IL-12 p40 GENE EXPRESSION: INHIBITORY PATHWAYS AND INFLAMMATORY BOWEL DISEASE

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The IL-12 family of heterodimeric cytokines, comprising IL-12, IL-23 and IL-27, is an integral component of the inflammatory response. The IL-12 p40 subunit, which is part of both IL-12 and IL-23, is expressed specifically in macrophages and dendritic cells in response to microbial stimuli which signal through toll-like receptors (TLRs) and nucleotide-oligomerization-binding domain (NOD) proteins. Dysregulated expression of IL-12 p40 could lead to prolonged, unresolved inflammation manifesting into chronic inflammatory disorders such as inflammatory bowel disease (IBD). Understandably, IL-12 p40 expression is tightly regulated. We have demonstrated the requirement of a complex comprising nuclear factor of activated T cells (NFAT) and interferon regulatory factor 8 (IRF8) in IL-12 p40 gene transcriptional regulation. Subsequently, IRF8 was shown to be a target for an important anti-inflammatory pathway activated by carbon monoxide (CO) and heme oxygenase-1 (HO-1) in murine IBD. This dissertation explored two molecular mechanisms of IL-12 p40 inhibition – targeting the transcription factor NFAT using a novel cell-permeable inhibitory peptide and phosphoinositide 3-kinase (PI3K) mediated inhibition of IL-12. Firstly, VIVIT peptide which prevents the nuclear translocation of NFAT was employed to examine the role of NFAT in macrophage gene expression. We observed that NFAT inhibition attenuated the expression of inflammatory cytokines including IL-12, IL-23 and TNF. Secondly, we studied PI3K mediated inhibition of IL-12 and characterized immune responses and macrophage defects in PI3K p110δ mutant mice which spontaneously develop IBD. We observed that the PI3K mutant mice

recapitulate certain aspects of human IBD, including a profound increase in several proinflammatory cytokines in the intestinal mucosa and in macrophages, such as IL-12, IL-23, IL-17, TNF-α and IFN-γ. Furthermore, macrophages from PI3K p110δ mutant mice were defective in IL-10 and C5a mediated inhibition of IL-12 p40.

Thus, the expression of proinflammatory cytokines is coordinately regulated by transcription factors (such as NFAT and IRF8) and signaling molecules (such as PI3K), and mechanisms limiting inflammation are crucial for maintenance of immune homeostasis. Manipulation of such inhibitory pathways is a potential therapeutic approach for treating chronic inflammatory disorders.

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1.0 INTRODUCTION

The seminal discovery that naïve $CD4^+$ T cells could differentiate down two divergent pathways, to form Th1 and Th2 cells with distinct cytokine profiles and effector functions (1) led to the search for factors that determined lineage decisions. Out of this search, interleukin-12 (IL-12) emerged as a cytokine critical for the development of Th1 cells. IL-12 was originally discovered in 1989, as a soluble factor capable of stimulating natural killer (NK) cells to produce IFN-γ (2). Since then, tremendous progress has been made towards understanding the role of IL-12 in immune responses.

1.1 IL-12 FAMILY OF CYTOKINES

IL-12 is a 70 kDa heterodimeric cytokine comprising two disulfide-linked subunits – p40 and p35 (2, 3). p35 shares homology with other single-chain cytokines such as IL-6 and granulocyte colony-stimulating factor (G-CSF) (4), whereas p40 is homologous to the hematopoietin receptor family, particularly to the extracellular domain of the IL-6 receptor α chain (5). It has been suggested that IL-12 might have evolved from a primitive cytokine of the IL-6 family and its receptor (3). The genes encoding p35 and p40 are located on separate chromosomes (6-8) and therefore their expression is independently regulated. Co-expression of both subunits is required to form the biologically active, secreted p70 heterodimer (9). IL-12 p40 is produced in much greater quantities than is required to form heterodimers (2, 10-12). Excess p40 is secreted as free monomers and homodimers (13). Homodimeric p40 has been ascribed immunomodulatory roles.

It can act as an antagonist by competing for the IL-12 receptor and dampen the immune response (14). In other cases it has been reported to be immunostimulatory (15, 16). However, homodimeric p40 has not been detected in human cells and is not considered a physiological IL-12 antagonist in humans (17).

Recently two other heterodimeric cytokines related to IL-12; IL-23 and IL-27, were identified (18, 19). IL-23 is heterodimer of IL-12 p40 and p19, which is homologous to p35 (19). IL-27 is comprised of the subunits Epstein-Barr virus-induced gene 3 (EBI3), which is homologous to p40; and a novel p28 protein, which is related to p35 (18, 20, 21). They are grouped together as the IL-12 family of heterodimeric cytokines, with IL-12 being the prototype member (22, 23).

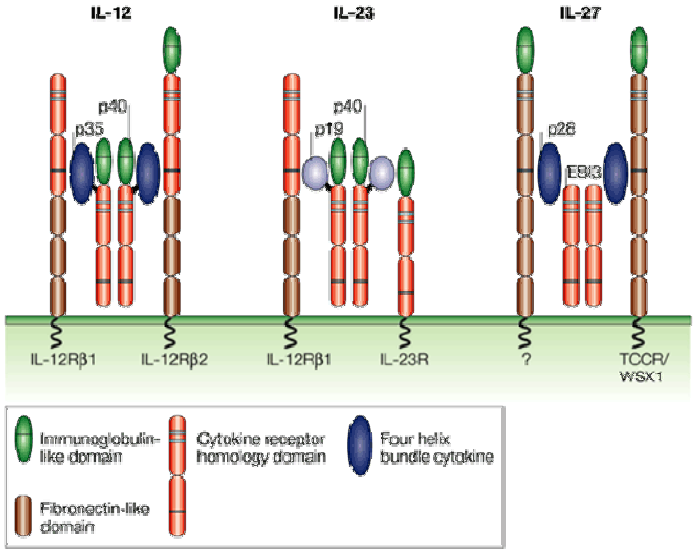


Figure 1. IL-12 is the prototype of a family of heterodimeric cytokines.

IL-12 p40 associates not only with p35 to form IL-12 but also with p19 to form IL-23. EBI3 is homologous to p40 and heterodimerises with p28, a p35 like molecule, to form IL-27. All three members of the IL-12 family play critical roles during a Th1 response. Adapted from Trinchieri G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat.Rev.Immunol. 3, 133-146*

Macrophages and dendritic cells (DCs) form an integral component of the innate immune system. These cells are the first to respond during an infection and initiate an inflammatory response characterized by secretion of soluble mediators such as cytokines and chemokines, alteration of vascular permeability, recruitment of other innate immune cells such as neutrophils and monocytes; eventually leading to the activation of the adaptive immune response. The cytokines produced during the inflammatory innate response direct the differentiation of naïve $CD4^+$ T cells toward either Th1 or Th2 responses, which are effective against intracellular or extracellular pathogens respectively (3).

Activation of innate immune cells occurs by the recognition of conserved molecular microbial patterns through germ-line encoded receptors, of which Toll-like receptors (TLRs) play a key role (24-26). Thus, macrophages and DCs perform crucial functions at the interface between innate and adaptive immune responses and the IL-12 family of cytokines is a critical molecular component of this interface linking the two arms of immunity (3). Given this important role, the expression of IL-12 family of cytokines needs to be tightly regulated to prevent chronic inflammatory and autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA) and inflammatory bowel disease (IBD).

1.1.1 IL-12

1.1.1.1 Biological functions

IL-12 is mainly produced by activated antigen presenting cells and phagocytes including dendritic cells, macrophages, monocytes and neutrophils and to a lesser extent by B cells (3). IL-12 is a cytokine with pleiotropic effects on various cell types. The most physiologically

important target cells are natural killer (NK), NKT and T cells, in which IL-12 induces several cytokines including granulocyte-macrophage colony stimulating factor (GMCSF), tumor necrosis factor (TNF) and particularly, IFN-γ (27, 28). Induction of IFN-γ is one of the most important biologic effects of IL-12 which coordinates the subsequent immune response and also mediates many of the proinflammatory properties of IL-12 (3, 29). IL-12 is an efficient inducer of IFN-γ at low concentrations and also synergizes with other stimuli such as IL-2, T-cell receptor (TCR) ligation and signaling through CD28 receptor for T cells; and IL-2 and immune complexes for NK cells (27, 30). IL-12 also synergizes with another unrelated cytokine IL-18 (earlier known as IFN-γ inducing factor-IGIF) in inducing IFN-γ in T (31, 32), NK (33) and B (34) cells. IL-12 and IL-18 can together induce IFN-γ production by macrophages, DCs and B cells, which normally are not considered to produce IFN-γ (35, 36). IFN-γ thus produced further stimulates the antimicrobial functions of phagocytes such as macrophages and DCs and synergizes with microbial products to enhance IL-12 production by these cells.

Besides its role in inducing IFN-γ production, IL-12 also induces the proliferation of hematopoietic precursors and stimulates the production of Th1 class of immunoglobulins such as IgG 2a by B cells (3).

The ability of IL-12 to dictate the outcome of T-helper responses exemplifies its role as a bridge between innate and adaptive immune responses. IL-12 directs Th0 precursor naïve $CD4^+$ T cells into the Th1 pathway and is critical for the development of Th1 cells (3, 37, 38). In addition, IL-12 and IFN-γ antagonize Th2 differentiation. Th1 cells in turn produce IFN-γ and promote cell-mediated immunity by inducing the differentiation of naïve CD8 T cells to form cytotoxic T lymphocytes (CTLs) (39) which are essential for immunity against intracellular pathogens (40-42). Accordingly, mice that are deficient for IL-12 p40 or p35 are highly

susceptible to infection by intracellular pathogens such as *Leishmania major* or *Toxoplasma gondii* (3). IL-12 also induces the proliferation and enhances the cytotoxicity of NK cells and CD8 T cells and plays an important role in anti-tumor immunity (3).

1.1.1.2 IL-12 receptor and signal transduction

The IL-12 receptor (IL-12R) is composed of two subunits – IL-12Rβ1 and IL-12Rβ2 – which are structurally related to the type I cytokine receptor superfamily and are homologous to gp130 (43, 44). Although each subunit has low affinity for IL-12, when co-expressed, a high affinity receptor is generated (43). IL-12R is mainly expressed on T cells and NK cells and also on DCs and B cells (35, 43, 45). Although NK cells express low levels of the receptor, IL-12R is only expressed in T cells upon activation. The expression of the β2 subunit is more stringently regulated than that of the β1 subunit and is thought to be a determinant of Th phenotype, since it is specifically expressed on Th1 cells (46, 47). Signaling through T cell receptor (TCR) synergizes with other stimuli such as IL-12, IFN-γ , TNF and co-stimulation through CD28 to upregulate the β2 subunit. IFN-γ induces the transcription factor T-bet, which has been shown to maintain the expression of IL-12Rβ2. Conversely, the Th2 cytokine IL-4 has been shown to inhibit the expression of the β2 subunit. Thus, reciprocal regulation of IL-12Rβ2 expression by IFN-γ and IL-4 may control Th1/Th2 differentiation (48). Furthermore, anti-inflammatory cytokines such as IL-10 and TGF-β have been shown to modulate the expression of IL-12Rβ2 subunit (49, 50). Collectively, the above studies suggest that responsiveness to IL-12 is an important means of immune regulation.

The IL-12Rβ2 chain is the signaling component of the receptor and the β1 chain is important for ligand binding (43, 51). Binding of IL-12 induces the Janus kinase (JAK)-STAT (signal transducer and activator of transcription) pathway leading to the activation of several STAT family members including STAT1, STAT3, STAT4 and STAT5 (52). However, the main biologic effects of IL-12 are mediated by STAT4, since STAT4 deficient mice demonstrate a phenotype similar to that of IL-12 p40 deficient mice (53, 54).

1.1.2 IL-23

IL-23 which is a heterodimer of covalently linked subunits – p19 and p40 – was identified as part of a bioinformatics search for novel IL-6 family members (19). The p19 protein is homologous to IL-12 p35 and similar to p35 is not biologically active on its own. Similar to IL-12, both p19 and p40 need to be coexpressed in a single cell to form bioactive IL-23 (19).

Similar to IL-12, IL-23 is produced mainly by DCs and macrophages upon stimulation of TLRs (55). Interestingly, some differences were observed in the induction of IL-12 and IL-23 by various TLRs. While p19 is induced by gram negative bacteria like *Escherichia coli*, *Bacteroides vulgatus* and *Veilonella parvula*, a stronger response was reported for stimulation with gram positive bacteria (56). Accordingly, stimulation of DCs with the TLR2 ligand peptidoglycan (which is more abundant in gram positive bacteria) induced greater amounts of IL-23 than the TLR4 ligand lipolysaccharide (LPS) (56, 57). In addition, unlike IL-12, maximum induction of IL-23 has been reported to be less dependent on IFN-γ (58). Such differences in the stimulation might result in the variant biologic activities of IL-23 compared to those of IL-12.

1.1.2.1 Biological functions

Accumulating evidence has established that similar to IL-12, IL-23 functions as a bridge between innate and adaptive immunity with overlapping and yet distinct effects (55). IL-23 biology is currently under intense investigation and much still needs to be understood about its biologic effects. Studies using p19 -/- mice and a careful re-analysis of p35 -/- (IL-12 deficient) and p40 -/- (IL-12 and IL-23 deficient) mice have revealed that IL-23 may play a greater role in chronic inflammatory and autoimmune compared to IL-12. IL-23 deficient (p19 -/-) mice have been shown to be resistant to experimentally induced autoimmunity such as experimental autoimmune encephalitis (EAE, a murine model for MS) (59) and collagen-induced arthritis (CIA, a murine model for RA) (60). Furthermore, p19 transgenic mice develop spontaneous severe multiorgan inflammation with overexpression of proinflammatory cytokines (61). These data suggest that IL-23 is more important than IL-12 in the perpetuation of chronic inflammation. In line with this hypothesis, IL-23 is reported to be a poor inducer of IFN-γ production and activates memory $CD4^+$ T cells more efficiently than naïve T cells (59, 60). Thus, unlike IL-12, IL-23 has been thought to function later during Th cell development, playing a key role in the maintenance of memory T cells, rather than directing Th lineage development.

However, recent reports have shown that IL-23 can direct the development of a distinct Th cell subset, characterized by the production of the proinflammatory cytokine – IL-17 (62, 63). This novel Th cell subset, designated Th17 is currently being investigated. It has been reported that TGF-β and not IL-23 is critical for the differentiation of $CD4^+$ T cells into Th17 cells; and that IL-23 plays a key role in the survival and maintenance of these cells (64). The dependence of Th17 cells on IL-23 is exemplified by the absence of this subset in IL-23 deficient, p19-/- and p40-/- mice (60). While the IL-12/IFN-γ pathway is important for host defense against

intracellular pathogens, IL-23/IL-17 pathway is shown to be important for immunity against extracellular pathogens such as *Klebsiella pneumoniae* (65). IL-17 has been shown to recruit and activate neutrophils (66, 67), induce proinflammatory cytokine production by various cell types and also drive the maturation of DCs (52). This IL-23-IL-17 axis has been described as an important pathway in a number of chronic inflammatory and autoimmune diseases (68, 69). IL-23 dependent Th17 cells are reported to drive autoimmunity in mouse models of EAE (70, 71) , CIA (60) and IBD (72). Thus, the role of IL-23 /IL-17 in host defense and autoimmunity is still being unfolded.

1.1.2.2 IL-23 receptor and signal transduction

IL-23 shares one of the receptor chains with IL-12. IL-23 receptor is composed of IL-12Rβ1 and a new second chain, IL23R. The IL23R is expressed on activated or memory T cells, NK cells, monocytes and DCs. Upon binding to its receptor, IL-23 induces the same JAK-STAT pathway as IL-12, but STAT3/STAT4 heterodimers seem to mediate the main biologic effects of IL-23 as opposed to STAT4 homodimers in the case of IL-12 (3, 23). This divergence in signaling might explain the unique properties of IL-23.

1.1.3 IL-27

IL-27 was discovered from a computational search for proteins homologous to p35 and IL-6 (18). IL-27 is a heterodimer of EBI3 (Epstein-Barr induced protein 3) and p28. EBI3 is homologous to IL-12 p40 and p28 is structurally related to IL-12 p35. Similar to IL-12, both EBI3 and p28 need to be coexpressed to express the secreted form of IL-27 (23, 52).

