and J. Neefjes. 2007. Activation of endosomal dynein motors by stepwise assembly of Rab7-RILP-p150Glued, ORP1L, and the receptor βlll spectrin. *J Cell Biol* 176:459-471.

Jostins, L., S. Ripke, R.K. Weersma, R.H. Duerr, D.P. McGovern, K.Y. Hui, J.C. Lee, L.P. Schumm, Y. Sharma, C.A. Anderson, J. Essers, M. Mitrovic, K. Ning, I. Cleynen, E. Theatre, S.L. Spain, S. Raychaudhuri, P. Goyette, Z. Wei, C. Abraham, J.P. Achkar, T. Ahmad, L. Amininejad, A.N. Ananthakrishnan, V. Andersen, J.M. Andrews, L. Baidoo, T. Balschun, P.A. Bampton, A. Bitton, G. Boucher, S. Brand, C. Buning, A. Cohain, S. Cichon, M. D'Amato, D. De Jong,

K.L. Devaney, M. Dubinsky, C. Edwards, D. Ellinghaus, L.R. Ferguson, D. Franchimont, K. Fransen, R. Gearry, M. Georges, C. Gieger, J. Glas, T. Haritunians, A. Hart, C. Hawkey, M. Hedl, X. Hu, T.H. Karlsen, L. Kupcinskas, S. Kugathasan, A. Latiano, D. Laukens, I.C. Lawrance, C.W. Lees, E. Louis, G. Mahy, J. Mansfield, A.R. Morgan, C. Mowat, W. Newman, O. Palmieri, C.Y. Ponsioen, U. Potocnik, N.J. Prescott, M. Regueiro, J.I. Rotter, R.K. Russell, J.D. Sanderson, M. Sans, J. Satsangi, S. Schreiber, L.A. Simms, J. Sventoraityte, S.R. Targan, K.D. Taylor, M. Tremelling, H.W. Verspaget, M. De Vos, C. Wijmenga, D.C. Wilson, J. Winkelmann, R.J. Xavier, S. Zeissig, B. Zhang, C.K. Zhang, H. Zhao, M.S. Silverberg, V. Annese, H. Hakonarson, S.R. Brant, G. Radford-Smith, C.G. Mathew, J.D. Rioux, E.E. Schadt, M.J. Daly, A. Franke, M. Parkes, S. Vermeire, J.C. Barrett, and J.H. Cho. 2012. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 491:119-124.

Jou, S.T., N. Carpino, Y. Takahashi, R. Piekorz, J.R. Chao, D. Wang, and J.N. Ihle. 2002. Essential, nonredundant role for the phosphoinositide 3-kinase p110δ in signaling by the B-cell receptor complex. *Mol Cell Biol* 22:8580-8591.

Kaiser, F., D. Cook, S. Papoutsopoulou, R. Rajsbaum, X. Wu, H.T. Yang, S. Grant, P. Ricciardi-Castagnoli, P.N. Tsichlis, S.C. Ley, and A. O'Garra. 2009. TPL-2 negatively regulates interferon-β production in macrophages and myeloid dendritic cells. *J Exp Med* 206:1863-1871.

Kamada, N., T. Hisamatsu, S. Okamoto, H. Chinen, T. Kobayashi, T. Sato, A. Sakuraba, M.T. Kitazume, A. Sugita, K. Koganei, K.S. Akagawa, and T. Hibi. 2008. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn's disease via IL-23/IFN-γ axis. *J Clin Invest* 118:2269-2280.

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

Kanai, F., H. Liu, S.J. Field, H. Akbary, T. Matsuo, G.E. Brown, L.C. Cantley, and M.B. Yaffe. 2001. The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat Cell Biol* 3:675-678.

Kanai, T., K. Uraushihara, T. Totsuka, Y. Nemoto, R. Fujii, T. Kawamura, S. Makita, D. Sawada, H. Yagita, K. Okumura, and M. Watanabe. 2006. Ameliorating effect of

saporin-conjugated anti-CD11b monoclonal antibody in a murine T-cell-mediated chronic colitis. *J Gastroenterol Hepatol* 21:1136-1142.

Kang, B.N., S.G. Ha, X.N. Ge, M. Reza Hosseinkhani, N.S. Bahaie, Y. Greenberg, M.N. Blumenthal, K.D. Puri, S.P. Rao, and P. Sriramarao. 2012. The p110δ subunit of PI3K regulates bone marrow-derived eosinophil trafficking and airway eosinophilia in allergen-challenged mice. *Am J Physiol Lung Cell Mol Physiol* 302:L1179-1191.

Kawai, T., and S. Akira. 2008. Toll-like receptor and RIG-I-like receptor signaling. *Ann N Y Acad Sci* 1143:1-20.

Kawai, T., and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 11:373-384.

Kawai, T., and S. Akira. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34:637-650.

Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K.J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 6:981-988.

Kemp, K., J. Griffiths, and K. Lovell. 2012. Understanding the health and social care needs of people living with IBD: a meta-synthesis of the evidence. *World J Gastroenterol* 18:6240-6249.

Kersse, K., M.J. Bertrand, M. Lamkanfi, and P. Vandenabeele. 2011. NOD-like receptors and the innate immune system: coping with danger, damage and death. *Cytokine Growth Factor Rev* 22:257-276.

Khan, K.J., T.A. Ullman, A.C. Ford, M.T. Abreu, A. Abadir, J.K. Marshall, N.J. Talley, and P. Moayyedi. 2011. Antibiotic therapy in inflammatory bowel disease: a systematic review and meta-analysis. *Am J Gastroenterol* 106:661-673.

Kim, C., S. Wilcox-Adelman, Y. Sano, W.J. Tang, R.J. Collier, and J.M. Park. 2008. Antiinflammatory cAMP signaling and cell migration genes co-opted by the anthrax *Bacillus*. *Proc Natl Acad Sci U S A* 105:6150-6155.

Kim, N., A. Saudemont, L. Webb, M. Camps, T. Ruckle, E. Hirsch, M. Turner, and F. Colucci. 2007. The p110δ catalytic isoform of PI3K is a key player in NK-cell development and cytokine secretion. *Blood* 110:3202-3208.

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

Kinchen, J.M., K. Doukoumetzidis, J. Almendinger, L. Stergiou, A. Tosello-Trampont, C.D. Sifri, M.O. Hengartner, and K.S. Ravichandran. 2008. A pathway for phagosome maturation during engulfment of apoptotic cells. *Nat Cell Biol* 10:556-566.

Kinnebrew, M.A., C.G. Buffie, G.E. Diehl, L.A. Zenewicz, I. Leiner, T.M. Hohl, R.A. Flavell, D.R. Littman, and E.G. Pamer. 2012. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. *Immunity* 36:276-287.