1.1.3.1 Biological functions

Initial studies with IL-27 receptor deficient or EBI3 deficient mice demonstrated that IL-27 has a role in Th1 responses. Both EBI3 and IL-27 receptor were shown to be involved in protective responses to *Leishmania major* (73, 74). IL-27 receptor deficient cells produced less IFN-γ than wild type (WT) cells and IL-27 receptor deficient mice were more susceptible to infection with the intracellular pathogen *Listeria monocytogenes* (74, 75). Accordingly, signaling through the IL-27 receptor has been shown to activate STAT1 and induce T-bet, a transcription factor critical for Th1 development; and inhibit the expression of GATA3, a Th2 inducing transcription factor (23). In addition, IL-27 is shown to induce the proliferation of naïve T cells and enhance IFN-γ production in synergy with IL-12 and IL-18 (18). Furthermore, IL-27 p28 antibodies were shown to ameliorate disease in animal models for RA and EAE (23). Thus, IL-27 was considered to be important for the development of Th1 cells and have a proinflammatory role. However, recent studies have shown that IL-27 can also have regulatory, immunosuppressive roles. IL-27 receptor deficient mice infected with *Toxoplasma gondii* initially develop protective immune responses, but fail to donwregulate the immune response at later stages, leading to a lethal T cell dependent inflammatory disease with excessive production of IFN-γ (76). In another study, IL-27 receptor deficient mice infected with Trypanosoma cruzi developed severe liver injury and increased mortality accompanied by enhanced production of inflammatory cytokines TNF, IL-6, IL-4 and IFN-γ (77). Hence elucidating the multiple roles of IL-27 in immunity would help in our understanding of Th cell development.

1.1.3.2 IL-27 receptor and signal transduction

IL-27 receptor (IL-27R) comprises of two receptor chains – the T cell cytokine receptor (TCCR) also known as WSX-1 (named after the WSXWS protein motif found in type I cytokine receptors) and gp130. WSX-1 is related to the IL-12R and gp130 is also used by IL-6 and other cytokines (52). WSX-1 is expressed highly on naïve T and NK cells; and also on mast cells, monocytes and dendritic cells. Signaling through IL-27R activates the JAK-STAT pathway leading to the activation of STAT1, 3 and 5 (52).

1.2 REGULATION OF IL-12

1.2.1 Positive regulation

Microbial products – including bacterial cell wall components (such as LPS, PGN), CpG containing bacterial DNA, baterial flagellin, viral RNA, intracellular parasites and fungi – that signal through TLRs are strong inducers of IL-12 in monocytes, macrophages and DCs (3). However, the relative efficiency of IL-12 induction depends on the levels of expression of the different TLRs that these products engage and the signaling pathways that they initiate.

TLRs are transmembrane proteins comprising highly conserved structural domains – an extracellular leucine-rich repeat (LRR) domain, a single transmembrane domain and a cytoplasmic tail consisting of the Toll-interleukin1 receptor (TIR) domain. The LRR is the ligand binding domain and the TIR domain transduces signals by binding to signaling proteins. At least eleven members of this expanding family of receptors have been identified in mice and humans (78). After ligand binding, TLRs dimerize and undergo conformational changes that help in the

recruitment of a set of TIR-domain containing adaptor proteins. Except TLR3 all TLRs identified so far signal through the adaptor protein myeloid differentiation primary-response protein 88 (MyD88), which in turn activates the recruitment of downstream kinases known as IL-1 receptor-associated kinases (IRAKs) 1, 2 and 4. TNF-receptor-associated factor 6 (TRAF6) is then activated which subsequently activates Iκ-B kinases (IKKs) resulting in the nuclear translocation of NF-κB and activation of proinflammatory genes. Mitogen-activated protein kinases (MAPKs) – c-jun N-terminal kinase (JNK), extracellular regulated kinase (ERK) and p38 are also activated by TLR signaling which in turn activate other transcription factors which induce inflammatory genes (78). In addition to the membrane bound TLRs, the cytosolic nucleotide-oligomerization binding domain (NOD) proteins act as intracellular pattern recognition receptors (PRRs), activated by specific microbial components. These proteins also activate NF-κB through a different signal transduction pathway that includes the kinase RICK/RIP2, and induce proinflammatory cytokine expression (79). The importance of NOD proteins in human disease is emphasized by the description of the family member NOD2 as the first susceptibility gene in human Crohn's disease (80, 81).

Signaling through TLR ligands alone induces low levels of IL-12 in macrophages and DCs. IFN-γ has been described to have a potent priming effect on IL-12 induction (82) and synergizes with TLR ligands resulting in maximal production of IL-12 heterodimer. This enhancement of IL-12 production by IFN-γ constitutes a positive feedback loop in Th1 responses. IFN-γ exerts its effects by induction of a family of transcription factors known as interferon regulatory factors (IRFs). IRF-1, 2 and 8 have been shown to be involved in the transcriptional regulation of p35 and p40 genes (83-88). Recently, IRF3 has been shown to play a selective role in IL-12 p35 expression (89). IRF-1 and 2 deficient mice are defective in both IL-

12 p35 and p40 expression; and IRF8 deficient mice display selective impairment of IL-12 p40 expression (90-92).

Apart from TLR ligands and IFN-γ, IL-12 production can also be induced via a T cell dependent pathway through interactions of CD40 ligand on activated T cells and CD40 on macrophages and DCs. Microbial stimulation of macrophages and DCs induces the expression of CD40 and makes them responsive for CD40L resulting in enhancement of IL-12 production (93-95).

Thus, optimal induction of IL-12 requires a combination of T cell independent (through microbes and IFN-γ) and T cell dependent (through CD40) stimuli.

1.2.2 Negative regulation

Although IL-12 is critical for host defense, appropriate downregulation of IL-12 is equally important to prevent persistent inflammation leading to various immune mediated inflammatory disorders such as MS, RA and IBD.

The anti-inflammatory cytokine IL-10 is a potent inhibitor of IL-12. IL-10 has been shown to inhibit the transcription of both IL-12 p35 and p40 in a protein synthesis dependent manner (96, 97). The molecular mechanism for IL-10 mediated inhibition of IL-12 is largely unknown. Some studies have reported the inhibition of nuclear translocation and DNA binding of NF-κB subunits by IL-10 (98-100). However, a recent study by Zhou et. al., demonstrated that IL-10 inhibits recruitment of RNA polymerase to the IL-12 p40 promoter but does not affect NF-κB complexes (101). The prominent role of IL-10 in regulating IL-12 is demonstrated by the development of uncontrolled, lethal, systemic inflammatory response upon infection of IL-10 deficitn mice with *Toxoplasma gondii* (102). These mice also develop chronic intestinal

inflammation (103). Apart from IL-10 other cytokines that have been reported to inhibit IL-12 are IL-4, TGF-β, IFNα/β and TNF (3).

It has been well established that signaling through G-protein coupled receptors (GPCRs) play an important role in the regulation of IL-12. GPCR ligands inhibit IL-12 production mainly through their induction of cyclic AMP (cAMP) (104). Prostaglandin E_2 (PGE_2) was one of the first GPCR ligands shown to inhibit IL-12 (105). Subsequently, other $G_{\alpha s}$-linked receptor ligands were also shown to inhibit IL-12. These include histamine receptor H_2, β_2 adrenergic receptors, adenosine receptor A2a and vasoactive intestinal peptide (VIP). Furthermore, ligation of GPCRs by cholera toxin (CT) has shown to inhibit IL-12 and IFN-γ *in vivo* and the β_2 adrenergic agonist salbutamol was effective in ameliorating disease in CIA model of arthritis (104). In addition to $G_{\alpha s}$-linked receptors, several ligands for $G_{\alpha i}$-linked receptors have also been shown to inhibit IL-12. These include chemokines and chemoattractants such as CCL2, CCL8, CCL7 and CCL13 (MCP-1 to 4) (104). Complement components C5a and formyl peptide fMLP which also signal through $G_{\alpha i}$-linked receptors inhibit IL-12. In accordance with an inhibitory role for GPCRs, $G_{i2\alpha}$ deficient mice display enhanced production of IL-12 and develop Th1 mediated colitis (106).

Activation of other non-GPCRs on macrophages and DCs also inhibits IL-12. These include activation of Fc receptors by immune complexes, activation of CR3 by iC3b, activation of CD47 by thrombospondin and ligation of CD46 by C3b (3, 107). Apart from cell surface receptors, nuclear receptors also inhibit IL-12. Ligands for glucocoticoid receptor, vitamin D receptor and peroxisomal proliferator activated receptor (PPAR) have been shown to inhibit IL-12 have anti-inflammatory effects (108). One of the mechanisms employed by some of the inhibitors of IL-12 is induction of IL-10, suggesting the importance of balance between pro and anti-inflammatory mediators in maintaining immune homeostasis.

Our group has demonstrated that nitric oxide (NO) suppresses IL-12 in macrophages and DCs (109). In our study NO inhibited TLR signaling by disrupting the interaction between IRAK and TRAF6. In addition, NO inhibited phosphorylation of I-κB, thus preventing the nuclear localization of NF-κB.

1.3 TRANSCRIPTIONAL REGULATION OF IL-12

IL-12 is mostly regulated at the level of transcription of the genes encoding the subunits p35 and p40; and the expression of both genes need to be coordinated in order to produce the biologically active heterodimer (6).

1.3.1 IL-12 p35

IL-12 p35 is ubiquitously expressed in many cell types, whereas IL-12 p40 is only expressed in cells that produce bioactive IL-12 – mainly macrophages and DCs (12). For this reason combined with the fact that IL-12 p40 heterodimerises with p19 to form IL-23, the regulation of IL-12 p40 has been studied in extensive detail compared to that of p35. In addition, analysis of IL-12 p35 regulation has been difficult due to its ubiquitous and low level of expression. However, in purified monocytes and DCs, it is found that both p40 and p35 genes are transcriptionally induced upon activation (110). Due to its low abundancy even in activated cells, it has been proposed that expression of p35 is the rate-limiting step for production of the bioactive heterodimer (111).

Analysis of both the murine and human IL-12 p35 promoters has revealed a complex picture (112-114). The human p35 gene has at least two transcription initiation sites; one is active in B-

lymphoblastoid cells and the other in monocytes. The latter generates a shorter mRNA, suggesting cell type specific differences in p35 regulation (114). The murine p35 gene is shown to possess multiple transcription initiation sites resulting in the generation of four isoforms. Differential usage of initiation sites was observed in resting and activated cells. The isoforms generated upon activation supported translation better than those formed in resting cells suggesting that p35 is also regulated by post-transcriptional mechanisms (115). Both human and murine p35 promoters contain binding sites for several transcription factors that include IRF-1, IRF-3, SP1, IRF8 and NF-κB family members c-Rel and p65 (83, 84, 89, 116, 117).

IL-12 p35 is also regulated at a post-translational level. Processing of the p35 pre-protein does not conform to the co-tranlocational model in that the removal of the signal peptide occurs through two sequential cleavages (118).

Thus, the expression of IL-12 p35 is regulated at several steps by complex mechanisms.

1.3.2 IL-12 p40

Extensive studies of the IL-12 p40 promoter by our group and others have revealed a complex mechanism of regulation characterized by many protein-protein and protein-DNA interactions. Initial characterization of the murine p40 promoter revealed an NF-κB element which was found to be necessary for induction of promoter activity by LPS (119). In another study, an ETS binding site was identified in both murine and human p40 promoters upstream of the NF-κB site. This site was shown to bind a multi-protein complex of which Ets-2 (a member of the Ets family of transcription factors), PU-1, IRF1, IRF2, IRF8 and c-Rel (a member of the NF-κB family) were major components and was thought to mediate the synergistic effect of IFN- γ on IL-12 p40 induction by LPS (85) . However, mutation of this site did not eliminate synergistic activation of

the promoter, indicating the presence of downstream sites (88, 120). Subsequently, an ISRE-like (interferon stimulated response element-like) element was identified downstream of the NF-κB site, which mediates promoter activation through ICSBP and IRF1 (87, 88, 121). Consistent with the promoter analysis, mice deficient for IRF1, IRF2 or IRF8 are compromised in their IL-12 p40 production. In fact, IRF8 deficient mice display a selective defect in IL-12 p40 expression and are susceptible to intracellular pathogens like *Toxoplasma gondii* (91, 92, 122). In addition, c-Rel deficient mice demonstrate severely diminished expression of IL-12 p40 suggesting the critical role of this NF-κB family member in p40 expression (123). Our group has performed a comprehensive analysis of the murine and human p40 promoter which revealed multiple sites responsible for promoter activation in response to LPS and heat killed *Listeria monocytogenes* (HKLM) (124). The most critical elements identified in this study were the C/EBP and NF-κB sites, which were shown to be functionally synergistic with each other. We have also demonstrated the presence of an AP-1 element in the p40 promoter which cooperates with the C/EBP site (125). Our recent study identified a novel composite element, spanning a region from -68 to -57 of the p40 promoter, which bound NFAT and IRF8. The involvement of NFAT, a prominent T-cell transcription factor, in IL-12 p40 gene expression in macrophages was a novel finding of this study (126). Furthermore, this element likely accounts in part for the biologically important phenomenon of synergistic activation of IL-12 p40 expression by TLR ligands and IFN-γ. The only known repressor element in the IL-12 p40 promoter was reported by Becker et. al. This sequence known as the GA-12 (GATA sequence in the IL-12 p40 promoter) element was identified between the NF-κB and the ETS sites and was occupied by a GA-12 binding protein (GAP12) in unstimulated cells. GAP12 binding was increased by two known inhibitors of IL-12

– IL-4 and PGE_2. Inhibition of IL-12 p40 by IL-4 was critically dependent on this sequence (127).

Furthermore, the IL-12 p40 promoter harbors a positioned nucleosome called nucleosome 1 in the proximal promoter region encompassing the NF-κB, C/EBP, AP1 and the NFAT/IRF8 sites. Upon activation with LPS and IFN-γ, nucleosome 1 was selectively remodeled (128). Thus, IL-12 p40 gene expression requires an inducible and highly selective remodeling event in addition to synergistic activation by transcription factors. It has been proposed one of the transcription factors required for p40 expression aids in selective recruitment of a chromatin remodeling complex to nucleosome 1. However, such a targeting factor is yet to be identified. It has been shown that this remodeling event is independent of c-Rel. (129).

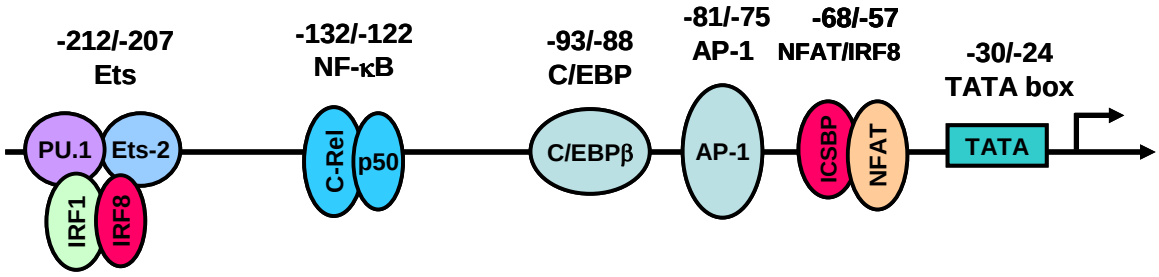


Figure 2. IL-12 p40 promoter

Schematic representation of the murine IL-12 p40 promoter

Targeting the binding of one or more of the above transcription factors is an attractive therapeutic strategy for treating chronic inflammatory diseases. In fact, several inhibitors of IL-12 production have been shown to attenuate transcription factor binding to the p40 promoter. 1,25-dihydroxyvitamin D_3 affects binding of NF-κB, Fc-receptor ligation affects PU-1 binding and VIP decreases binding of both proteins (130-132).

1.4 IL-12 AND INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disease of the intestine of unknown etiology. Both polygenetic predisposition and environmental factors are thought to initiate and propagate the disease. The current hypothesis is that IBD results from an uncontrolled, exaggerated mucosal immune response to common, non-pathogenic, intestinal microflora (133). The pathogenesis of CD is predominantly driven by an excessive Th1 response characterized by an increased secretion of IL-12, IFN-γ and/or TNF and is considered a Th1 mediated disease. UC is considered non-Th1 like with Th2 cytokines - IL-4, IL-5, and IL-13 - and TNF playing an important role (134-137).

The development and characterization of several animal models of chronic intestinal inflammation has greatly furthered our understanding of mucosal immunity and the pathophysiology of IBD (138). A skewed Th1 or, less frequently, Th2 cytokine response is a common feature of these animal models, underscoring the importance of negative regulatory pathways. These models can be broadly divided into four groups – spontaneous models (e.g., C3H/HeJBir mice), induced models (e.g., trinitrobenzene sulfonic acid – TNBS – model), adoptive transfer model (e.g., transfer of $CD4^{+}CD45RB^{hi}$ T cells into immunodeficient mice) and genetically engineered models (e.g., IL-10 -/- mice and STAT4 transgenic mice). These models recapitulate various aspects of human IBD and have been used extensively to generate valuable pre-clinical data for the development of novel therapeutics for IBD treatment.

Although IBD was initially thought to result from dysregulated acquired immune responses, recent progress has brought to light the critical role of innate immunity in the establishment of the initial inductive phase of disease, with the adaptive response playing a role in the perpetuation of inflammation in the later effector phase (137). The gene encoding NOD2

(an innate immune pattern recognition receptor) was the first described susceptibility gene for IBD (80, 81). In addition, several innate immune proinflammatory cytokines have been described to play a role in the pathogenesis of IBD and have also been targeted for therapy; the most common of which are TNF-α and IL-12 antagonists (137).

The role of IL-12 in IBD has been clearly established in both human and murine IBD. Both IL-12 p35 and p40 mRNA are upregulated in the mucosal tissue samples from CD patients (139, 140). In addition, secretion of the IL-12 p70 heterodimer was also enhanced by cells cultured ex vivo from mucosal tissue biopsies of CD patients (139-141). Furthermore, expression of the IL-12 receptor β2 chain was increased in active CD (142, 143). A recent study showed that transcripts for IL-23 p19, IL-27 p28 and EBI3 were enhanced in colonic mucosa of CD patients, implicating other IL-12 family members – IL-23 and IL-27 – in human IBD (144). Upregulated expression of the IL-23 dependent cytokine IL-17 has also been shown in CD (145, 146). Anti-IL-12 p40 monoclonal antibody (mAb) has been undergoing clinical trials in Crohn's disease patients. Initial analyses reveal exciting results. It was reported that lamina propria macrophages from CD patients secreted elevated levels of IL-12, IL-23 and IL-6 and treatment with anti-IL-12 p40 mAb downregulated these cytokines (147, 148).

Administration of IL-12p40 neutralising antibodies ameliorated mucosal inflammation in mouse models of colitis and IL-12 p40 -/- mice were resistant to colitis (149-151). In addition, constitutive high expression of p40 was demonstrated in the terminal ileum of IL-12 p40 promoter transgenic mice (152). Protein levels for IL-12 p40 and IL-23 p19/p40 were also shown to be elevated in the terminal ileum of this mouse model, suggesting that high bacterial load in the terminal ileum activates p40 gene transcription (152).

Collectively, the data suggest a critical role for IL-12 family members in IBD and understanding the molecular regulation of these cytokines would provide valuable insight into disease pathogenesis and reveal novel therapeutic approaches.

1.5 NFAT/IRF8 INTERACTIONS IN IL-12 P40 GENE EXPRESSION AND IBD

The focus of our group has been to understand the molecular regulation of IL-12 p40, with the long term goal being a better understanding of regulation of gene expression in the immune system. Towards this aim, we have extensively characterized the IL-12 p40 promoter. Our initial analysis revealed that multiple control elements were involved in heat-killed *Listeria monocytogenes* (HKLM) - and LPS-activated transcription of the p40 gene. These elements include a C/EBP binding site between -96 and -88 (124), and an AP-1 site from -81 to -75 (125). Furthermore, functional studies suggested that several other *cis*-acting elements may regulate promoter activation by bacteria and bacterial products (125). Accordingly, subsequent analysis demonstrated the presence of a novel composite element in the promoter proximal region of the IL-12 p40 promoter which was shown to bind a complex comprising NFAT and IRF8 as its major constituents.

NFAT family of transcription factors plays a key role in cytokine gene expression in T cells during an immune response. Five NFAT family members have been identified so far, four of which are calcium-regulated and require the calcium/calmodulin-dependent phosphatase, calcineurin for their nuclear localization. This is inhibited by the immunosuppressive agents, cyclosporin A (CsA) and FK506 (tacrolimus) (153). Although the role of NFAT in T cells has

been well studied, little is known about its functions in other cells of the immune system, specifically macrophages.

Our study was the first to conclusively prove a role for NFAT in IL-12 p40 gene expression in particular and macrophage gene expression in general. In a follow up study, we demonstrated a functional role for IRF8 in pathways inhibiting IL-12 p40 and inflammation in a mouse model for IBD – IL-10 deficient mice.

This section presents some of the data from the above studies which have been published (154, 155).

1.5.1 Characterization of NFAT/IRF8 interactions at the IL-12 p40 promoter

IFN-γ provides a potent priming signal for induction of IL-12 by macrophages and dendritic cells (86) and it is has been shown that signaling through TLR or CD40, on antigen presenting cells, alone is not sufficient for optimal production of IL-12 (156). This priming effect of IFN-γ on IL-12 has also been shown at the level of p40 promoter activity (86). Although interferon regulatory factors (IRFs) 1, 2 and 8 have been shown to be involved in the transcriptional induction of IL-12 p40 promoter (83, 85-88), the identification of an IFN-γ responsive site on the p40 promoter remains to be performed. Ma et. al., demonstrated the presence of an Ets element which binds IRFs 1, 2 and 8 along with other transcription factors (Ets-2, PU-1 and c-Rel) as a multi-protein complex. This site was shown to mediate the synergistic effect of IFN-γ on IL-12 p40 promoter. However, mutation of this site did not completely eliminate this synergy, indicating the presence of other sites (85). One of the sites which appeared to have functional significance was the region between -74 and -57, downstream of the C/EBP site. Mutation of this region considerably reduced the promoter activity and also abolished the DNase I footprint observed with HKLM

treated nuclear extracts (124). A bioinformatic search for potential transcription factor binding sites suggested that the region from -63 to -54 had strong homology to a consensus NFAT binding site and the region from -74 to -62 had a weaker homology to an ISRE (interferon stimulated response element) site, which is a binding site for IRF family members.

The functional significance of this region of the IL-12 p40 promoter upon activation by LPS and IFN-γ was tested in a transient transfection system. We transfected RAW 264.7 macrophage cells with promoter-luciferase constructs containing the wild type (WT) promoter sequence (-101 to +55) or sequences mutated in the putative NFAT (-62 to -57) or the IRF binding site (-68 to -62). We observed activation of the WT promoter sequence with LPS and strong synergistic activation with IFN-γ. In cells transfected with the mutated promoter constructs, LPS induced promoter activation was diminished. Interestingly, we observed a significant abrogation of the synergistic effect of IFN-γ on LPS induced promoter activity (Fig.3).

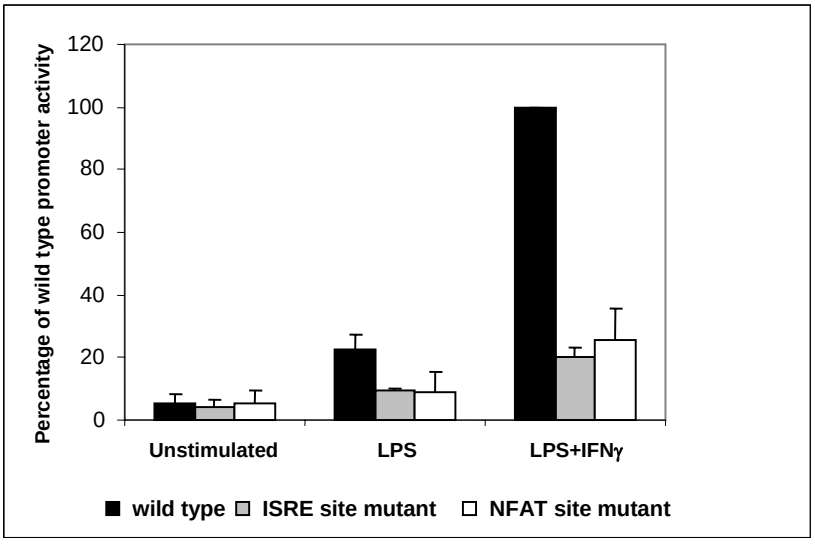


Figure 3. Characterization of a functional element at -73 to -54 region of the IL-12 p40 promoter.

To determine effects of mutants on synergistic promoter activation by LPS and IFN-γ, RAW264.7 cells were transiently transfected with murine IL-12 p40 promoter-luciferase plasmids (WT, NFAT site mutant and ISRE

site mutant) and a CMV-β-galactosidase reporter. After 24 h, cells were stimulated with LPS (5 μg/ml) with or without IFN-γ (10 ng/ml) for 10–12 h. Luciferase activity was normalized to β-galactosidase activity, and results expressed as percentage of promoter activity compared with LPS plus IFN-γ activation for the wild type IL-12 p40 promoter-luciferase plasmid (100%). Each result represents the mean ± standard deviation from four experiments.

In order to characterize specific DNA-protein interactions in this region, electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts from LPS or LPS+IFN-γ activated RAW 264.7 cells. Mutation of NFAT or ISRE site, which demonstrated decreased promoter activity in functional assays, also abrogated protein-DNA complex formation in EMSA, confirming the functional relevance of this protein-DNA interaction. Supershift assays indicated the presence of NFAT and IRF8 (also known as ICSBP- interferon consensus sequence binding protein) in the complex (154).

Chromatin immunoprecipitation reactions, which detect *in vivo* protein-DNA interactions, were utilized to confirm *in vitro* results. In these studies, proteins cross-linked to DNA in intact nuclei were immunoprecipitated as sheared chromatin fragments with specific antibodies. The IL-12 p40 promoter region from -146 to -50 was then detected by PCR amplification. We observed that both NFAT and IRF8 associate with the endogenous IL-12 p40 promoter in LPS and IFN- γ activated RAW 264.7 cells. NFAT binds to the promoter even in unactivated cells, indicating constitutive nuclear localization of NFAT. This is in agreement with a report demonstrating the presence of constitutive calcineurin activity in macrophages (157). Treatment of cells with the calcineurin/NFAT inhibitor, cyclosporin A (CsA), prevents DNA binding of both NFAT and IRF8. Thus, DNA binding of IRF8 requires NFAT (Fig.4).

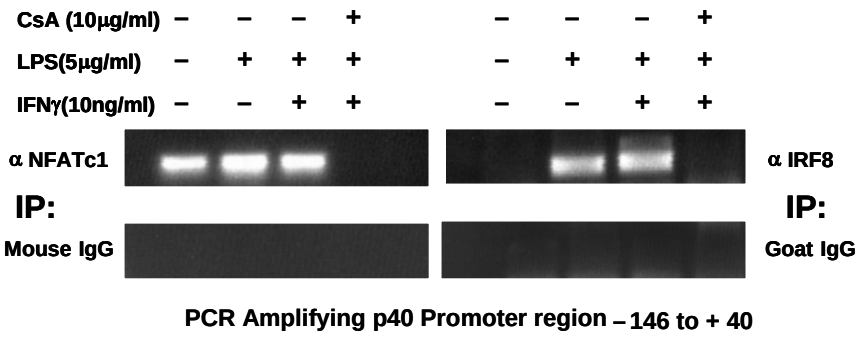


Figure 4. NFAT and IRF8 bind to the endogenous p40 promoter in RAW 264.7 cells.

RAW 264.7 cells were either untreated or activated by LPS, IFN- γ /LPS and Cyclosporin A/IFN-γ/LPS for 4 hours. Formaldehyde was added to 1% final concentration for 30 minutes to cross-link protein-DNA interactions, followed by which cell lysate was sonicated to shear chromatin. Antibodies against NFATc1, IRF8 or species matched control antibody were used to precipitate protein-DNA complexes. The immunoprecipitated IL-12 p40 promoter was amplified using specific PCR primers after reversal of crosslinking and protease digestion . Shown here is a representative result of three independent experiments.

Co-immunoprecipitation reactions of endogenous NFAT and ICSBP demonstrated DNA-independent interaction between the two proteins in LPS and IFN- γ activated RAW 264.7 cells (Fig.5).

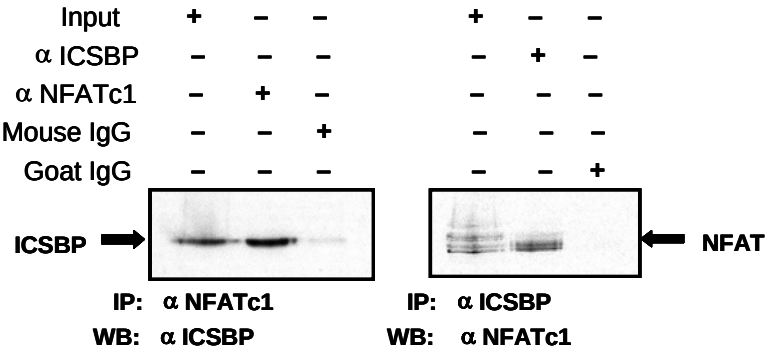


Figure 5. Demonstration of an *in vivo* interaction between NFAT and ICSBP.

Nuclear extracts from IFN-γ/LPS treated RAW264.7 cells were immunoprecipitated by antibodies to NFATc1 (left), or ICSBP (right). Immunoblots with antibodies against ICSBP (left) and NFATc1 (right) demonstrate a strong interaction. Shown here is a representative result of three independent experiments.

In order to test functional interactions between NFAT and IRF8 in IL-12 p40 promoter activation, NFAT or IRF8 expression plasmid were co-transfected into RAW 264.7 cells with

promoter luciferase constructs (WT or NFAT site mutant). We observed that expression of IRF8 alone, but not NFAT alone induces promoter activity (Fig. 6). However, expression of NFAT and IRF8 together, synergistically induced promoter activity. Mutation of the NFAT site abrogated this synergy. Thus, NFAT and IRF8 can function in synergy to activate the IL-12 p40 promoter.

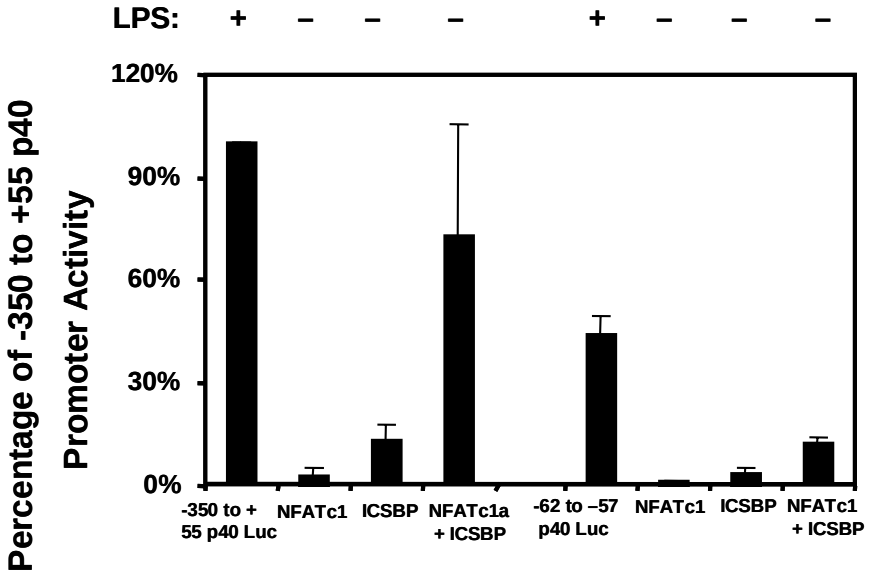


Figure 6. ICSBP and NFATc1 synergistically activate the IL-12 p40 promoter in RAW 264.7 cells.

ICSBP and NFATc1 expression plasmids were co-transfected into RAW 264.7 cells with IL-12 p40 promoter reporter constructs (wild type or NFAT site mutated) and CMV-β-galactosidase reporter using Superfect. 24 hours after transfection, cell lysates were obtained and assayed for luciferase activity, which was normalized with β-galactosidase activity. Cells transfected with the IL-12 p40 luciferase reporter and empty expression vector were stimulated with LPS (5μg/ml) prior to harvesting. Results are reported as percent promoter activity compared to NFAT+ICSBP induced activation (100%). Each result represents mean $\pm$ sem (n=3).

A functional role for NFAT in endogenous IL-12 p40 mRNA was determined by transfecting RAW 264.7 cells with a plasmid for dominant negative NFAT, which lacks the ability for nuclear translocation. We observed that endogenous IL-12 p40 mRNA expression in response to LPS and IFN-γ was reduced in RAW 264.7 cells transfected with a dominant negative construct of NFAT, as assessed by RT-PCR.

In summary, we demonstrate the presence of a composite NFAT/IRF8 element in the proximal region of the IL-12 p40 promoter that is functionally important for promoter activation by LPS and LPS plus IFN-γ. DNA binding of NFAT and IRF8 is demonstrated on the endogenous promoter by chromatin immunoprecipitation. NFAT is required for IRF8 binding to this region. Overexpression of NFAT and IRF8 synergistically activates the p40 promoter. A physical association between NFAT and ICSBP in the absence of DNA is detected by co-immunoprecipitation of endogenous proteins. Finally, a dominant negative NFAT molecule attenuates LPS- and IFN γ activated endogenous IL-12 p40 mRNA expression.

1.5.2 Description of IRF8 as a target for the anti-inflammatory effect of heme oxygenase (HO) – 1 in IBD

Inflammatory bowel disease is a chronic inflammatory disease of the intestine, the etiology of which is unknown. Nevertheless, it is clear that the disease is caused by a complex interplay of genetic, immunologic and environmental factors. The human IBDs are subdivided into two distinct but overlapping entities, Crohn's disease (CD) and ulcerative colitis (UC) (133).