Klaasen, H.L., P.J. Van der Heijden, W. Stok, F.G. Poelma, J.P. Koopman, M.E. Van den Brink, M.H. Bakker, W.M. Eling, and A.C. Beynen. 1993. Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice. *Infect Immun* 61:303-306.

Kobayashi, T., K. Matsuoka, S.Z. Sheikh, H.Z. Elloumi, N. Kamada, T. Hisamatsu, J.J. Hansen, K.R. Doty, S.D. Pope, S.T. Smale, T. Hibi, P.B. Rothman, M. Kashiwada, and S.E. Plevy. 2011. NFIL3 is a regulator of IL-12 p40 in macrophages and mucosal immunity. *J Immunol* 186:4649-4655.

Kobayashi, T., K. Matsuoka, S.Z. Sheikh, S.M. Russo, Y. Mishima, C. Collins, E.F. deZoeten, C.L. Karp, J.P. Ting, R.B. Sartor, and S.E. Plevy. 2012. IL-10 regulates *Il12b* expression via histone deacetylation: implications for intestinal macrophage homeostasis. *J Immunol* 189:1792-1799.

Kok, K., B. Geering, and B. Vanhaesebroeck. 2009. Regulation of phosphoinositide 3-kinase expression in health and disease. *Trends Biochem Sci* 34:115-127.

Koyasu, S. 2003. The role of PI3K in immune cells. *Nat Immunol* 4:313-319.

Krishnamoorthy, N., T.B. Oriss, M. Paglia, M. Fei, M. Yarlagadda, B. Vanhaesebroeck, A. Ray, and P. Ray. 2008. Activation of c-Kit in dendritic cells regulates T helper cell differentiation and allergic asthma. *Nat Med* 14:565-573.

Kromann, N., and A. Green. 1980. Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. *Acta Med Scand* 208:401-406.

Kropf, P., J.M. Fuentes, E. Fahnrich, L. Arpa, S. Herath, V. Weber, G. Soler, A. Celada, M. Modolell, and I. Muller. 2005. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis *in vivo*. *Faseb J* 19:1000-1002.

Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75:263-274.

Kullberg, M.C., A.G. Rothfuchs, D. Jankovic, P. Caspar, T.A. Wynn, P.L. Gorelick, A.W. Cheever, and A. Sher. 2001. *Helicobacter hepaticus*-induced colitis in interleukin-10-deficient mice: cytokine requirements for the induction and maintenance of intestinal inflammation. *Infect Immun* 69:4232-4241.

Kullberg, M.C., J.M. Ward, P.L. Gorelick, P. Caspar, S. Hieny, A. Cheever, D. Jankovic, and A. Sher. 1998. *Helicobacter hepaticus* triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and interferon-γ-dependent mechanism. *Infect Immun* 66:5157-5166.

Kuo, C.C., W.T. Lin, C.M. Liang, and S.M. Liang. 2006. Class I and III phosphatidylinositol 3-kinase play distinct roles in TLR signaling pathway. *J Immunol* 176:5943-5949.

Kuroki, F., M. Iida, T. Matsumoto, K. Aoyagi, K. Kanamoto, and M. Fujishima. 1997. Serum n3 polyunsaturated fatty acids are depleted in Crohn's disease. *Dig Dis Sci* 42:1137-1141.

Langrish, C.L., Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein, and D.J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233-240.

LeibundGut-Landmann, S., O. Gross, M.J. Robinson, F. Osorio, E.C. Slack, S.V. Tsoni, E. Schweighoffer, V. Tybulewicz, G.D. Brown, J. Ruland, and C. Reis e Sousa.

2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol* 8:630-638.

Leverrier, Y., K. Okkenhaug, C. Sawyer, A. Bilancio, B. Vanhaesebroeck, and A.J. Ridley. 2003. Class I phosphoinositide 3-kinase p110β is required for apoptotic cell and Fcγ receptor-mediated phagocytosis by macrophages. *J Biol Chem* 278:38437-38442.

Levine, B., and V. Deretic. 2007. Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol* 7:767-777.

Lewis, K.L., M.L. Caton, M. Bogunovic, M. Greter, L.T. Grajkowska, D. Ng, A. Klinakis, I.F. Charo, S. Jung, J.L. Gommerman, I.I. Ivanov, K. Liu, M. Merad, and B. Reizis. 2011. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity* 35:780-791.

Li, Y., H. Zhu, and M.A. Trush. 1999. Detection of mitochondria-derived reactive oxygen species production by the chemilumigenic probes lucigenin and luminol. *Biochim Biophys Acta* 1428:1-12.

Liew, F.Y., D. Xu, E.K. Brint, and L.A. O'Neill. 2005. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol* 5:446-458.

Lin, E.Y., J.F. Li, L. Gnatovskiy, Y. Deng, L. Zhu, D.A. Grzesik, H. Qian, X.N. Xue, and J.W. Pollard. 2006. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res* 66:11238-11246.

Ling, Y.M., M.H. Shaw, C. Ayala, I. Coppens, G.A. Taylor, D.J. Ferguson, and G.S. Yap. 2006. Vacuolar and plasma membrane stripping and autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. *J Exp Med* 203:2063-2071.

Liu, D., and J.E. Uzonna. 2010. The p110δ isoform of phosphatidylinositol 3-kinase controls the quality of secondary anti-*Leishmania* immunity by regulating expansion and effector function of memory T cell subsets. *J Immunol* 184:3098-3105.

Liu, D., T. Zhang, A.J. Marshall, K. Okkenhaug, B. Vanhaesebroeck, and J.E. Uzonna. 2009a. The p110δ isoform of phosphatidylinositol 3-kinase controls susceptibility

to *Leishmania major* by regulating expansion and tissue homing of regulatory T cells. *J Immunol* 183:1921-1933.

Liu, K., G.D. Victora, T.A. Schwickert, P. Guermonprez, M.M. Meredith, K. Yao, F.F. Chu, G.J. Randolph, A.Y. Rudensky, and M. Nussenzweig. 2009b. *In vivo* analysis of dendritic cell development and homeostasis. *Science* 324:392-397.

Liu, Y.J., V. Soumelis, N. Watanabe, T. Ito, Y.H. Wang, W. Malefyt Rde, M. Omori, B. Zhou, and S.F. Ziegler. 2007. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol* 25:193-219.

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

Loo, Y.M., and M. Gale, Jr. 2011. Immune signaling by RIG-I-like receptors. *Immunity* 34:680-692.

Low, P.C., R. Misaki, K. Schroder, A.C. Stanley, M.J. Sweet, R.D. Teasdale, B. Vanhaesebroeck, F.A. Meunier, T. Taguchi, and J.L. Stow. 2010. Phosphoinositide 3-kinase δ regulates membrane fission of Golgi carriers for selective cytokine secretion. *J Cell Biol* 190:1053-1065.

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

Mackaness, G.B. 1977. Cellular immunity and the parasite. *Adv Exp Med Biol* 93:65-73.

Mahalingam, S., and B.A. Lidbury. 2002. Suppression of lipopolysaccharide-induced antiviral transcription factor (STAT-1 and NF-κB) complexes by antibody-dependent enhancement of macrophage infection by Ross River virus. *Proc Natl Acad Sci U S A* 99:13819-13824.

Maheshwari, A., D.R. Kelly, T. Nicola, N. Ambalavanan, S.K. Jain, J. Murphy-Ullrich, M. Athar, M. Shimamura, V. Bhandari, C. Aprahamian, R.A. Dimmitt, R. Serra, and R.K. Ohls. 2011. TGF-β2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. *Gastroenterology* 140:242-253.

Maillard, M.H., V. Cotta-de-Almeida, F. Takeshima, D.D. Nguyen, P. Michetti, C. Nagler, A.K. Bhan, and S.B. Snapper. 2007. The Wiskott-Aldrich syndrome protein is required for the function of CD4(+)CD25(+)Foxp3(+) regulatory T cells. *J Exp Med* 204:381-391.

Mantis, N.J., N. Rol, and B. Corthesy. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol* 4:603-611.

Marjuki, H., A. Gornitzky, B.M. Marathe, N.A. Ilyushina, J.R. Aldridge, G. Desai, R.J. Webby, and R.G. Webster. 2011. Influenza A virus-induced early activation of ERK and PI3K mediates V-ATPase-dependent intracellular pH change required for fusion. *Cell Microbiol* 13:587-601.

Marks, D.J., K. Miyagi, F.Z. Rahman, M. Novelli, S.L. Bloom, and A.W. Segal. 2009. Inflammatory bowel disease in CGD reproduces the clinicopathological features of Crohn's disease. *Am J Gastroenterol* 104:117-124.

Marks, D.J., C.R. Seymour, G.W. Sewell, F.Z. Rahman, A.M. Smith, S.A. McCartney, and S.L. Bloom. 2010. Inflammatory bowel diseases in patients with adaptive and complement immunodeficiency disorders. *Inflamm Bowel Dis* 16:1984-1992.

Marques, R., and I.G. Boneca. 2011. Expression and functional importance of innate immune receptors by intestinal epithelial cells. *Cell Mol Life Sci* 68:3661-3673.

Martin, M., K. Rehani, R.S. Jope, and S.M. Michalek. 2005. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat Immunol* 6:777-784.

Martins, A.J., P. Colquhoun, G. Reid, and S.O. Kim. 2009. Reduced expression of basal and probiotic-inducible G-CSF in intestinal mononuclear cells is associated with inflammatory bowel disease. *Inflamm Bowel Dis* 15:515-525.

Maslowski, K.M., A.T. Vieira, A. Ng, J. Kranich, F. Sierro, D. Yu, H.C. Schilter, M.S. Rolph, F. Mackay, D. Artis, R.J. Xavier, M.M. Teixeira, and C.R. Mackay. 2009. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* 461:1282-1286.

Matsuzawa, T., B.H. Kim, A.R. Shenoy, S. Kamitani, M. Miyake, and J.D. Macmicking. 2012. IFN-γ elicits macrophage autophagy via the p38 MAPK signaling pathway. *J Immunol* 189:813-818.

Matteoli, G., E. Mazzini, I.D. Iliev, E. Mileti, F. Fallarino, P. Puccetti, M. Chieppa, and M. Rescigno. 2010. Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. *Gut* 59:595-604.

Maxwell, M.J., E. Tsantikos, A.M. Kong, B. Vanhaesebroeck, D.M. Tarlinton, and M.L. Hibbs. 2012. Attenuation of phosphoinositide 3-kinase δ signaling restrains autoimmune disease. *J Autoimmun* 38:381-391.

Mazmanian, S.K., J.L. Round, and D.L. Kasper. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 453:620-625.

McCoy, C.E., F.J. Sheedy, J.E. Qualls, S.L. Doyle, S.R. Quinn, P.J. Murray, and L.A. O'Neill. 2010. IL-10 inhibits miR-155 induction by toll-like receptors. *J Biol Chem* 285:20492-20498.

Medina-Contreras, O., D. Geem, O. Laur, I.R. Williams, S.A. Lira, A. Nusrat, C.A. Parkos, and T.L. Denning. 2011. CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice. *J Clin Invest* 121:4787-4795.

Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437:1167-1172.

Miles, S.A., S.M. Conrad, R.G. Alves, S.M. Jeronimo, and D.M. Mosser. 2005. A role for IgG immune complexes during infection with the intracellular pathogen *Leishmania*. *J Exp Med* 201:747-754.

Moayyedi, P., S. O'Mahony, P. Jackson, D.A. Lynch, M.F. Dixon, and A.T. Axon. 1997. Small intestine in lymphocytic and collagenous colitis: mucosal morphology, permeability, and secretory immunity to gliadin. *J Clin Pathol* 50:527-529.

Molenaar, R., M. Knippenberg, G. Goverse, B.J. Olivier, A.F. de Vos, T. O'Toole, and R.E. Mebius. 2011. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. *J Immunol* 186:1934-1942.

Molodecky, N.A., I.S. Soon, D.M. Rabi, W.A. Ghali, M. Ferris, G. Chernoff, E.I. Benchimol, R. Panaccione, S. Ghosh, H.W. Barkema, and G.G. Kaplan. 2012.

Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 142:46-54 e42; quiz e30.

Morgan, X.C., T.L. Tickle, H. Sokol, D. Gevers, K.L. Devaney, D.V. Ward, J.A. Reyes, S.A. Shah, N. LeLeiko, S.B. Snapper, A. Bousvaros, J. Korzenik, B.E. Sands, R.J. Xavier, and C. Huttenhower. 2012. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol* 13:R79.

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

Mouchemore, K.A., N.G. Sampaio, M.W. Murrey, E.R. Stanley, B.J. Lannutti, and F.J. Pixley. 2013. Specific inhibition of PI3K p110δ inhibits CSF-1-induced macrophage spreading and invasive capacity. *Febs J*

Mowat, A.M., and C.C. Bain. 2011. Mucosal macrophages in intestinal homeostasis and inflammation. *J Innate Immun* 3:550-564.

Mucida, D., Y. Park, G. Kim, O. Turovskaya, I. Scott, M. Kronenberg, and H. Cheroutre. 2007. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317:256-260.