Epidemiological observations have demonstrated a protective effect of cigarette smoking in patients with UC (158, 159). However, the immunologic mechanisms for this effect remain unclear. Several studies have focused on nicotine as a mediator of this protective effect. Therapeutic trial in UC patients treated with nicotine gum and transdermal nicotine has been inconclusive (160, 161). One component of cigarette smoke which has been shown to have anti-inflammatory effects is carbon monoxide (CO) and we studied the effects of CO in our study.

Mammalian cells generate CO endogenously as a product of heme degradation by the heme oxygenase (HO) enzymes (162). Two primary isoforms of HO exist: HO-1 is ubiquitously

expressed and highly inducible, whereas HO-2 is constitutively expressed primarily in the central nervous system and vascular beds (163). HO-1 is induced by a variety of agents causing oxidative stress and inflammation (164).

The protective role of CO has been demonstrated in murine models of sepsis, postoperative ileus and xenotransplantation (165-167). In acute inflammation, it has been shown that CO inhibits the expression of inflammatory cytokines. Macrophages activated with LPS in the presence of CO secreted significantly less TNF (168). In addition, CO significantly increased LPS induced secretion of the anti-inflammatory cytokine IL-10. In this study we address the role of CO and HO-1 in mucosal immunity and chronic inflammation. Given the significance of IL-12 and its subunit IL-12 p40 in IBD (52, 149-151), investigated the effect of CO/HO-1 pathway on IL-12 p40 gene expression.

The immunomodulatory effect of CO was studied in a murine model of Th1 mediated colitis – IL-10 -/- mice. Mice were exposed to 250 ppm of CO from 8 to 12 wk of age (n=12) and compared with a control group (n=8) exposed to ambient air. Hematoxylin and eosin stained colonic sections of CO and ambient air – treated mice were graded by a pathologist blinded to the treatment groups using a standard scoring system (155), and results were presented as a sum total of four averaged scores from five regions of the large intestine. CO-exposed mice demonstrated a 60% improvement in histological scores compared to air exposed mice (Fig. 7), which suggests that CO ameliorates colitis in IL-10 -/- mice. We also observed that IL-12 p40 protein secretion is attenuated in intestinal explants from CO treated IL-10 -/- mice, compared to those from ambient air treated mice (data not shown).

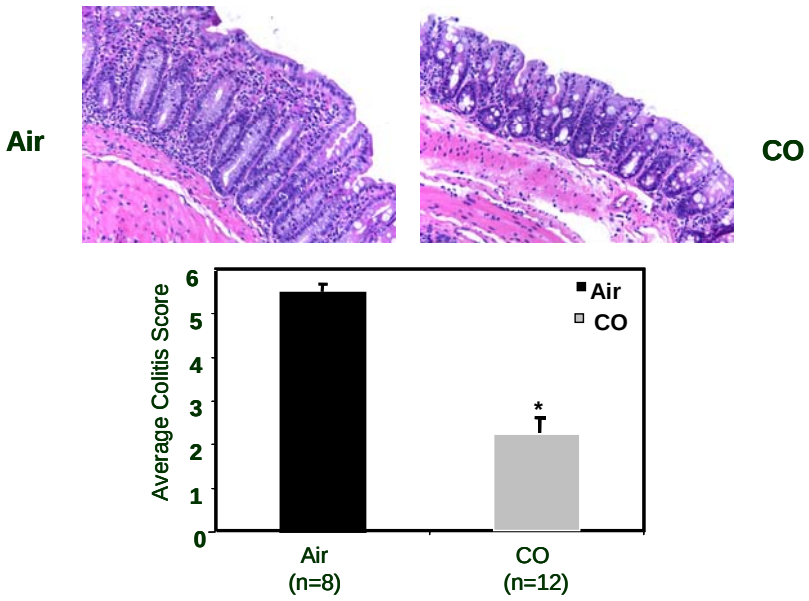


Figure 7. CO abrogates colitis in IL-10 -/-mice.

IL-10-/- mice were housed in ambient air (*n* =8) or a chamber maintaining a constant concentration of 250 ppm CO (*n*=12) from 8 through 12 wk of life. (A) Representative hematoxylin and eosin staining and histologic colitis ofIL-10 -/- colonic tissue. Left, ambient air; right, CO-treated. (B) Colitis scores were significantly decreased in CO-exposed mice compared with control mice. *, $P < 0.01$

We then investigated the molecular effects of CO on IL-12 p40 expression in murine bone-marrow derived macrophages. Since IFN-γ is required for optimal IL-12 p40 expression, macrophages were stimulated with LPS alone or LPS plus IFN-γ. It was observed that exposure of cells to 250 ppm of CO selectively inhibits the synergistic induction of IL-12 p40 by LPS and IFN-γ (Fig. 8a). This effect was also observed at the level of p40 mRNA (data not shown), suggesting that CO modulates IFN-γ signaling in macrophages. Since HO-1 has been shown to mediate the protective effects of CO in a model of acute liver failure, we investigated the involvement of HO-1 in CO mediated inhibition of IL-12 p40 in *hmox*$^{-/-}$ mice. In support of our hypothesis, bone marrow derived macrophages from *hmox*$^{-/-}$ mice displayed abrogation of CO mediated inhibition of LPS and IFN-γ induced IL-12 p40 (Fig. 8b). This suggests that HO-1 is required for the inhibitory effects of CO on IFN-γ signaling in macrophages.

(a.) (b.)

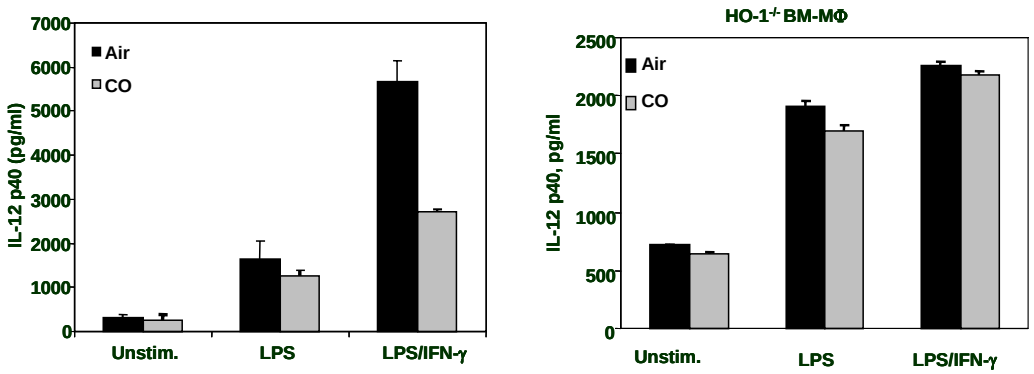


Figure 8. CO inhibits LPS and IFN-γ induced IL-12 p40 expression in a HO-1 dependent manner.

BM-derived macrophages from WT (a.) and *hmox*$^{-/-}$ (b.) mice were activated with 1 μg/ml LPS and 10 ng/ml IFN-γ in the presence or absence of 250 ppm CO. IL-12 p40 protein was measured in supernatants at 24 h. Each result represents the mean ± SD of triplicate assays and is representative of three independent experiments.

IL-12 p40 is transcriptionally regulated through a number of cis-acting elements that respond to bacterial products and IFN-γ (124). To study inhibition of LPS/IFN-γ induced IL-12 p40 transcription by CO, J774 murine macrophage cells were transiently transfected with a murine IL-12 p40 promoter fused to a luciferase reporter gene and then activated with IFN-γ plus LPS in the presence or absence of CO at 250 ppm for 24 h. CO selectively inhibits IL-12 p40 promoter activity synergistically induced by LPS/IFN-γ (Fig. 9a). IFN-γ signaling activated IRF family of transcription factors that bind to ISRE sequences found in promoters of IFN-γ inducible genes. Therefore, we determined whether CO modulates LPS/IFN-γ activation of a promoter that contains a consensus ISRE. J774 cells were transiently transfected with a plasmid containing a multimerized consensus ISRE fused to a luciferase reporter gene. Cells exposed to 250 ppm CO and activated with LPS/IFN-γ showed significantly augmented ISRE reporter activity (Fig. 9b).

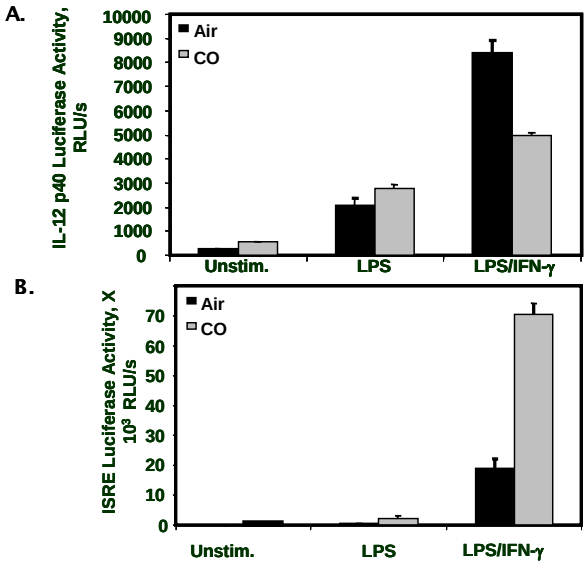


Figure 9. CO alters IFN-γ signaling in macrophages.

J774 murine macrophages were transiently transfected with an IL-12 p40 promoter-luciferase reporter construct (A) or (B) a multimerized ISRE-luciferase reporter plasmid. Cells were cultured in 250 ppm CO after the addition of 1μg/ml LPS or 1 μg/ml LPS plus 10 ng/ml IFN-γ for 18 h. Results are expressed as relative light units normalized to activity from a cotransfected constitutively active β-galactosidase reporter plasmid.

Having shown that CO antagonizes IFN-g responses for IL-12 p40 and augments the same for ISRE, we hypothesized CO modulates IRF family of transcription factors. IRF8 which has been shown to be critical for IL-12 p40 expression and also a repressor at ISRE sequences was considered a logical target of CO (154). Murine splenocytes were stimulated with LPS and IFN-γ and were cultured in ambient air or 250 ppm of CO for 8h. As determined by western blot analysis of nuclear extracts, CO inhibits LPS and IFN-γ induced IRF8 protein expression compared to air exposed cells (Fig. 8). CO did not inhibit other IRFs (data not shown). In order to conclusively prove that CO mediated inhibition of IL-12 p40 is dependent on IRF8 inhibition; we expressed IRF8 in bone marrow derived macrophages by retroviral transduction of an IRF8 expression vector. Cells were also transduced with an empty vector. Retrovirally transduced cells were stimulated with LPS and IFN-γ and cultured in either ambient air or 250 ppm of CO for 24h. Consistent with our hypothesis, the inhibitory effects of CO on LPS and IFN-γ stimulated

IL-12 p40 is completely abrogated in IRF8-vector transduced BM-derived macrophages compared to control cells (Fig. 10). Thus, CO inhibits LPS and IFN-γ induced IL-12 p40 through IRF8.

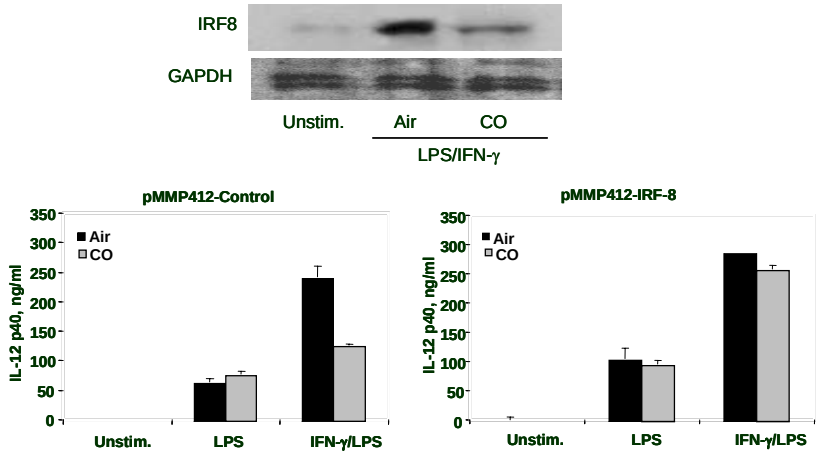


Figure 10. CO selectively inhibits LPS/IFN-γ induced IRF-8 in murine macrophages.

(Top panel) Western blot analysis of IRF8 in murine splenocytes. Lane 1, unstimulated; lanes 2 and 3 stimulated with LPS/IFN-γ for 8 h in the absence or presence of 250 ppm CO, respectively. (Bottom panel) BM-derived macrophages were transduced with retrovirus IRF-8 or empty virus as control. 48 h after infection, cells were seeded in 24-well plates at 10^6 cells/ml, stimulated with 1 μg/ml LPS or LPS and 10 ng/ml IFN-γ, and incubated for 24 h in the absence or presence of 250 ppm CO. Supernatants were then collected, and IL-12 p40 protein secretion was measured by ELISA. Each result is representative of three independent experiments.

In summary, CO exposure at low concentrations ameliorates Th1-mediated colitis. The molecular effects of CO on IL-12 p40 regulation were studied and it was observed that CO specifically inhibits the synergistic effect of IFN-γ on LPS induced IL-12 p40 in murine macrophages. This inhibitory effect of CO was dependent on HO-1 and was found to be mediated through inhibition of IRF8.

Hence, this study demonstrated for the first time that the transcription factor IRF8 which is critical for IL-12 production could be targeted by anti-inflammatory pathways.

These studies lead us to study other pathways which inhibit IL-12 p40, which may converge on the NFAT-IRF8 element, in order to identify novel therapeutic targets.

1.6 SPECIFIC AIMS

Studies on the pathogenesis of several chronic inflammatory diseases such as IBD, rheumatoid arthritis, diabetes and multiple sclerosis have revealed common underlying immunologic mechanisms. This has prompted extensive research on different aspects of an inflammatory response including cell migration, identity and function of cytokines and inflammatory mediators, signaling pathways and transcription factors involved in their expression and their role in the induction of innate and adaptive immune responses. Precise and coordinated resolution of inflammation is crucial to prevent chronic inflammation and maintain immune homeostasis. Although much is known about positive regulation of inflammation; inhibitory pathways involved in the resolution of inflammation are less well characterized. The studies in this dissertation focused on inhibition of expression of IL-12 p40 and other proinflammatory mediators in macrophages as critical events during the innate immune response. Specifically we studied interactions at a composite element in the IL-10 p40 promoter that binds the transcription factors NFAT and IRF8 as an important target of inhibitory signal transduction pathways. This was approached in two ways: (1.) use of a novel cell-permeable peptide to inhibit a transcription factor – NFAT involved in IL-12 p40 expression (2.) study of PI3K mediated inhibition of IL-12

1.6.1 Role of NFAT in proinflammatory gene expression in macrophages

The role of NFAT family of transcription factors in T cell mediated immune responses is well established. However, their role in regulating innate immune responses has been less studied. Our previous study demonstrated that NFAT and IRF8 are important for IL-12 p40 gene expression in macrophages (154). In addition, NFAT/IRF8 sites were also observed in the

promoters of iNOS and IL-18 (Zhu C. and Plevy S., unpublished data). In a subsequent study we demonstrated the involvement of IRF8 as a target for antiinflammatory pathways. Here, we extend our observations and investigate the role of NFAT in proinflammatory gene expression in macrophages. Experiments performed in Chapter 2 employed a novel cell permeable peptide – TAT-VIVIT to inhibit the nuclear translocation of NFAT. The purpose of the aim was to demonstrate a functional role for NFAT in IL-12 p40 gene expression in murine macrophages and also identify other NFAT target genes.

1.6.2 Characterization of chronic IBD and innate immune defects in PI3K p110δ mutant mice

Phosphoinositide 3-kinase (PI3K) has recently emerged as an important molecule involved in dampening TLR signaling and inflammatory responses. The negative regulatory role of PI3K is highlighted by the fact that mice harboring a point mutation in the p110δ subunit of PI3K spontaneously develop IBD (169). Here, we characterize the histopathologic and immunologic features of IBD in these mice and also investigate macrophage specific defects. In addition, experiments performed in Chapter 3 address the role of PI3K in negative regulation of IL-12 and other inflammatory genes.