Muller, U., W. Stenzel, G. Kohler, C. Werner, T. Polte, G. Hansen, N. Schutze, R.K. Straubinger, M. Blessing, A.N. McKenzie, F. Brombacher, and G. Alber. 2007. IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with *Cryptococcus neoformans*. *J Immunol* 179:5367-5377.

Munitz, A., E.T. Cole, A. Beichler, K. Groschwitz, R. Ahrens, K. Steinbrecher, T. Willson, X. Han, L. Denson, M.E. Rothenberg, and S.P. Hogan. 2010. Paired immunoglobulin-like receptor B (PIR-B) negatively regulates macrophage activation in experimental colitis. *Gastroenterology* 139:530-541.

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

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

Murray, L.A., Q. Chen, M.S. Kramer, D.P. Hesson, R.L. Argentieri, X. Peng, M. Gulati, R.J. Homer, T. Russell, N. van Rooijen, J.A. Elias, C.M. Hogaboam, and E.L. Herzog. 2011. TGF-β driven lung fibrosis is macrophage dependent and blocked by serum amyloid P. *Int J Biochem Cell Biol* 43:154-162.

Nakagawa, I., A. Amano, N. Mizushima, A. Yamamoto, H. Yamaguchi, T. Kamimoto, A. Nara, J. Funao, M. Nakata, K. Tsuda, S. Hamada, and T. Yoshimori. 2004. Autophagy defends cells against invading group A *Streptococcus*. *Science* 306:1037-1040.

Nathan, C., and A. Ding. 2010. Nonresolving inflammation. *Cell* 140:871-882.

Nell, S., S. Suerbaum, and C. Josenhans. 2010. The impact of the microbiota on the pathogenesis of IBD: lessons from mouse infection models. *Nat Rev Microbiol* 8:564-577.

Newberry, R.D., J.S. McDonough, W.F. Stenson, and R.G. Lorenz. 2001. Spontaneous and continuous cyclooxygenase-2-dependent prostaglandin E2 production by stromal cells in the murine small intestine lamina propria: directing the tone of the intestinal immune response. *J Immunol* 166:4465-4472.

Niess, J.H., S. Brand, X. Gu, L. Landsman, S. Jung, B.A. McCormick, J.M. Vyas, M. Boes, H.L. Ploegh, J.G. Fox, D.R. Littman, and H.C. Reinecker. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307:254-258.

Ogawa, M., T. Yoshimori, T. Suzuki, H. Sagara, N. Mizushima, and C. Sasakawa. 2005. Escape of intracellular *Shigella* from autophagy. *Science* 307:727-731.

Okkenhaug, K., A. Bilancio, G. Farjot, H. Priddle, S. Sancho, E. Peskett, W. Pearce, S.E. Meek, A. Salpekar, M.D. Waterfield, A.J. Smith, and B. Vanhaesebroeck. 2002. Impaired B and T cell antigen receptor signaling in p110δ PI 3-kinase mutant mice. *Science* 297:1031-1034.

Okkenhaug, K., D.T. Patton, A. Bilancio, F. Garcon, W.C. Rowan, and B. Vanhaesebroeck. 2006. The p110δ isoform of phosphoinositide 3-kinase controls clonal expansion and differentiation of Th cells. *J Immunol* 177:5122-5128.

Olesen, M., S. Eriksson, J. Bohr, G. Jarnerot, and C. Tysk. 2004. Microscopic colitis: a common diarrhoeal disease. An epidemiological study in Orebro, Sweden, 1993-1998. *Gut* 53:346-350.

Onyiah, J.C., S.Z. Sheikh, N. Maharshak, E.C. Steinbach, S.M. Russo, T. Kobayashi, L.C. Mackey, J.J. Hansen, A.J. Moeser, J.F. Rawls, L.B. Borst, L.E. Otterbein, and S.E. Plevy. 2013. Carbon monoxide and heme oxygenase-1 prevent intestinal inflammation in mice by promoting bacterial clearance. *Gastroenterology* 144:789-798.

Palmen, M.J., C.D. Dijkstra, M.B. van der Ende, A.S. Pena, and E.P. van Rees. 1995. Anti-CD11b/CD18 antibodies reduce inflammation in acute colitis in rats. *Clin Exp Immunol* 101:351-356.

Palmer, C.D., F.Z. Rahman, G.W. Sewell, A. Ahmed, M. Ashcroft, S.L. Bloom, A.W. Segal, and A.M. Smith. 2009. Diminished macrophage apoptosis and reactive oxygen species generation after phorbol ester stimulation in Crohn's disease. *PLoS One* 4:e7787.

Papakonstanti, E.A., O. Zwaenepoel, A. Bilancio, E. Burns, G.E. Nock, B. Houseman, K. Shokat, A.J. Ridley, and B. Vanhaesebroeck. 2008. Distinct roles of class IA PI3K isoforms in primary and immortalised macrophages. *J Cell Sci* 121:4124-4133.

Pardi, D.S., E.V. Loftus, Jr., T.C. Smyrk, P.P. Kammer, W.J. Tremaine, C.D. Schleck, W.S. Harmsen, A.R. Zinsmeister, L.J. Melton, 3rd, and W.J. Sandborn. 2007. The epidemiology of microscopic colitis: a population based study in Olmsted County, Minnesota. *Gut* 56:504-508.

Park, K.T., and D. Bass. 2011. Inflammatory bowel disease-attributable costs and cost-effective strategies in the United States: a review. *Inflamm Bowel Dis* 17:1603-1609.

Patton, D.T., O.A. Garden, W.P. Pearce, L.E. Clough, C.R. Monk, E. Leung, W.C. Rowan, S. Sancho, L.S. Walker, B. Vanhaesebroeck, and K. Okkenhaug. 2006. Cutting edge: the phosphoinositide 3-kinase p110δ is critical for the function of CD4+CD25+Foxp3+ regulatory T cells. *J Immunol* 177:6598-6602.

Patton, D.T., M.D. Wilson, W.C. Rowan, D.R. Soond, and K. Okkenhaug. 2011. The PI3K p110δ regulates expression of CD38 on regulatory T cells. *PLoS One* 6:e17359.

Paz, S., Q. Sun, P. Nakhaei, R. Romieu-Mourez, D. Goubau, I. Julkunen, R. Lin, and J. Hiscott. 2006. Induction of IRF-3 and IRF-7 phosphorylation following activation of the RIG-I pathway. *Cell Mol Biol (Noisy-le-grand)* 52:17-28.

Pineton de Chambrun, G.P., J. Torres, A. Darfeuille-Michaud, and J.F. Colombel. 2012. The role of anti(myco)bacterial interventions in the management of IBD: is there evidence at all? *Dig Dis* 30:358-367.

Py, B.F., M.M. Lipinski, and J. Yuan. 2007. Autophagy limits *Listeria monocytogenes* intracellular growth in the early phase of primary infection. *Autophagy* 3:117-125.

Qin, J., Y. Li, Z. Cai, S. Li, J. Zhu, F. Zhang, S. Liang, W. Zhang, Y. Guan, D. Shen, Y. Peng, D. Zhang, Z. Jie, W. Wu, Y. Qin, W. Xue, J. Li, L. Han, D. Lu, P. Wu, Y. Dai, X. Sun, Z. Li, A. Tang, S. Zhong, X. Li, W. Chen, R. Xu, M. Wang, Q. Feng, M. Gong, J. Yu, Y. Zhang, M. Zhang, T. Hansen, G. Sanchez, J. Raes, G. Falony, S. Okuda, M. Almeida, E. LeChatelier, P. Renault, N. Pons, J.M. Batto, Z. Zhang, H. Chen, R. Yang, W. Zheng, H. Yang, J. Wang, S.D. Ehrlich, R. Nielsen, O. Pedersen, and K. Kristiansen. 2012. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490:55-60.

Qualls, J.E., A.M. Kaplan, N. van Rooijen, and D.A. Cohen. 2006. Suppression of experimental colitis by intestinal mononuclear phagocytes. *J Leukoc Biol* 80:802-815.

Qualls, J.E., H. Tuna, A.M. Kaplan, and D.A. Cohen. 2009. Suppression of experimental colitis in mice by CD11c+ dendritic cells. *Inflamm Bowel Dis* 15:236-247.

Rada, B., C. Hably, A. Meczner, C. Timar, G. Lakatos, P. Enyedi, and E. Ligeti. 2008. Role of Nox2 in elimination of microorganisms. *Semin Immunopathol* 30:237-253.

Rada, B., and T.L. Leto. 2008. Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. *Contrib Microbiol* 15:164-187.

Rahman, F.Z., D.J. Marks, B.H. Hayee, A.M. Smith, S.L. Bloom, and A.W. Segal. 2008. Phagocyte dysfunction and inflammatory bowel disease. *Inflamm Bowel Dis* 14:1443-1452.

Rajamaki, K., T. Nordstrom, K. Nurmi, K.E. Akerman, P.T. Kovanen, K. Oorni, and K.K. Eklund. 2013. Extracellular acidosis is a novel danger signal alerting innate immunity via the NLRP3 inflammasome. *J Biol Chem* 288:13410-13419.

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

Randis, T.M., K.D. Puri, H. Zhou, and T.G. Diacovo. 2008. Role of PI3Kδ and PI3Kγ in inflammatory arthritis and tissue localization of neutrophils. *Eur J Immunol* 38:1215-1224.

Read, S., and F. Powrie. 2001. Induction of inflammatory bowel disease in immunodeficient mice by depletion of regulatory T cells. *Curr Protoc Immunol* Chapter 15:Unit 15 13.

Reif, K., K. Okkenhaug, T. Sasaki, J.M. Penninger, B. Vanhaesebroeck, and J.G. Cyster. 2004. Cutting edge: differential roles for phosphoinositide 3-kinases, p110γ and p110δ, in lymphocyte chemotaxis and homing. *J Immunol* 173:2236-2240.

Rescigno, M., and A. Di Sabatino. 2009. Dendritic cells in intestinal homeostasis and disease. *J Clin Invest* 119:2441-2450.

Rich, K.A., C. Burkett, and P. Webster. 2003. Cytoplasmic bacteria can be targets for autophagy. *Cell Microbiol* 5:455-468.

Rimoldi, M., M. Chieppa, V. Salucci, F. Avogadri, A. Sonzogni, G.M. Sampietro, A. Nespoli, G. Viale, P. Allavena, and M. Rescigno. 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol* 6:507-514.

Rioux, J.D., R.J. Xavier, K.D. Taylor, M.S. Silverberg, P. Goyette, A. Huett, T. Green, P. Kuballa, M.M. Barmada, L.W. Datta, Y.Y. Shugart, A.M. Griffiths, S.R. Targan, A.F. Ippoliti, E.J. Bernard, L. Mei, D.L. Nicolae, M. Regueiro, L.P. Schumm, A.H. Steinhart, J.I. Rotter, R.H. Duerr, J.H. Cho, M.J. Daly, and S.R. Brant. 2007.

Genome-wide association study identifies new susceptibility loci for Crohn's disease and implicates autophagy in disease pathogenesis. *Nat Genet* 39:596-604.

Rivollier, A., J. He, A. Kole, V. Valatas, and B.L. Kelsall. 2012. Inflammation switches the differentiation program of Ly6C(hi) monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *J Exp Med* 209:139-155.

Robinson, J.M. 2008. Reactive oxygen species in phagocytic leukocytes. *Histochem Cell Biol* 130:281-297.

Rodon, J., R. Dienstmann, V. Serra, and J. Tabernero. 2013. Development of PI3K inhibitors: lessons learned from early clinical trials. *Nat Rev Clin Oncol* 10:143-153.

Rolf, J., S.E. Bell, D. Kovesdi, M.L. Janas, D.R. Soond, L.M. Webb, S. Santinelli, T. Saunders, B. Hebeis, N. Killeen, K. Okkenhaug, and M. Turner. 2010. Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. *J Immunol* 185:4042-4052.

Rossol, M., M. Pierer, N. Raulien, D. Quandt, U. Meusch, K. Rothe, K. Schubert, T. Schoneberg, M. Schaefer, U. Krugel, S. Smajilovic, H. Brauner-Osborne, C. Baerwald, and U. Wagner. 2012. Extracellular Ca2+ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nat Commun* 3:1329.

Round, J.L., S.M. Lee, J. Li, G. Tran, B. Jabri, T.A. Chatila, and S.K. Mazmanian. 2011. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* 332:974-977.

Ruane, D.T., and E.C. Lavelle. 2011. The role of CD103(+) dendritic cells in the intestinal mucosal immune system. *Front Immunol* 2:25.

Ruas, L.P., E.S. Bernardes, M.L. Fermino, L.L. de Oliveira, D.K. Hsu, F.T. Liu, R. Chammas, and M.C. Roque-Barreira. 2009. Lack of galectin-3 drives response to *Paracoccidioides brasiliensis* toward a Th2-biased immunity. *PLoS One* 4:e4519.

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

Rutella, S., G. Bonanno, L. Pierelli, A. Mariotti, E. Capoluongo, A.M. Contemi, F. Ameglio, A. Curti, D.G. De Ritis, M.T. Voso, A. Perillo, S. Mancuso, G. Scambia, R.M. Lemoli, and G. Leone. 2004. Granulocyte colony-stimulating factor promotes the generation of regulatory DC through induction of IL-10 and IFN-α. *Eur J Immunol* 34:1291-1302.