2.0 ROLE OF NFAT IN PROINFLAMMATORY GENE EXPRESSION IN MACROPHAGES

Nuclear factor of activated T cells (NFAT) is important for the development and function of diverse cell types, including immune and non-immune cells. However, their role in macrophage-specific gene expression is relatively unknown. We had previously reported that NFAT is important for IL-12 p40 gene expression in the macrophage cell-line RAW 264.7 where NFAT regulated the interleukin (IL)-12 p40 promoter as a complex with interferon regulatory factor 8 (IRF8). In this study, a synthetic, cell-permeable NFAT inhibitory peptide (VIVIT) was employed to elucidate the role of NFAT proteins in mediating inflammatory responses in murine macrophages. Inhibition of NFAT by VIVIT attenuated LPS or LPS and IFN-γ induced IL-12 p40 protein and mRNA expression. Accordingly, VIVIT decreased secretion of IL-12 p70 and IL-23. TNF secretion was also inhibited by VIVIT treatment. VIVIT inhibited the DNA binding of both NFAT and IRF8 to the IL-12 p40 promoter and inhibition of IL-12 p40 was independent of IL-10 induction. NFAT also played a role in the expression of inducible nitric oxide synthase (iNOS) gene and nitric oxide secretion in murine macrophages. Thus, NFAT inhibition induces immunosuppression through innate as well as adaptive immune mechanisms.

2.1 INTRODUCTION

Macrophages lie at the critical interface between innate and adaptive immunity, performing effector functions such as phagocytosis, microbial killing, antigen presentation to T-helper (Th) cells and production of inflammatory mediators. Microbial stimulation of macrophages through toll-like receptors (TLRs) leads to a cascade of signaling events culminating in the production of various proinflammatory cytokines including IL-12, IL-23 and TNF; and antimicrobial mediators such as oxygen radicals, nitric oxide (NO) and proteases, all of which aid in the clearance of the pathogen. Expression of these mediators needs to be tightly regulated, failure of which could underlie the pathogenesis of chronic inflammatory diseases including rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease (IBD) (170). Hence, understanding the molecular regulation of genes encoding cytokines and antimicrobial products in macrophages will provide important insight into the role of innate immunity in infectious and inflammatory diseases.

The IL-12 family of heterodimeric cytokines (IL-12, IL-23 and IL-27) has been implicated in the pathogenesis of various chronic inflammatory disorders (149, 171-173). IL-12 and IL-23 are heterodimeric cytokines composed of a common p40 subunit in addition to a p35 and p19 subunit, respectively. Both IL-12 and IL-23 are involved in maintaining a Th1 response (3, 22). IL-23 has recently been shown to be involved in the development and maintenance of an inflammatory T cell population that produces IL-17 (Th17 cells) (64), also involved in autoimmune and inflammatory diseases (66). IL-12 p40 is expressed specifically in macrophages and dendritic cells (174) and is highly induced by microbial constituents such as LPS, CpG rich bacterial DNA, lipoproteins and T-cell dependent cognate interactions via CD40-CD40 ligand (3). Detailed molecular characterization of IL-12 p40 promoter activation by microbes and

cytokines has described complex regulation through numerous protein DNA and protein protein interactions (124, 125, 154). Recently, our group described a novel composite element in the IL-12 p40 promoter that interacts with members of the nuclear factor of activated T cells (NFAT) and the interferon regulatory factor (IRF) families of transcription factors (154). This element is involved in the synergistic induction of IL-12 p40 promoter activity by bacterial products and interferon-gamma (IFN-γ).

The NFAT family of transcription factors plays a key role in cytokine gene expression in T cells during an immune response. Five NFAT family members have been identified so far, four of which are calcium-regulated and require the calcium/calmodulin-dependent phosphatase, calcineurin for their nuclear localization. More recently, NFAT has been described to mediate gene expression in other cells of the immune system and other organ systems (175). Although the role of NFAT in T cells has been well studied, little is known about its functions in other cells of the immune system, specifically macrophages. In this study, we characterized the role of NFAT in macrophage-specific gene expression using a small cell permeable peptide inhibitor, VIVIT, which prevents NFAT-calcineurin interaction without affecting the phosphatase activity of calcineurin (176). Inhibition of NFAT by VIVIT, but not by a control inactive peptide (VEET), attenuated the expression of LPS or LPS plus IFN-γ induced IL-12 p40 mRNA and protein expression in bone marrow derived macrophages (BMDMs). VIVIT also reduced the DNA binding of NFAT to the NFAT/IRF8 site in IL-12 p40 promoter. In addition, the expression of iNOS mRNA and NO production was also inhibited by VIVIT. Furthermore, VIVIT also inhibited the secretion of IL-12 p70, IL-23 and TNF, suggesting a role for NFAT in proinflammatory gene expression in macrophages.

2.2 MATERIALS AND METHODS

2.2.1 Mice

7 to 10 week old IL-10 -/- mice on a C57BL/6 background and C57BL/6 wild type mice were purchased from the Jackson Laboratories. All animals were housed in specific pathogen free (spf) conditions in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. At the end of the study period, animals were euthanized using excess CO_2 inhalation, followed by which femurs were collected and bone marrow cells were isolated.

2.2.2 Reagents

LPS from *Salmonella enteritidis* was purchased from Sigma. FK506 was purchased from Alexis Biochemicals. Recombinant murine (rm) IFN-γ and GM-CSF were purchased from R&D Systems. Antibodies to NFATc1 (K18) and IRF8 (H-70) were purchased from Santa Cruz Biotechnology.

2.2.3 Peptide synthesis

TAT-VIVIT (YGRKKRRQRRR-GGG-MAGPVIVITGPHEE) and TAT-VEET (YGRKKRRQRRR-GGG-MAGPVIVITGPHEE) peptides were synthesized and purified using high performance liquid chromatography at the peptide synthesizing Facility of the University of Pittsburgh.

2.2.4 Bone marrow macrophage culture

Bone marrow derived macrophages (BMDMs) were prepared as described (155). Briefly, bone marrow cells obtained from WT or IL-10 -/- mice were seeded at ~1 x 10^6 cells/ml per 150mm culture dish in 25 ml culture medium (RPMI 1640 supplemented with penicillin (100 units/ml), streptomycin (100 units/ml), L-glutamine, and 10% heated-inactivated fetal bovine serum) containing rmGM-CSF (20 ng/ml). On day 3, 25 ml of culture medium containing rmGM-CSF (20 ng/ml) was added to the plates. Cells were harvested on day 7 for further experiments.

2.2.5 Electrophoretic mobility shift assay

Nuclear extracts from BMDMs were prepared using the NE-PER kit purchased from Pierce Biotechnology, by following the manufacturer's instructions. For EMSA, 200 ng of double-stranded oligonucleotide DNA probes were labeled with [α-^{32}P]dGTP and [α-^{32}P]dCTP by Klenow, and purified with Quick Spin columns (Sephadex G-50) (Roche). The sequence of the EMSA probe spanning the NFAT/IRF8 site of the IL-12 p40 promoter is 5'-gatcTCAGTTTCTACTTTGGGTTTCCATCAGAAAGT. EMSAs were performed as described previously (154).

2.2.6 RNA isolation, RT and real-time PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen). cDNA was derived from 1.5 μg total RNA by reverse transcription (RT) using Superscript II (Invitrogen) and Random hexamers (Invitrogen). Real-time PCR was performed as described previously (155) on the cDNA obtained

to detect IL-12 p40 and iNOS mRNA levels which were expressed as relative fold increase in transcript level with respect to unstimulated cells.

2.2.7 Cytokine ELISA

Murine IL-12 p40, IL-12 p70, IL-10 and TNF immunoassay kits (R & D Systems) were used according to manufacturer's instructions.

2.2.8 Nitrite Determination

Nitrite concentration in culture, a measure of NO synthesis, was assayed by a standard Greiss Reaction adapted to a microplate system as described previously (109). $NaNO_2$ is used as a standard, and the data are presented as a concentration of nitrite.

2.3 RESULTS

2.3.1 Involvement of NFAT in IL-12 p40 expression

We first investigated the role of NFAT in IL-12 p40 gene expression using the inhibitor FK506. We first utilized FK506 (177), as another clinically relevant NFAT inhibitor cyclosporine A has been shown to activate NF-κB, a transcription factor also involved in IL-12 p40 gene expression (178, 179). In bone marrow derived macrophages (BMDMs), FK506 inhibits IL-12 p40 protein secretion induced by LPS alone or LPS plus IFN-γ, in a dose dependent manner (Fig.11 A).

Real-time PCR analysis for IL-12 p40 mRNA demonstrated a similar dose-dependent inhibition of p40 mRNA by FK506 (Fig.11 B).

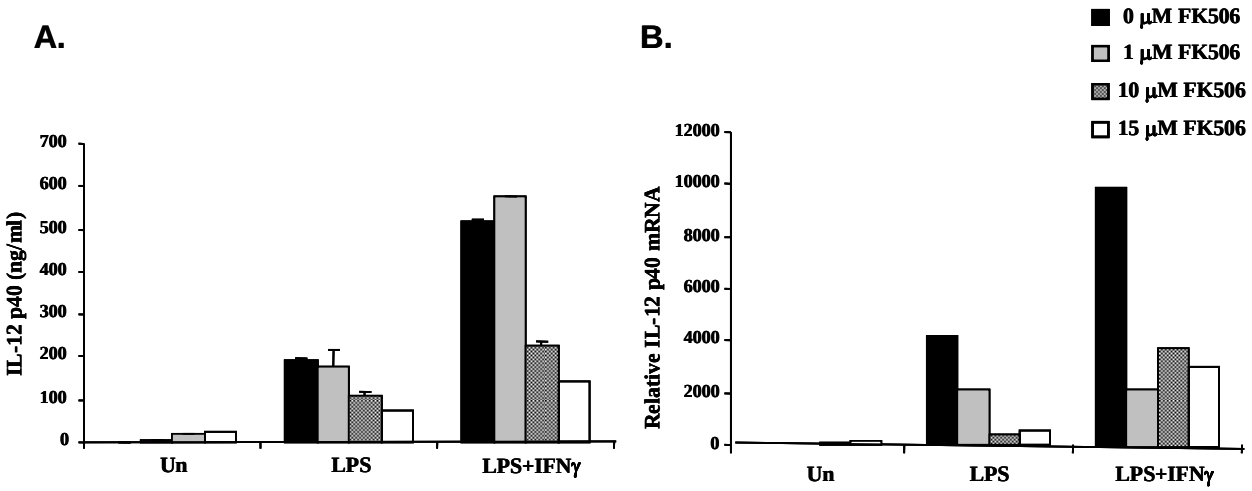


Figure 11. FK506 inhibits IL-12 p40 protein and mRNA expression.

(A.) Murine bone marrow-derived macrophages (BMDMs) were either untreated (un) or pretreated with the indicated concentrations of FK506 for 1 h followed by LPS (1 μg/ml) and/or IFN-γ (10 ng/ml) for 24 h. IL-12 p40 protein secretion was assayed from supernatants by ELISA. (B.) BMDMs were either untreated (un) or pretreated with the indicated concentrations of FK506 for 1 h followed by 1 h treatment with IFN-γ (10 ng/ml, where indicated) prior to LPS (1 μg/ml) treatment for 4h. Cells were harvested and total RNA was assayed for IL-12 p40 mRNA levels by real-time RT PCR. Each result represents the mean $\pm$ standard deviation (sd) for duplicate assays and is representative of three independent experiments.

We next utilized the selective peptide inhibitor of NFAT, VIVIT, to validate findings with FK506. Protein transduction domains (PTDs) have been employed for peptide delivery *in vitro* and *in vivo* (180, 181) and VIVIT was rendered cell-permeable by means of conjugation to the PTD of the HIV TAT protein. TAT-VIVIT inhibited IL-12 p40 protein secretion in a dose-dependent manner in LPS and LPS plus IFN-γ treated BMDMs (Fig. 12 A). Inhibition was also observed at the level of IL-12 p40 mRNA, as assessed by real-time RT PCR (Fig. 12 C). A

control related inactive peptide sequence which was also conjugated to the TAT domain, TAT-VEET, did not inhibit IL-12 p40 protein (Fig. 12 B) or mRNA (Fig. 12 C).

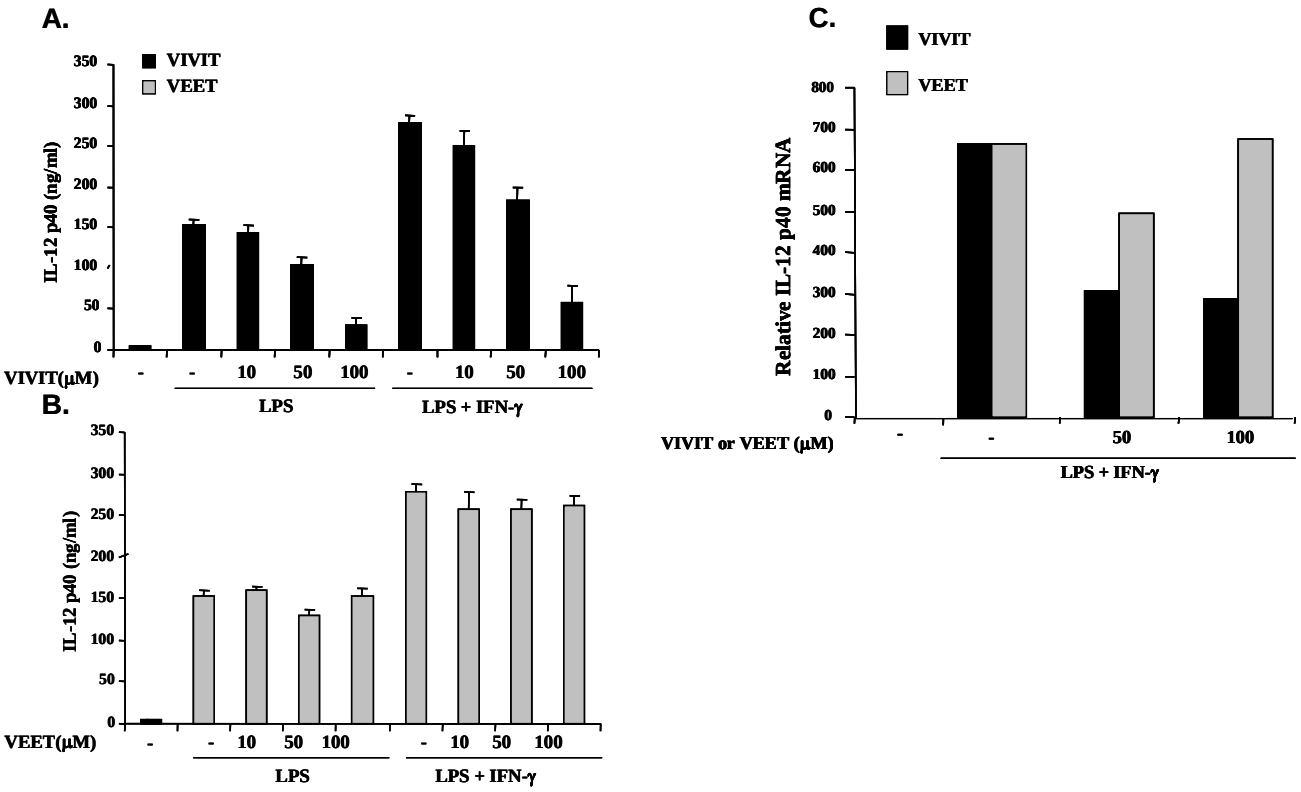


Figure 12. TAT-VIVIT inhibits IL-12 p40 protein and mRNA expression.

(A.) Murine bone marrow-derived macrophages (BMDMs) were either untreated or pretreated with the indicated concentrations of TAT-VIVIT (A.) or the control, scrambled peptide, TAT-VEET (B.) for 1 h followed by LPS (1 μg/ml) and/or IFN-γ (10 ng/ml) for 24 h. IL-12 p40 protein secretion was assayed from supernatants by ELISA. (C.) BMDMs were either untreated or pretreated with the indicated concentrations of TAT-VIVIT or TAT-VEET for 1 h followed by 1 h treatment with IFN-γ (10 ng/ml) prior to LPS (1 μg/ml) treatment for 4h. Cells were harvested and total RNA was assayed for IL-12 p40 mRNA levels by real-time RT PCR. Each result represents the mean ± sd for duplicate assays and is representative of three independent experiments.

To confirm the efficacy of TAT-VIVIT on NFAT nuclear translocation, we analyzed the DNA binding of NFAT to the NFAT/IRF8 site in the IL-12 p40 promoter by EMSA using nuclear extracts from murine BMDMs. Treatment of BMDMs with LPS and IFN-γ induced

DNA-protein complex formation on an oligonucleotide probe containing the NFAT/IRF8 site, spanning the region from -88 to -54 with respect to the transcription start site of the murine IL-12 p40 promoter (Fig 13, lane 2). The presence of NFAT and IRF8 in this complex was confirmed by supershift using anti-NFAT and anti-IRF8 antibodies. Anti-NFAT antibody generated a supershift whereas anti-IRF8 antibody abrogated complex formation, as shown previously (154). TAT-VIVIT treatment of cells reduced the induction of DNA-protein complex formed upon LPS and IFN-γ stimulation (Fig. 13, compare lanes 2 and 5). Supershift using antibodies revealed reduced levels of NFAT (Fig. 13, compare lanes 3 and 6) and IRF8 (Fig. 13, compare lanes 4 and 7) present in the complex. Treatment of cells with the control peptide did not affect DNA-protein complex formation. The above results demonstrate that the VIVIT peptide effectively prevented nuclear translocation of NFAT leading to reduced DNA-protein complex formation *in vitro*. In addition, these results also suggest that VIVIT inhibits IRF8 DNA binding as well, confirming our previous report that NFAT and IRF8 exist as a complex in macrophages.