Saito, T., R. Hirai, Y.M. Loo, D. Owen, C.L. Johnson, S.C. Sinha, S. Akira, T. Fujita, and M. Gale, Jr. 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc Natl Acad Sci U S A* 104:582-587.

Sakai, K., H. Suzuki, H. Oda, T. Akaike, Y. Azuma, T. Murakami, K. Sugi, T. Ito, H. Ichinose, S. Koyasu, and M. Shirai. 2006. Phosphoinositide 3-kinase in nitric oxide synthesis in macrophage: critical dimerization of inducible nitric-oxide synthase. *J Biol Chem* 281:17736-17742.

Sartor, R.B. 2004. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 126:1620-1633.

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

Sartor, R.B. 2010. Genetics and environmental interactions shape the intestinal microbiome to promote inflammatory bowel disease versus mucosal homeostasis. *Gastroenterology* 139:1816-1819.

Schaljo, B., F. Kratochvill, N. Gratz, I. Sadzak, I. Sauer, M. Hammer, C. Vogl, B. Strobl, M. Muller, P.J. Blackshear, V. Poli, R. Lang, P.J. Murray, and P. Kovarik. 2009. Tristetraprolin is required for full anti-inflammatory response of murine macrophages to IL-10. *J Immunol* 183:1197-1206.

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

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

Schroder, K., and J. Tschopp. 2010. The inflammasomes. *Cell* 140:821-832.

Schulz, O., E. Jaensson, E.K. Persson, X. Liu, T. Worbs, W.W. Agace, and O. Pabst. 2009. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med* 206:3101-3114.

Scott, C.C., P. Cuellar-Mata, T. Matsuo, H.W. Davidson, and S. Grinstein. 2002. Role of 3-phosphoinositides in the maturation of *Salmonella*-containing vacuoles within host cells. *J Biol Chem* 277:12770-12776.

Segal, B.H., L. Romani, and P. Puccetti. 2009. Chronic granulomatous disease. *Cell Mol Life Sci* 66:553-558.

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

Seth, R.B., L. Sun, C.K. Ea, and Z.J. Chen. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF-3. *Cell* 122:669-682.

Sheikh, S.Z., and S.E. Plevy. 2010. The role of the macrophage in sentinel responses in intestinal immunity. *Curr Opin Gastroenterol* 26:578-582.

Shirey, K.A., L.E. Cole, A.D. Keegan, and S.N. Vogel. 2008. *Francisella tularensis* live vaccine strain induces macrophage alternative activation as a survival mechanism. *J Immunol* 181:4159-4167.

Sica, A., and A. Mantovani. 2012. Macrophage plasticity and polarization: *in vivo* veritas. *J Clin Invest* 122:787-795.

Siddiqui, K.R., S. Laffont, and F. Powrie. 2010. E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis. *Immunity* 32:557-567.

Sinclair, L.V., D. Finlay, C. Feijoo, G.H. Cornish, A. Gray, A. Ager, K. Okkenhaug, T.J. Hagenbeek, H. Spits, and D.A. Cantrell. 2008. Phosphatidylinositol 3-OH-kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking. *Nat Immunol* 9:513-521.

Singh, S.B., A.S. Davis, G.A. Taylor, and V. Deretic. 2006. Human IRGM induces autophagy to eliminate intracellular *Mycobacteria*. *Science* 313:1438-1441.

Skvorak, K., B. Vissel, and G.E. Homanics. 2006. Production of conditional point mutant knockin mice. *Genesis* 44:345-353.

Smallie, T., G. Ricchetti, N.J. Horwood, M. Feldmann, A.R. Clark, and L.M. Williams. 2010. IL-10 inhibits transcription elongation of the human TNF gene in primary macrophages. *J Exp Med* 207:2081-2088.

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

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

Smythies, L.E., R. Shen, D. Bimczok, L. Novak, R.H. Clements, D.E. Eckhoff, P. Bouchard, M.D. George, W.K. Hu, S. Dandekar, and P.D. Smith. 2010. Inflammation anergy in human intestinal macrophages is due to Smad-induced IκBα expression and NF-κB inactivation. *J Biol Chem* 285:19593-19604.

So, L., S.S. Yea, J.S. Oak, M. Lu, A. Manmadhan, Q.H. Ke, M.R. Janes, L.V. Kessler, J.M. Kucharski, L.S. Li, M.B. Martin, P. Ren, K.A. Jessen, Y. Liu, C. Rommel, and D.A. Fruman. 2013. Selective inhibition of phosphoinositide 3-kinase p110α preserves lymphocyte function. *J Biol Chem* 288:5718-5731.

Sokol, H., and P. Seksik. 2010. The intestinal microbiota in inflammatory bowel diseases: time to connect with the host. *Curr Opin Gastroenterol* 26:327-331.

Sollid, L.M. 2004. Intraepithelial lymphocytes in celiac disease: license to kill revealed. *Immunity* 21:303-304.

Soond, D.R., E. Bjorgo, K. Moltu, V.Q. Dale, D.T. Patton, K.M. Torgersen, F. Galleway, B. Twomey, J. Clark, J.S. Gaston, K. Tasken, P. Bunyard, and K. Okkenhaug.

2010. PI3K p110δ regulates T cell cytokine production during primary and secondary immune responses in mice and humans. *Blood* 115:2203-2213.

Spence, S., A. Fitzsimons, C.R. Boyd, J. Kessler, D. Fitzgerald, J. Elliott, J.N. Gabhann, S. Smith, A. Sica, E. Hams, S.P. Saunders, C.A. Jefferies, P.G. Fallon, D.F. McAuley, A. Kissenpfennig, and J.A. Johnston. 2013. Suppressors of cytokine signaling 2 and 3 diametrically control macrophage polarization. *Immunity* 38:66-78.

Stables, M.J., S. Shah, E.B. Camon, R.C. Lovering, J. Newson, J. Bystrom, S. Farrow, and D.W. Gilroy. 2011. Transcriptomic analyses of murine resolution-phase macrophages. *Blood* 118:e192-208.

Stewart, M., C.N. Andrews, S. Urbanski, P.L. Beck, and M. Storr. 2011. The association of coeliac disease and microscopic colitis: a large population-based study. *Aliment Pharmacol Ther* 33:1340-1349.

Strasser, D., K. Neumann, H. Bergmann, M.J. Marakalala, R. Guler, A. Rojowska, K.P. Hopfner, F. Brombacher, H. Urlaub, G. Baier, G.D. Brown, M. Leitges, and J. Ruland. 2012. Syk kinase-coupled C-type lectin receptors engage protein kinase C-Σ to elicit Card9 adaptor-mediated innate immunity. *Immunity* 36:32-42.