Collectively, the above results in primary murine macrophages corroborate our previous study which revealed the NFAT/IRF8 site in the IL-12 p40 promoter (154) and provide a functional basis for the role of NFAT in IL-12 p40 expression in murine bone-marrow derived macrophages.

2.3.2 Inhibition of IL-12 p40 by VIVIT is independent of IL-10

IL-10 is a potent anti-inflammatory cytokine which inhibits macrophage activation and the proinflammatory response (182). Importantly, IL-10 inhibits IL-12 p40 transcription (97). Therefore, we determined whether inhibition of IL-12 p40 by VIVIT was mediated by

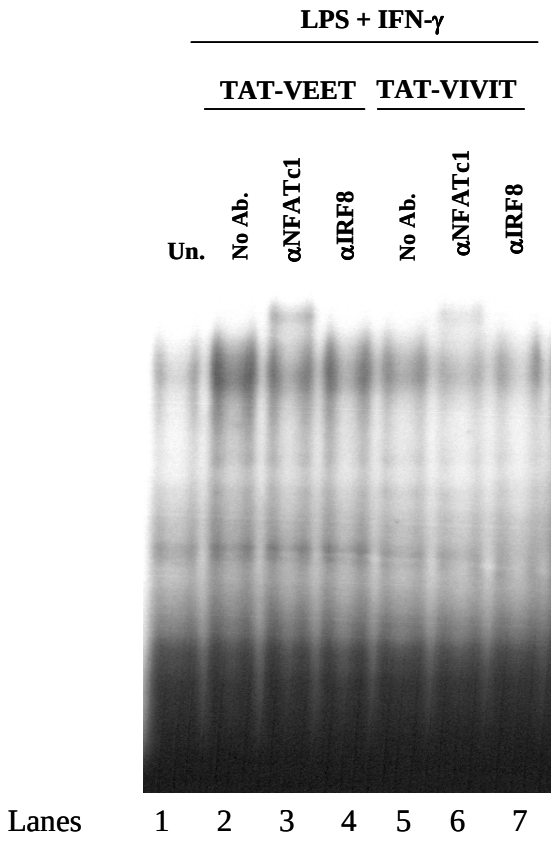


Figure 13. TAT-VIVIT reduces DNA binding to the NFAT/IRF8 site in the murine IL-12 p40 promoter.

BMDMs were either untreated (lane 1) or pretreated with TAT-VIVIT (75 μM, lanes 5-7) or TAT-VEET(75 μM, lanes 2-4) for 1 h followed by 1 h treatment with IFN-γ (10 ng/ml) prior to LPS (1 μg/ml) treatment for 4h. Cells were harvested and nuclear extracts were prepared for EMSA. ^{32}P labeled oligonucleotide probe spanning the NFAT/IRF8 site of the IL-12 p40 promoter (154) was incubated with 10 μg of nuclear extracts on ice for 30 min prior to electrophoresis. For supershift assays (lanes 3,4 and 6,7), 10 μg nuclear extract was incubated with 3 μg of anti-NFAT c1 (lanes 3 and 6) or anti-IRF8 (lanes 4 and 7) antibody for 30 min on ice prior to addition of the ^{32}P labeled probe and 30 min incubation on ice followed by electrophoresis. The above result is representative of three independent experiments.

induction of IL-10. IL-10 levels were evaluated in cell free supernatants of BMDMs treated with the indicated concentrations of TAT-VIVIT (Fig. 14 A). Inhibition of NFAT does not induce IL-10 in LPS or LPS plus IFN-γ treated BMDMs. IFN-γ inhibited LPS induced IL-10,

as previously described (183). Furthermore, TAT-VIVIT inhibits IL-12 p40 in BMDMs derived from IL-10 deficient mice in a dose dependent manner (Fig. 14 B), substantiating that VIVIT inhibits IL-12 p40 expression through IL-10 independent pathways.

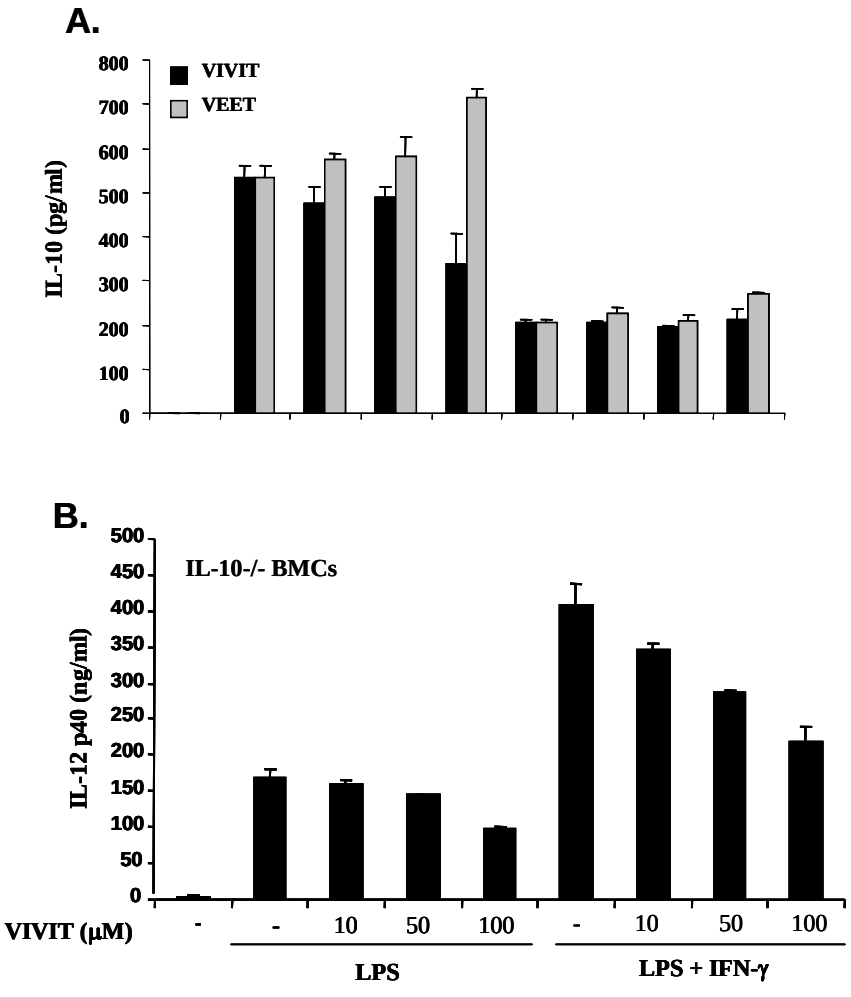


Figure 14. Inhibition of IL-12 p40 by TAT-VIVIT is independent of IL-10.

(A.) Murine BMDMs were either untreated or pretreated with the indicated concentrations of TAT-VIVIT (black bars) or TAT-VEET (grey bars) for 1 h followed by LPS (1 μg/ml) and/or IFN-γ (10 ng/ml) for 24 h. IL-10 protein secretion was assayed from supernatants by ELISA. (B.) BMDMs from IL-10 -/- mice were either untreated or pretreated with the indicated concentrations of TAT-VIVIT for 1 h followed by LPS (1 μg/ml) and/or IFN-γ (10 ng/ml) for 24 h. IL-12 p40 protein secretion was assayed from supernatants by ELISA. Each result represents mean ± sd of duplicate assays and is representative of three independent experiments.

2.3.3 NFAT is involved in inducible nitric oxide synthase (iNOS) expression

We have previously demonstrated the presence of NFAT/IRF8 composite element in the murine iNOS promoter (Chen Zhu, Scott Plevy, unpublished data) and also established that IRF8 and IRF1 are essential for induction of the iNOS gene (184). To examine the role of NFAT in iNOS expression, we assayed nitric oxide production by BMDMs treated with the indicated concentrations of TAT-VIVIT. Inhibition of NFAT attenuated nitric oxide production (Fig. 15 B), and real-time RT-PCR analysis revealed that iNOS mRNA was inhibited in a dose dependent manner (Fig. 15 B). Thus, these results suggest that NFAT mediates the expression of iNOS and nitric oxide production in BMDMs.

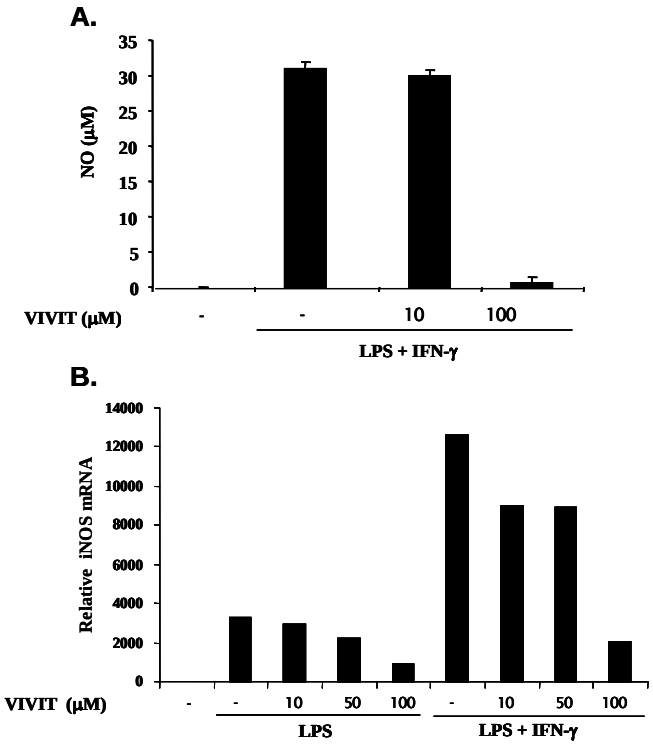


Figure 15. TAT-VIVIT inhibits nitric oxide secretion and iNOS mRNA expression.

(A.) BMDMs from WT mice were either untreated or pretreated with the indicated concentrations of TAT-VIVIT for 1 h followed by LPS (1 μg/ml) and IFN-γ (10 ng/ml) for 24 h. Nitric oxide secetion was assayed from supernatants by Greiss reaction. (B.) BMDMs were either untreated or pretreated with the indicated concentrations

of TAT-VIVIT for 1 h followed by 1 h treatment with IFN-γ (10 ng/ml) prior to LPS (1 μg/ml) treatment for 4h. Cells were harvested and total RNA was assayed for iNOS mRNA levels by real-time RT PCR. Each result represents the mean ± sd for duplicate assays and is representative of three independent experiments.

2.3.4 NFAT is important for induction of IL-12 p70, IL-23 and TNF

Since IL-12 p40 heterodimerizes with IL-12 p35 and p19 to form IL-12 p70 and IL-23 respectively, levels of bioactive cytokines were determined in BMDMs treated with TAT-VIVIT and stimulated with LPS or LPS plus IFN-γ. It was observed that VIVIT inhibited both IL-12 p70 (Fig. 16 A) and IL-23 (Fig. 16 B) in a dose-dependent manner, while the control peptide had no effect (data not shown). NFAT has been shown to play a role in TNF expression in T cells (185). We examined the effect of NFAT inhibition on TNF secretion in VIVIT-treated BMDMs. VIVIT peptide suppressed the induction of TNF by LPS or LPS plus IFN-γ (Fig. 16 C), while the control peptide did not (data not shown). These results show that NFAT is important for expression of several key inflammatory mediators in murine BMDMs.

2.4 DISCUSSION

In summary, data presented in this study provide evidence for a role of NFAT in regulating the expression of proinflammatory genes in macrophages. NFAT positively regulates the expression of the IL-12 p40 gene, thereby controlling the levels of bioactive heterodimers of IL-12 p40 - IL-12 p70 and IL-23.

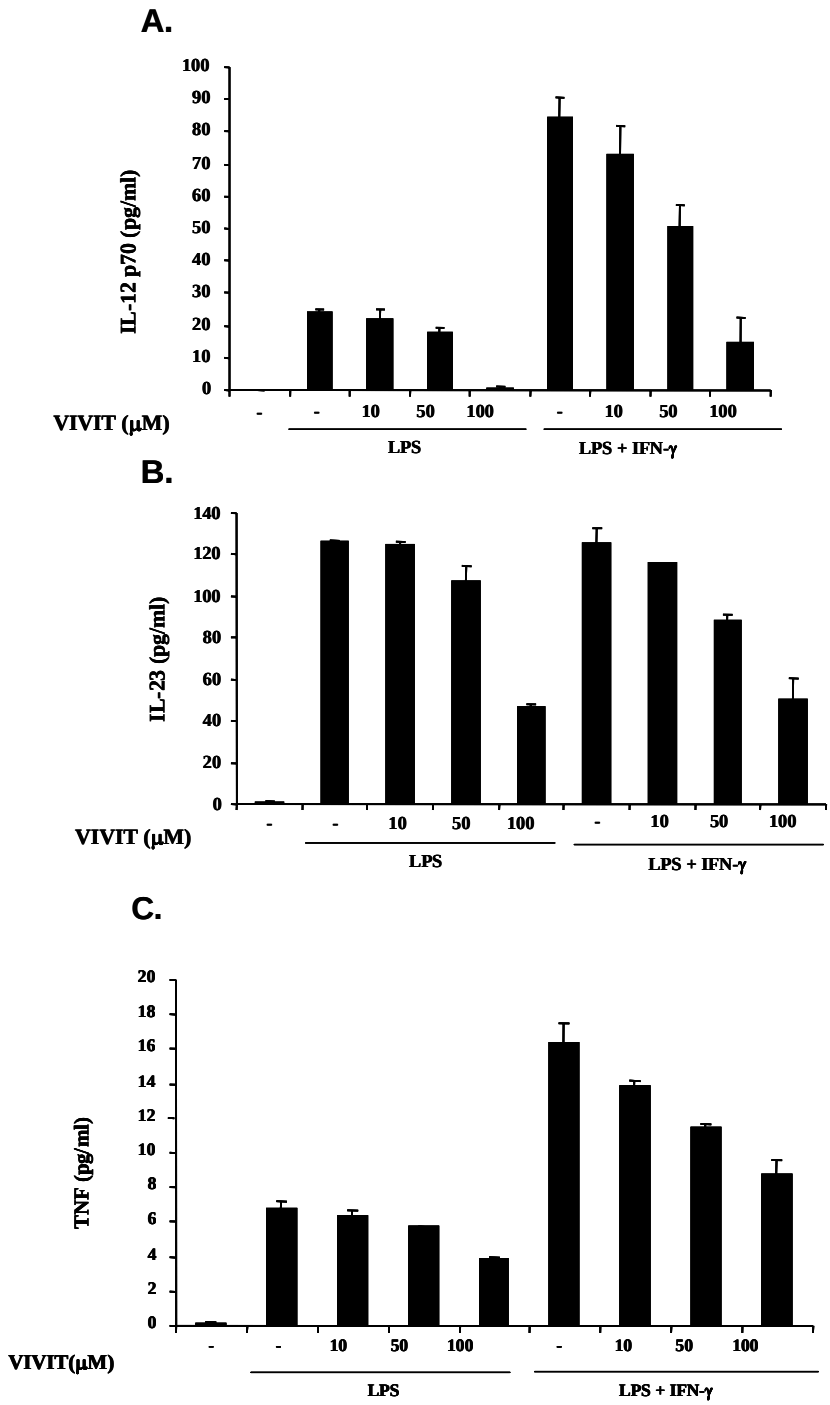


Figure 16. TAT-VIVIT inhibits proinflammatory cytokine expression in murine macrophages.

(A.) BMDMs from WT mice were either untreated or pretreated with the indicated concentrations of TAT-VIVIT for 1 h followed by LPS (1 μg/ml) and/or IFN-γ (10 ng/ml) for 24 h. IL-12 p70 (A.), IL-23 (B.) and TNF (C.) protein secretion was assayed from supernatants by ELISA. Each result represents mean $\pm$ sd of duplicate assays and is representative of three independent experiments.

This inhibition was found to be independent of IL-10. In addition to regulating IL-12 family of cytokines, NFAT also controls the expression of TNF in murine bone-marrow macrophages. Furthermore, NFAT also regulated the expression of the iNOS gene and nitric oxide production. Thus, NFAT, likely functioning as a complex with IRF8 as described in our previous study; or with other interacting partners has a general role in programming macrophage gene expression.

The NFAT family is a complex family of transcription factors with five proteins and each member has different alternatively spliced isoforms. Although, NFAT was first identified in T-cells, it is now known to be important for the development and functioning of several organs including the nervous system, heart, blood vessels, kidney, bone and muscle (175). Recent studies have also shown the importance of NFAT in regulating gene expression in other immune cells such as B cells (186), mast cells (187), eosinophils (188), basophils (189) and NK cells (190). Although NFAT proteins have been shown to be involved in the differentiation of monocyte/macrophage lineage cells into osteoclasts (191), our knowledge of NFAT functions in macrophages *per se* is very limited. Ca^{2+}/calcineurin signaling has been implicated in macrophage biology. In a report by Conboy et. al, Ca^{2+}/calcineurin signaling inhibited macrophage cytokine production (157). In another report, Ca^{2+} flux induced by Fcγ receptors resulted in inhibition of IL-12 production in response to LPS (192). However, several reports have shown the opposite effect. Calcium homeostasis and flux has been shown to be important for antigen processing by macrophages (193) and calcineurin signaling has been reported to mediate iNOS expression and NO secretion by murine macrophages (194). Ca^{2+} was also reported to be involved in TNF induction by LPS (195). Moreover, the African swine fever virus protein, A238L has been shown to inhibit inflammatory gene expression in infected macrophages by binding to calcineurin and inhibiting NFAT-dependent gene expression (196).

Furthermore, in a recent study by Jeffrey et. al. (197), macrophages deficient for the dual specificity phosphatase- PAC-1, displayed decreased activation of NFAT and diminished expression of proinflammatory mediators. Macrophages express NFAT family members (154, 191) and in our previous study we have demonstrated the requirement of NFATc1 and c2 in IL-12 p40 gene expression in a macrophage cell line (154). Here, we extend our observations and show that NFAT is important for general proinflammatory gene expression by primary murine macrophages using the selective inhibitory peptide-VIVIT.

NFAT proteins are involved in the induction and regulation of Th1 and Th2 responses. NFATc1 and c2 deficient mice display impaired Th1 and Th2 responses (198, 199). In fact, NFATc2 deficient mice are more susceptible to infection by the gram-positive intracellular pathogen, *Leishmania major* (200). Furthermore, sustained NFATc1 signaling has been shown to promote a Th1-like pattern of gene expression in murine $CD4^+$ T cells (201), suggesting that the extent of NFAT signaling can affect the balance of Th1 versus Th2 responses. Appropriate regulation of this balance is a prerequisite to avoid chronic inflammatory diseases. Dysregulation of the innate immune system is thought to be a major factor in the pathogenesis of these diseases (170). Despite the critical role for NFAT in controlling the outcome of immune responses, very little is known about its involvement in chronic inflammatory and autoimmune diseases. A recent study demonstrated increased nuclear localization of NFATc2 in keratinocytes of human psoriatic lesions compared to normal skin (202). Our study provides a basis for further understanding NFAT function in immune mediated inflammatory disorders.

The immunosuppressive agents CsA and FK506 inhibit the phosphatase activity of calcineurin, thus preventing nuclear localization of NFAT (203). They have been extensively used to treat autoimmune diseases, but are fraught with significant side effects including nephro-

and neuro-toxicity (204, 205). These agents also inhibit other pathways downstream of calcineurin which could lead to undesired clinical side effects (204, 205). The peptide inhibitor VIVIT has therapeutic potential due to its high affinity and selectivity. VIVIT peptide, including the cell permeable version, has been employed *in vivo* in mouse models of transplantation (206) and cardiac hypertrophy (207). Given the data from our study that NFAT plays an important role in inflammatory gene expression in macrophages, the TAT-VIVIT peptide could prove invaluable for developing novel therapeutic approaches for chronic chronic inflammatory diseases where both innate and adaptive immune response play a role in pathogenesis.

3.0 CHARATERIZATION OF INNATE IMMUNE DEFECTS AND IBD IN PI3K p110δ MUTANT MICE

Innate immune responses mediated by Toll-like receptors (TLRs) and nucleotide-oligomerization-binding-domain (NOD) proteins, while being crucial for host defense against pathogens; need to be tightly regulated to prevent chronic inflammatory diseases like inflammatory bowel disease (IBD). Phosphoinositide-3 kinase (PI3K) is an important negative regulator of TLR signaling. Initial characterization of mice with a targeted mutation in the p110δ subunit of PI3K revealed defects in B- and T-cell signaling as well as chronic, focal colitis. Here we have further characterized the histopathological features of IBD in these mice and investigated innate immune defects. Spontaneous colitis is demonstrated at 8 wks of age with disease severity increasing with age. Histologic analysis revealed a marked increase in the number of intraepithelial lymphocytes in the colonic crypts, in addition to leukocytic infiltration of the lamina propria and crypt abscesses. A proinflammatory mucosal and systemic cytokine profile was observed characterized by overexpression of IL-12, TNF, IFN-γ and IL-17. Dysregulated immune responses were observed in macrophages, including hyperresponsiveness to TLR ligands and defects in IL-10 and C5a mediated inhibition of IL-12 p40. Furthermore, PI3K mutant macrophages demonstrated impaired responses to the NOD2 ligand, muramyl dipeptide (MDP). In conclusion, this study further confirms the critical role of PI3K in dampening inflammation and presents a novel mouse model which could provide valuable insight into disease pathogenesis in human IBD.

3.1 INTRODUCTION

The gastrointestinal tract coexists with more than 10^{13} resident commensal bacteria in a delicate state of homeostasis maintained through a complex network of interactions between the bacteria, epithelial cells and the innate immune cells of the mucosal immune system. Both commensal and pathogenic bacteria are recognized through conserved molecular microbial patterns by pattern-recognition receptors (PRRs), of which Toll-like receptors (TLRs) and nucleotide-oligomerization-binding-domain (NOD) proteins form an integral component (78, 79, 208, 209). TLRs are membrane bound receptors whereas NOD proteins are cytosolic. Signaling through both these receptors leads to the activation of NF-kB, culminating in the induction of proinflammatory cytokines including IL-12, IL-23, IFN-γ and TNF resulting in the elimination of pathogens (78, 79). Although the mechanism by which the host distinguishes commensal from pathogenic bacteria is unknown, it has been shown that under normal conditions TLR signaling initiated by commensal microflora is protective (210). However, dysregulation of this response can lead to a chronic state of intestinal inflammation manifesting into diseases like Crohn's disease (CD) and Ulcerative colitis (UC) which comprise human inflammatory bowel diseases (IBD) (133). Both CD and UC are exemplified by inappropriate innate immune responses to normal intestinal microflora (211-213). The critical role for innate immunity in IBD has been substantiated by the recent description of *Nod2* (*CARD15*) as a susceptibility gene in CD (80, 81). Therefore, it is imperative that TLR and NOD signaling is strictly regulated in order to prevent unwanted stimulation to enteric flora, while effectively responding to potential microbial pathogens. It is also crucial that inflammation thus initiated is resolved in a timely manner to prevent perpetuation of the immune response leading to chronic inflammation. Several molecules

which dampen inflammation have been described, of which phosphoinositide-3 kinases (PI3Ks) have emerged as an important negative regulator of TLR signaling (214-216).

Class I_A PI3Ks are a family of heterodimeric enzymes consisting of a regulatory subunit (p85, p55 or p50) and a catalytic subunit (p110α, p110β or p110δ) (217, 218). While p110α and p110β are expressed ubiquitously, p110δ isoform is preferentially expressed in leukocytes (219). Dendritic cells from mice lacking the p85α regulatory subunit of Class I_A PI3K produce enhanced amounts of the proinflammatory cytokine IL-12 leading to a skewed Th1 response. As a result, these mice are resistant to *Leishmania major* infection, suggesting that PI3K is directly involved in regulating Th1 versus Th2 balance *in vivo* (220). However, the immunomodulatory effects of PI3K on innate immune responses in mucosal inflammation and macrophages are unclear.

The clearest role of PI3K in Th1 mediated chronic inflammation was described in a mouse harboring a point mutation in the p110δ catalytic subunit of PI3K (169). These mice demonstrated several B and T cell defects including improper maturation, impaired antigen receptor signaling and severely impaired humoral immune responses. Notably, these mice developed chronic segmental intestinal inflammation.

In light of the fact that the human p110δ gene maps to the IBD7 susceptibility locus on chromosome 1p36 (221, 222), in this study we further characterize the development of chronic IBD in PI3K p110δ mutant mice and investigate the role of PI3K in the regulation of TLR signaling in macrophages. Histologic analysis of colitis revealed several novel features, one of which was the presence of intraepithelial lymphocytes. A skewed mucosal Th1 response was observed with overexpression of several signature Th1 cytokines including IL-12, TNF, IFN-γ and IL-17. A defect was observed in the IL-10 and C5a mediated inhibition of IL-12 p40.

Finally, impaired responses to the NOD2 ligand MDP were observed in the mutant macrophages. Thus, the PI3K p110δ mutant mice demonstrate innate immune defects that may elucidate pathogenesis of human IBD.

3.2 MATERIALS AND METHODS

3.2.1 Mice

PI3K p110δ mutant mice which were on the C57BL/6 background were generated by B. Vanhaesebroeck (Ludwig Institute for Cancer Research, London, UK). C57BL/6 wild type mice were obtained from The Jackson Laboratory. All animals were housed in specific pathogen free conditions (spf) in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. At the end of the study period, animals were killed using excess CO_2 inhalation. Immediately, afterward spleen, colon, small bowel and femurs were collected. Splenocytes and bone marrow derived macrophages were cultured as described previously (109).

3.2.2 Reagents

Lipopolysaccharide (LPS) from *Salmonella enteritidis* was purchased from Sigma and repurified by modified phenol extraction as described in (223). CpG DNA was obtained from Integrated DNA Technologies. Synthetic bacterial lipoprotein (sBLP) was purchased from EMC

Microcollections. Peptidoglycan (PGN), muramyldipeptide (MDP), wortmannin and recombinant human C5a were purchased from Sigma. IL-10, GM-CSF and IFN-γ were obtained from R & D Systems.

3.2.3 Generation of elicited peritoneal macrophages

C57BL/6 mice were injected i.p. with 3ml sterile thioglycollate (0.3%). After 5 days, animals were harvested and elicited macrophages were obtained by peritoneal gavage using RPMI with 1% Penicillin/Streptomycin. Cells were washed once with the same medium, resuspended in complete medium (RPMI with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin) and incubated at 37º C for 2h to allow for cells to adhere. Adherent peritoneal macrophages were then collected and stimulated.

3.2.4 Cytokine ELISAs

Murine IL-12 p40, IL-12 p70, IL-10, IFN-γ and TNF immunoassay kits (R & D Systems) and IL-23 (eBioscience) were used according to manufacturers' instructions. IL-17, MIP1a, RANTES, KC and G-CSF levels were determined by multiplex ELISA.

3.2.5 Western blot analysis

Western blot analyses were performed on nuclear or whole cell extracts as noted in Results (Fig. 4 and 5) and described previously (109). Antibodies to p-JNK, p-p38, p-ERK, JNK2, p38, ERK, p-STAT3, STAT3, IRF8, STAT1 were obtained from Santa Cruz Biotechnology.

3.2.6 Nitrite Determination

Nitrite concentration in culture, a measure of NO synthesis, was assayed by a standard Greiss Reaction adapted to a microplate system as described previously (224). $NaNO_2$ is used as a standard, and the data are presented as a concentration of nitrite.

3.2.7 RNA extraction and RT PCR or real-time RT PCR analysis

Total RNA was extracted using TRIZOL reagent (Invitrogen). cDNA was derived from 1.5 μg total RNA by reverse transcription (RT) using Superscript II (Invitrogen) and Random hexamers (Invitrogen). PCR was performed on RT products as described (124) to detect IL-12 p40 NOD2 and β-actin. The following primer sequences were used for NOD2 - Upstream primer: 5'GCTCCATGGCCAAGCTCCTT3' and downstream: 5'CTGCACCCCGGTAGGTGATG 3'. Real-time PCR was performed as described previously (155) on the cDNA obtained to detect IL-12 p40 and IRF8 mRNA which were expressed as relative fold increase in transcript level with respect to unstimulated cells. IRF8 primers were: LUX: 5'GACGAGAGAGCACAGCGTAACCTCG5C 3' and its unlabeled counterpart: 5'TGGATGCTTCCATCTTCAAGG 3'.

3.2.8 Intestinal tissue explant cultures and histology

Colonic explant cultures were performed as described previously (155). 24h culture supernatants were assayed for cytokine ELISA. Slides were prepared for hematoxylin and Eosin staining as described (155) and histologic analysis was performed by a pathologist blinded to the study groups (A. Sepulveda).

3.2.9 p-AKT ELISA

A cell-based ELISA (CASE kit, Superarray Bioscience Corporation) was utilized to determine the phosphorylation levels of the downstream target of PI3K: AKT. The ELISA was performed according to the manufacturer's instructions.

3.3 RESULTS

3.3.1 PI3K p110δ mutant mice spontaneously develop colitis

In order to characterize the histological features of colitis in PI3K p110δ mutant mice, colonic sections were examined at the indicated ages and compared with those from wild type animals. A histological scoring system was used to assess the severity of the induced inflammation. Briefly, the criteria used to classify cases into grades 0 to 4 was as follows: *Grade 0* included cases characterized by (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 a *grade 2* was attributed. *Grade 3* was given when: (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, (b) or 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 all wild type mice groups revealed minimal mucosal inflammation with one or less mitosis per 10 HPF, and was scored as grade 0 to 1 (Figs. 17 and 18). Definite colitis was detected in all PI3K mutant mice. The severity of colitis was mainly characterized by increased number of intraepithelial lymphocytes, occasional apoptotic bodies, and marked increase in the number of mitosis in the colonic crypts. There was a patchy increase in lamina propria lymphocytes and rare neutrophils in the lamina propria, accompanying the increasing grade of colitis (Fig. 18). The colonic crypt architecture was overall well preserved with only focal disruption of the tubular architecture in a rare case that developed focal crypt abscesses (Fig. 17).

Macroscopically, colons from PI3K mutant mice were shorter in length and thicker than those from WT mice (Fig. 19 A). A small percentage (about 10%) of mice also developed rectal prolapse (Fig. 19 B).

Significant increases in the inflammatory scores correlated with increasing mice age (Fig. 19 C and D). Histologic colitis was detected starting at 8 weeks of age and peak grades were reached by the oldest mice. The percentage of fields demonstrating no histological inflammation (grade 0) significantly decreased and that depicting severe inflammation (grade 3 to 4) significantly increased (Fig. 19 C) with age. The mean colitis score also increased with age (Fig. 19 D). A characteristic feature among these most severe cases was the presence of numerous intraepithelial lymphocytes throughout the colonic mucosa (Fig. 18). The intraepithelial lymphocytosis predominantly involved the deep portion of the colonic crypts. Immunohistochemical analysis for CD3 protein revealed the presence of numerous $CD3^+$ IELs in

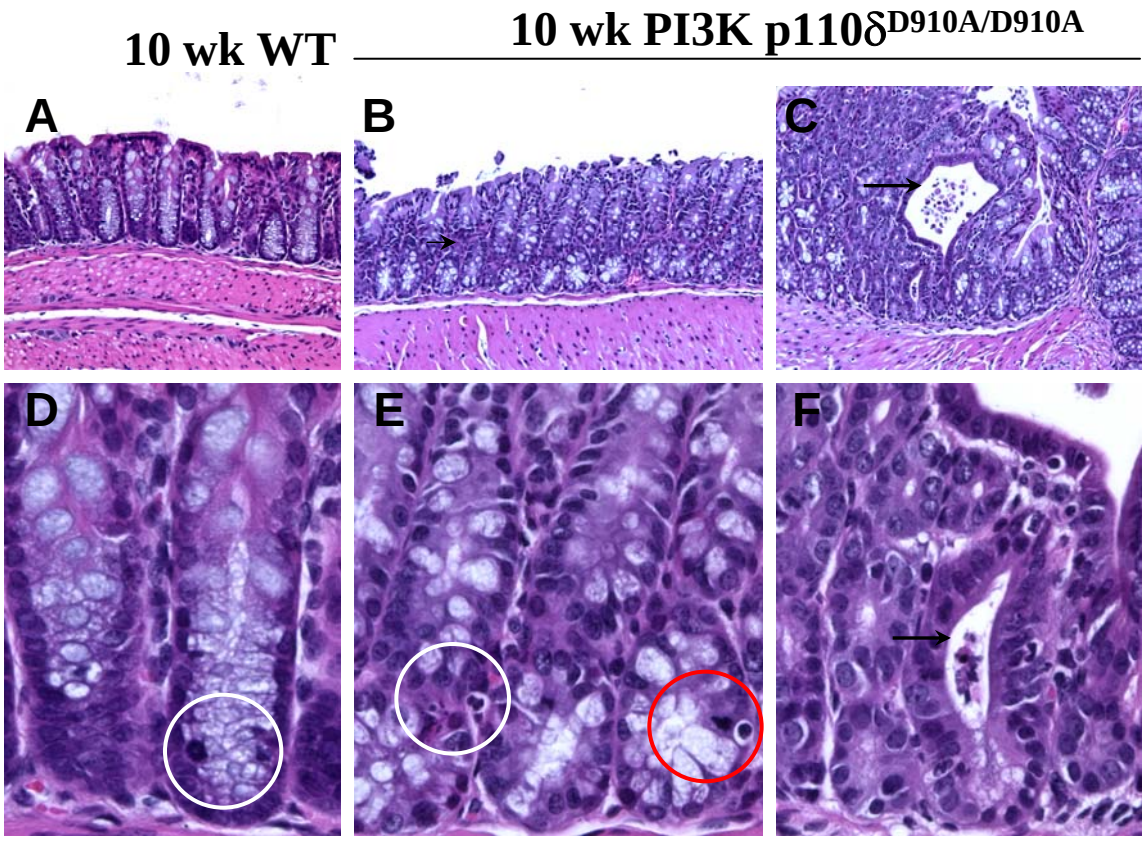
the colonic crypts of mutant mice compared to WT mice (Fig. 17 G and H). Interestingly, a significant increase in mucosal thickening was not observed, but a definitive reduction of crypt goblet cells, and some areas of relatively attenuated mucosa was observed, at increasing levels as the mice aged (Fig. 18). No significant increase in sub-epithelial collagen or lamina propria fibrosis was observed. There were no granulomas in any sections examined.