Sun, C.M., J.A. Hall, R.B. Blank, N. Bouladoux, M. Oukka, J.R. Mora, and Y. Belkaid. 2007. Small intestine lamina propria dendritic cells promote *de novo* generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 204:1775-1785.

Suzuki, K., B. Meek, Y. Doi, M. Muramatsu, T. Chiba, T. Honjo, and S. Fagarasan. 2004. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci U S A* 101:1981-1986.

Swann, J.B., M.D. Vesely, A. Silva, J. Sharkey, S. Akira, R.D. Schreiber, and M.J. Smyth. 2008. Demonstration of inflammation-induced cancer and cancer immunoediting during primary tumorigenesis. *Proc Natl Acad Sci U S A* 105:652-656.

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

Tanemura, S., H. Momose, N. Shimizu, D. Kitagawa, J. Seo, T. Yamasaki, K. Nakagawa, H. Kajiho, J.M. Penninger, T. Katada, and H. Nishina. 2009. Blockage by SP600125 of Fcε receptor-induced degranulation and cytokine gene expression in mast cells is mediated through inhibition of phosphatidylinositol 3-kinase signalling pathway. *J Biochem* 145:345-354.

Tassi, I., M. Cella, S. Gilfillan, I. Turnbull, T.G. Diacovo, J.M. Penninger, and M. Colonna. 2007. p110γ and p110δ phosphoinositide 3-kinase signaling pathways synergize to control development and functions of murine NK cells. *Immunity* 27:214-227.

Taylor, B.C., C. Zaph, A.E. Troy, Y. Du, K.J. Guild, M.R. Comeau, and D. Artis. 2009. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med* 206:655-667.

te Velde, A.A., Y. van Kooyk, H. Braat, D.W. Hommes, T.A. Dellemijn, J.F. Slors, S.J. van Deventer, and F.A. Vyth-Dreese. 2003. Increased expression of DC-SIGN(+)IL-12(+)IL-18(+) and CD83(+)IL-12(-)IL-18(-) dendritic cell populations in the colonic mucosa of patients with Crohn's disease. *Eur J Immunol* 33:143-151.

Teshima, C.W., L.A. Dieleman, and J.B. Meddings. 2012. Abnormal intestinal permeability in Crohn's disease pathogenesis. *Ann N Y Acad Sci* 1258:159-165.

Tezuka, H., Y. Abe, M. Iwata, H. Takeuchi, H. Ishikawa, M. Matsushita, T. Shiohara, S. Akira, and T. Ohteki. 2007. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. *Nature* 448:929-933.

Thi, E.P., and N.E. Reiner. 2012. Phosphatidylinositol 3-kinases and their roles in phagosome maturation. *J Leukoc Biol* 92:553-566.

Ting, J.P., J.A. Duncan, and Y. Lei. 2010. How the noninflammasome NLRs function in the innate immune system. *Science* 327:286-290.

Travassos, L.H., L.A. Carneiro, M. Ramjeet, S. Hussey, Y.G. Kim, J.G. Magalhaes, L. Yuan, F. Soares, E. Chea, L. Le Bourhis, I.G. Boneca, A. Allaoui, N.L. Jones, G. Nunez, S.E. Girardin, and D.J. Philpott. 2010. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol* 11:55-62.

Tumitan, A.R., L.G. Monnazzi, F.R. Ghiraldi, E.M. Cilli, and B.M. Machado de Medeiros. 2007. Pattern of macrophage activation in *Yersinia*-resistant and *Yersinia*-susceptible strains of mice. *Microbiol Immunol* 51:1021-1028.

Uddin, M., and B.D. Levy. 2011. Resolvins: natural agonists for resolution of pulmonary inflammation. *Prog Lipid Res* 50:75-88.

Uematsu, S., K. Fujimoto, M.H. Jang, B.G. Yang, Y.J. Jung, M. Nishiyama, S. Sato, T. Tsujimura, M. Yamamoto, Y. Yokota, H. Kiyono, M. Miyasaka, K.J. Ishii, and S. Akira. 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat Immunol* 9:769-776.

Uno, J.K., K.N. Rao, K. Matsuoka, S.Z. Sheikh, T. Kobayashi, F. Li, E.C. Steinbach, A.R. Sepulveda, B. Vanhaesebroeck, R.B. Sartor, and S.E. Plevy. 2010. Altered macrophage function contributes to colitis in mice defective in the phosphoinositide-3 kinase subunit p110δ. *Gastroenterology* 139:1642-1653, 1653 e1641-1646.

Valatas, V., J. He, A. Rivollier, G. Kolios, K. Kitamura, and B.L. Kelsall. 2013. Host-dependent control of early regulatory and effector T cell differentiation underlies the genetic susceptibility of RAG2-deficient mouse strains to transfer colitis. *Mucosal Immunol* 6:601-611.

Vanhaesebroeck, B., J. Guillermet-Guibert, M. Graupera, and B. Bilanges. 2010. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* 11:329-341.

Vanhaesebroeck, B., S.J. Leevers, K. Ahmadi, J. Timms, R. Katso, P.C. Driscoll, R. Woscholski, P.J. Parker, and M.D. Waterfield. 2001. Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* 70:535-602.

Vanhaesebroeck, B., M.J. Welham, K. Kotani, R. Stein, P.H. Warne, M.J. Zvelebil, K. Higashi, S. Volinia, J. Downward, and M.D. Waterfield. 1997. p110δ, a novel phosphoinositide 3-kinase in leukocytes. *Proc Natl Acad Sci U S A* 94:4330-4335.

Varol, C., A. Vallon-Eberhard, E. Elinav, T. Aychek, Y. Shapira, H. Luche, H.J. Fehling, W.D. Hardt, G. Shakhar, and S. Jung. 2009. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity* 31:502-512.

Vassiliou, E.K., O.M. Kesler, J.H. Tadros, and D. Ganea. 2008. Bone marrow-derived dendritic cells generated in the presence of resolvin E1 induce apoptosis of activated CD4+ T cells. *J Immunol* 181:4534-4544.

Vautier, S., G. Sousa Mda, and G.D. Brown. 2010. C-type lectins, fungi and Th17 responses. *Cytokine Growth Factor Rev* 21:405-412.

Verstege, M.I., F.J. ten Kate, S.M. Reinartz, C.M. van Drunen, F.J. Slors, W.A. Bemelman, F.A. Vyth-Dreese, and A.A. te Velde. 2008. Dendritic cell populations in colon and mesenteric lymph nodes of patients with Crohn's disease. *J Histochem Cytochem* 56:233-241.

Vong, L., J.G. Ferraz, R. Panaccione, P.L. Beck, and J.L. Wallace. 2010. A pro-resolution mediator, prostaglandin D(2), is specifically up-regulated in individuals in long-term remission from ulcerative colitis. *Proc Natl Acad Sci U S A* 107:12023-12027.

Wang, H., J. Brown, Z. Gu, C.A. Garcia, R. Liang, P. Alard, E. Beurel, R.S. Jope, T. Greenway, and M. Martin. 2011a. Convergence of the mammalian target of rapamycin complex 1 and glycogen synthase kinase 3β signaling pathways regulates the innate inflammatory response. *J Immunol* 186:5217-5226.

Wang, S., E.J. Villablanca, J. De Calisto, D.C. Gomes, D.D. Nguyen, E. Mizoguchi, J.C. Kagan, H.C. Reinecker, N. Hacohen, C. Nagler, R.J. Xavier, B. Rossi-Bergmann, Y.B. Chen, R. Blomhoff, S.B. Snapper, and J.R. Mora. 2011b. MyD88-dependent TLR1/2 signals educate dendritic cells with gut-specific imprinting properties. *J Immunol* 187:141-150.

Wang, S.L., Z.R. Wang, and C.Q. Yang. 2012. Meta-analysis of broad-spectrum antibiotic therapy in patients with active inflammatory bowel disease. *Exp Ther Med* 4:1051-1056.

Watanabe, N., K. Ikuta, K. Okazaki, H. Nakase, Y. Tabata, M. Matsuura, H. Tamaki, C. Kawanami, T. Honjo, and T. Chiba. 2003. Elimination of local macrophages in intestine prevents chronic colitis in interleukin-10-deficient mice. *Dig Dis Sci* 48:408-414.

Weichhart, T., G. Costantino, M. Poglitsch, M. Rosner, M. Zeyda, K.M. Stuhlmeier, T. Kolbe, T.M. Stulnig, W.H. Horl, M. Hengstschlager, M. Muller, and M.D. Saemann. 2008. The TSC-mTOR signaling pathway regulates the innate inflammatory response. *Immunity* 29:565-577.

Weichhart, T., and M.D. Saemann. 2009. The multiple facets of mTOR in immunity. *Trends Immunol* 30:218-226.

Weisser, S.B., H.K. Brugger, N.S. Voglmaier, K.W. McLarren, N. van Rooijen, and L.M. Sly. 2011. SHIP-deficient, alternatively activated macrophages protect mice during DSS-induced colitis. *J Leukoc Biol* 90:483-492.

Weylandt, K.H., J.X. Kang, B. Wiedenmann, and D.C. Baumgart. 2007. Lipoxins and resolvins in inflammatory bowel disease. *Inflamm Bowel Dis* 13:797-799.

Williams, J.J., G.G. Kaplan, S. Makhija, S.J. Urbanski, M. Dupre, R. Panaccione, and P.L. Beck. 2008. Microscopic colitis-defining incidence rates and risk factors: a population-based study. *Clin Gastroenterol Hepatol* 6:35-40.

Winter, S.E., M.G. Winter, M.N. Xavier, P. Thiennimitr, V. Poon, A.M. Keestra, R.C. Laughlin, G. Gomez, J. Wu, S.D. Lawhon, I.E. Popova, S.J. Parikh, L.G. Adams, R.M. Tsolis, V.J. Stewart, and A.J. Baumler. 2013. Host-derived nitrate boosts growth of *E. coli* in the inflamed gut. *Science* 339:708-711.

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

Xiong, H., C. Zhu, F. Li, R. Hegazi, K. He, M. Babyatsky, A.J. Bauer, and S.E. Plevy. 2004. Inhibition of interleukin-12 p40 transcription and NF-κB activation by nitric oxide in murine macrophages and dendritic cells. *J Biol Chem* 279:10776-10783.

Xu, L.G., Y.Y. Wang, K.J. Han, L.Y. Li, Z. Zhai, and H.B. Shu. 2005. VISA is an adapter protein required for virus-triggered IFN-β signaling. *Mol Cell* 19:727-740.

Yamaguchi, T., K. Ihara, T. Matsumoto, Y. Tsutsumi, A. Nomura, S. Ohga, and T. Hara. 2001. Inflammatory bowel disease-like colitis in glycogen storage disease type 1b. *Inflamm Bowel Dis* 7:128-132.

Yamamori, T., O. Inanami, H. Nagahata, and M. Kuwabara. 2004. Phosphoinositide 3-kinase regulates the phosphorylation of NADPH oxidase component p47(phox) by controlling cPKC/PKCδ but not Akt. *Biochem Biophys Res Commun* 316:720-730.

Yamamoto, M., T. Okamoto, K. Takeda, S. Sato, H. Sanjo, S. Uematsu, T. Saitoh, N. Yamamoto, H. Sakurai, K.J. Ishii, S. Yamaoka, T. Kawai, Y. Matsuura, O. Takeuchi, and S. Akira. 2006. Key function for the Ubc13 E2 ubiquitin-conjugating enzyme in immune receptor signaling. *Nat Immunol* 7:962-970.

Yan, D., X. Wang, L. Luo, X. Cao, and B. Ge. 2012. Inhibition of TLR signaling by a bacterial protein containing immunoreceptor tyrosine-based inhibitory motifs. *Nat Immunol* 13:1063-1071.

Yap, G.S., Y. Ling, and Y. Zhao. 2007. Autophagic elimination of intracellular parasites: convergent induction by IFN-γ and CD40 ligation? *Autophagy* 3:163-165.

Yates, R.M., A. Hermetter, G.A. Taylor, and D.G. Russell. 2007. Macrophage activation downregulates the degradative capacity of the phagosome. *Traffic* 8:241-250.

Yen, E.F., and D.S. Pardi. 2012. Non-IBD colitides (eosinophilic, microscopic). *Best Pract Res Clin Gastroenterol* 26:611-622.

Ying, H., H. Fu, M.L. Rose, A.M. McCormack, P. Sarathchandra, K. Okkenhaug, and F.M. Marelli-Berg. 2012. Genetic or pharmaceutical blockade of phosphoinositide 3-kinase p110δ prevents chronic rejection of heart allografts. *PLoS One* 7:e32892.

Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y.M. Loo, M. Gale, Jr., S. Akira, S. Yonehara, A. Kato, and T. Fujita. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 175:2851-2858.

Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5:730-737.

Yu, Y., S. Nagai, H. Wu, A.S. Neish, S. Koyasu, and A.T. Gewirtz. 2006. TLR5-mediated phosphoinositide 3-kinase activation negatively regulates flagellin-induced proinflammatory gene expression. *J Immunol* 176:6194-6201.

Zhang, H.H., A.I. Lipovsky, C.C. Dibble, M. Sahin, and B.D. Manning. 2006. S6K1 regulates GSK3 under conditions of mTOR-dependent feedback inhibition of Akt. *Mol Cell* 24:185-197.

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