In general, inflammation scores appeared to be higher in the proximal and transverse than in the distal portion of the colon. The inflammatory infiltrate was in general restricted to the mucosa. Only few cases presented with crypt abscess formation (grade 4) (Fig. 17). One of the cases displaying crypt abscesses was a 10-week-old mutant mouse, whereas other cases with grade grade 4 colitis were found among the oldest mice. No areas of dysplasia or carcinoma were identified.

Three cases of small bowel samples were also examined. All three cases displayed an increased number of intraepithelial lymphocytes but no significant disruption of the normal small bowel architecture was appreciated. No definitive villous blunting was seen. One case presented a crypt abscess, which accounted for a focal grade 4 area. Similar to the colon, no dysplasia or cancer was identified in the small bowel.

In summary, the histopathological features observed in PI3K mutant mice are histologically reminiscent of lymphocytic colitis (225) and perhaps inflammatory bowel disease. In contrast to lymphocytic colitis in humans, most of the intraepithelial lymphocytes in the mouse model occur deep in the colonic crypts, whereas, in humans intraepithelial lymphocytosis characteristically involves the surface epithelium in addition to the length of the crypts.

Figure 17


10 wk WT
10 wk PI3K p110δD910A/D910A
A
B
C
D
E
F

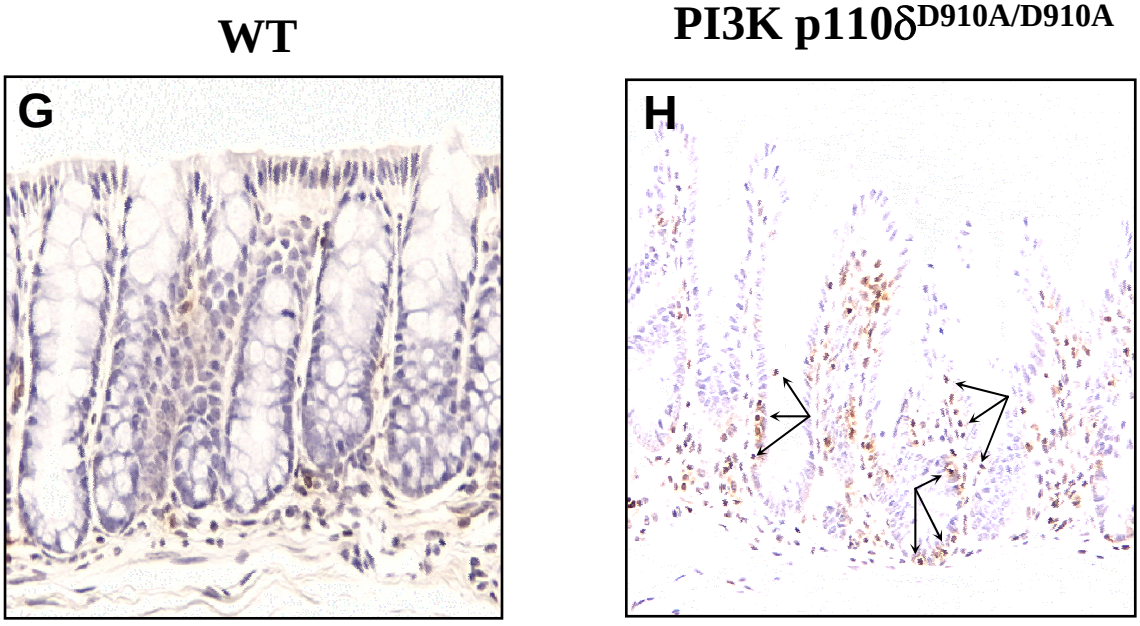

WT
PI3K p110δD910A/D910A
G
H

Figure 18

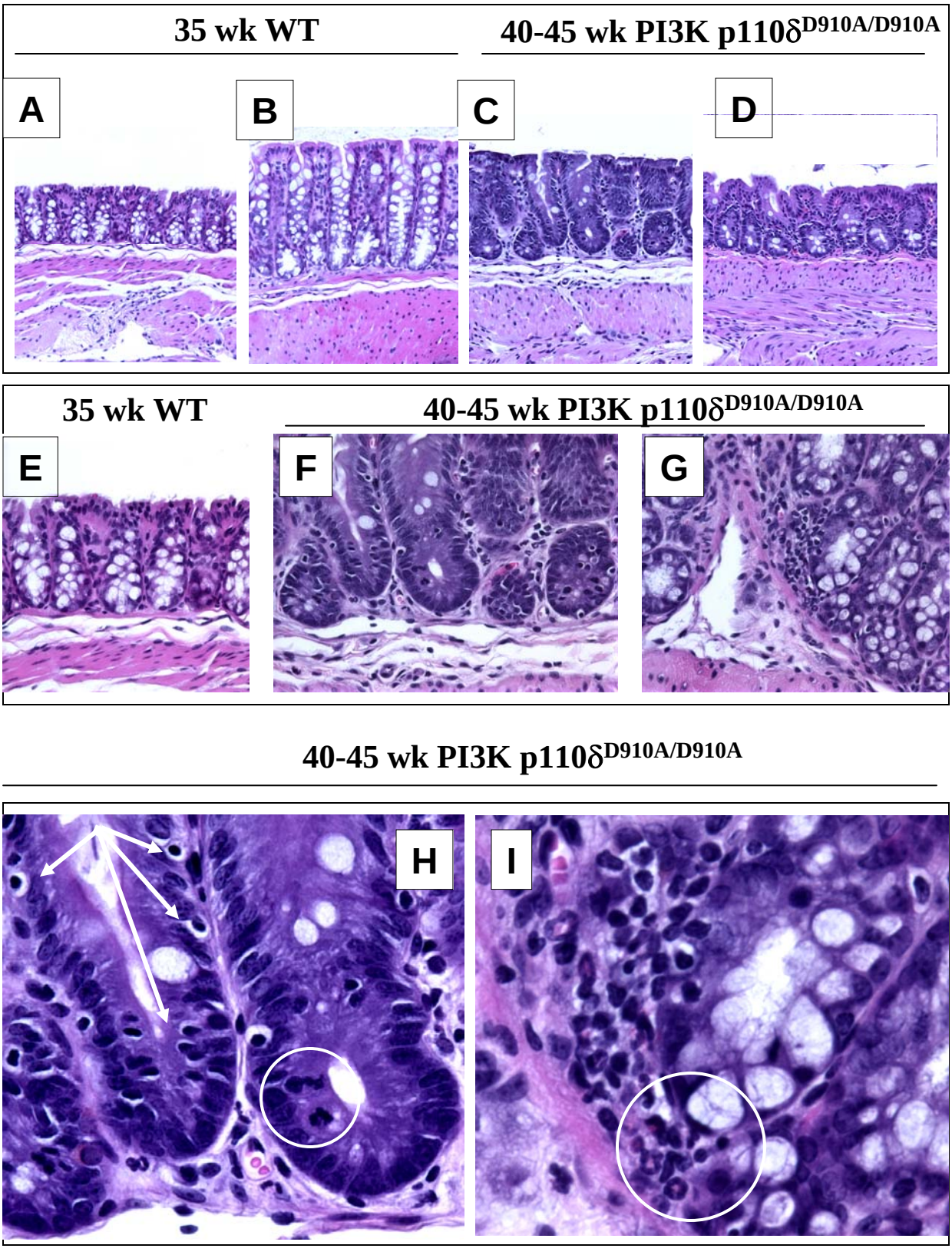

35 wk WT
40-45 wk PI3K p110δ$^{D910A/D910A}$
A
B
C
D
35 wk WT
40-45 wk PI3K p110δ$^{D910A/D910A}$
E
F
G
40-45 wk PI3K p110δ$^{D910A/D910A}$
H
I

Figure 17. PI3K p110δ mutant mice spontaneously develop colitis with markedly increased number of IELs in the colonic crypts.

(A-F) The colons of PI3K p110δ mutant mice were examined at the indicated ages and compared with those from wild type animals. The paraffin embedded tissues were cut at 5 microns and hematoxylin and eosin stained sections were coded and classified in various age groups for blinded microscopic analysis of the mucosal lesions. (A and D.) Representative staining of colonic sections from 10 wk old WT mice reveal minimal mucosal inflammation with 1 or less mitotic bodies (white circle, D.) in the crypts. (B and E) Colonic sections from 10 wk old PI3K mutant mice demonstrating mild inflammation with leukocytic infiltration of the lamina propria (black arrow, B.) and apoptotic bodies (white circle, E.) and intraepithelial lymphocytes (red circle, E.) in the crypts. (C and F) Occasional cases of focal crypt abscesses were observed (black arrows). (G and H) Representative immunohistochemical analysis of colonic sections from WT (G.) and PI3K mutant (H.) mice for CD3. Marked increase in CD3 positive IELs (black arrows, H) is observed in PI3K mutant sections as opposed to WT (G).

Figure 18. Histopathologic severity of colitis in PI3K mutant mice increases with age.

Shown here are representative hematoxylin and eosin staining of colonic sections from older WT and PI3K mutant mice. (A, B and E) Colons from 35 wk old WT mice display minimal inflammation. (C, D, F-I.) Low power (C and D) and high power (F-I) fields of colonic sections from 40-45 wk PI3K mutant mice demonstrating marked reduction of goblet cells, increased leukocytic infiltration of the lamina propria including neutrophils (white circle, I), increase in mitotic bodies (white circle, H) and increase in the number of IELs in the crypts (white arrows, H) .

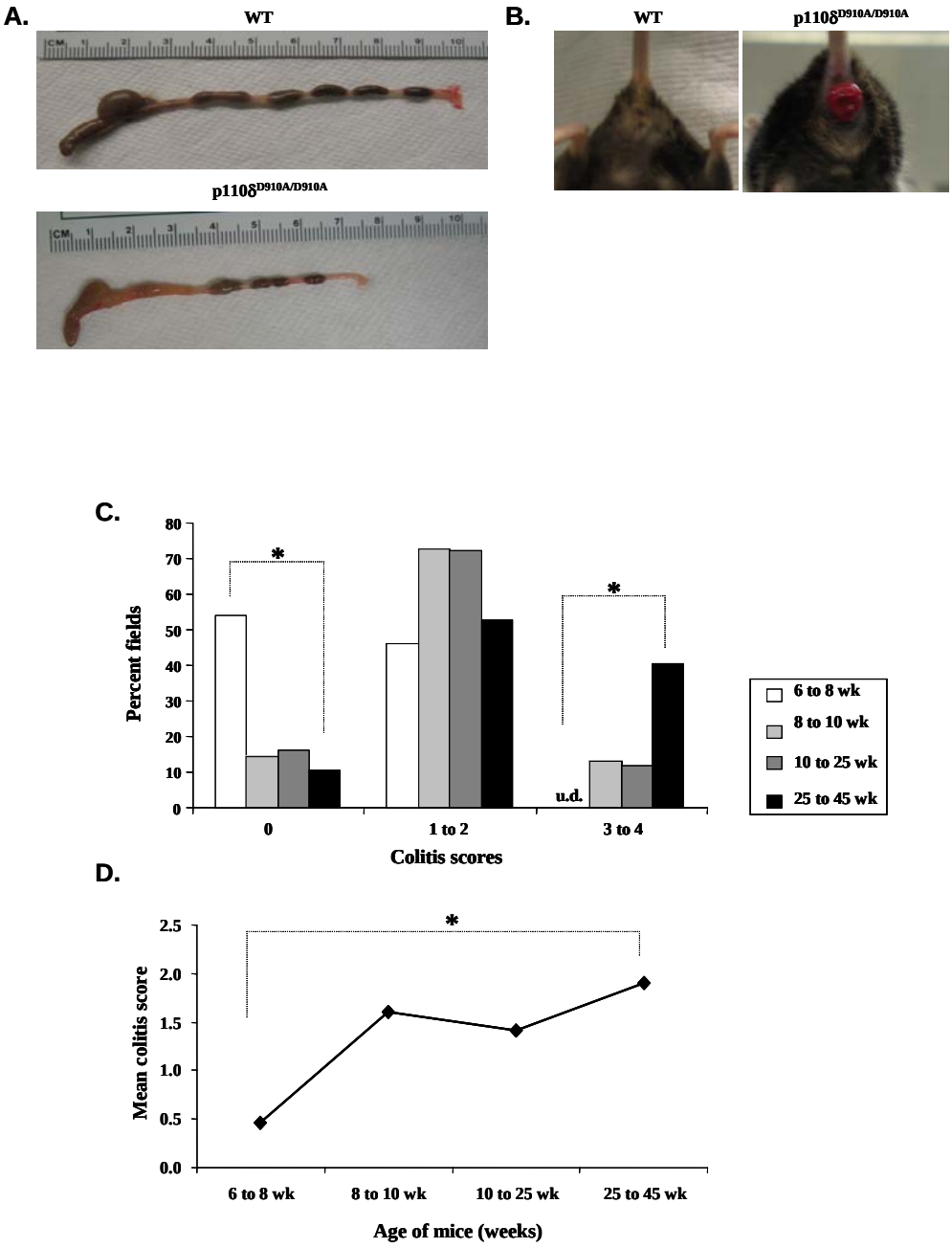


Figure 19. Assessment of colitis in PI3K p110δ mutant mice.

(A.) Representative colons from 25 wk C57Bl/6 WT and PI3K p110d mutant (p110dD910A/D910A) mice. The mutant mice had shorter colons which were mostly inflamed in the proximal and the transverse regions. (B.) Rectal prolapse was observed in about 10% of the PI3K mutant mice. (C. and D) Age wise comparison of histological scores of colonic sections from PI3K mutant mice. Results are represented as percentage of microscopic fields in each age group with score 0, 1 to 2 or 3 to 4 (C.) or mean colitis scores of each group (D.). The percent fields with severe score (3 to 4) and the mean colitis score increased significantly with age. *, $P<0.01$

3.3.2 PI3K p110δ mutant mice display mucosal and systemic Th1 cytokine profile

In order to characterize the mucosal cytokine profile of PI3K p110δ mutant mice, spontaneous secretion of cytokines was measured in cultures of colonic mucosal tissue from intestinal explants obtained from 15 week old PI3K mutant and age and strain matched wild type (WT) mice. Intestinal explants of PI3K mutant mice secrete significantly elevated amounts of the Th1 inducing cytokines – IL-12 p40, IL-12 p70 and TNF (Fig. 20 A). They also secrete markedly increased amounts of IL-17 (Fig. 20 A), suggesting the involvement of the recently discovered Th17 cells (62, 63). Furthermore, while IFN-γ levels were undetectable in supernatants from WT intestinal explants, a profound increase in IFN-γ level was observed in PI3K mutant colonic explant cultures (Fig. 20 A). No differences were observed in intestinal IL-10 levels between WT and mutant mice. PI3K mutant intestinal explants also secreted high amounts of growth factors and chemokines – G-CSF, MIP1-α, RANTES and KC (Fig. 20 A). Collectively, these results suggest that PI3K mutant mice display a proinflammatory Th1 mucosal cytokine profile.

Although the PI3K p110δ mutant mice have been shown to display a defect in T and B-cell signaling, we were interested in studying defects in innate immune cells of these mice. We therefore characterized the cytokine profile of splenocytes obtained from PI3K mutant and WT mice. In agreement with the intestinal cytokine profile, splenocytes obtained from PI3K mutant macrophages secreted markedly elevated levels of IL-12 p40 (Fig. 20 B). We also observed increase in TNF production. Similar to the intestinal cytokine profile, we observed strikingly enhanced secretion of IFN-γ by PI3K mutant splenocytes (Fig. 20 B).

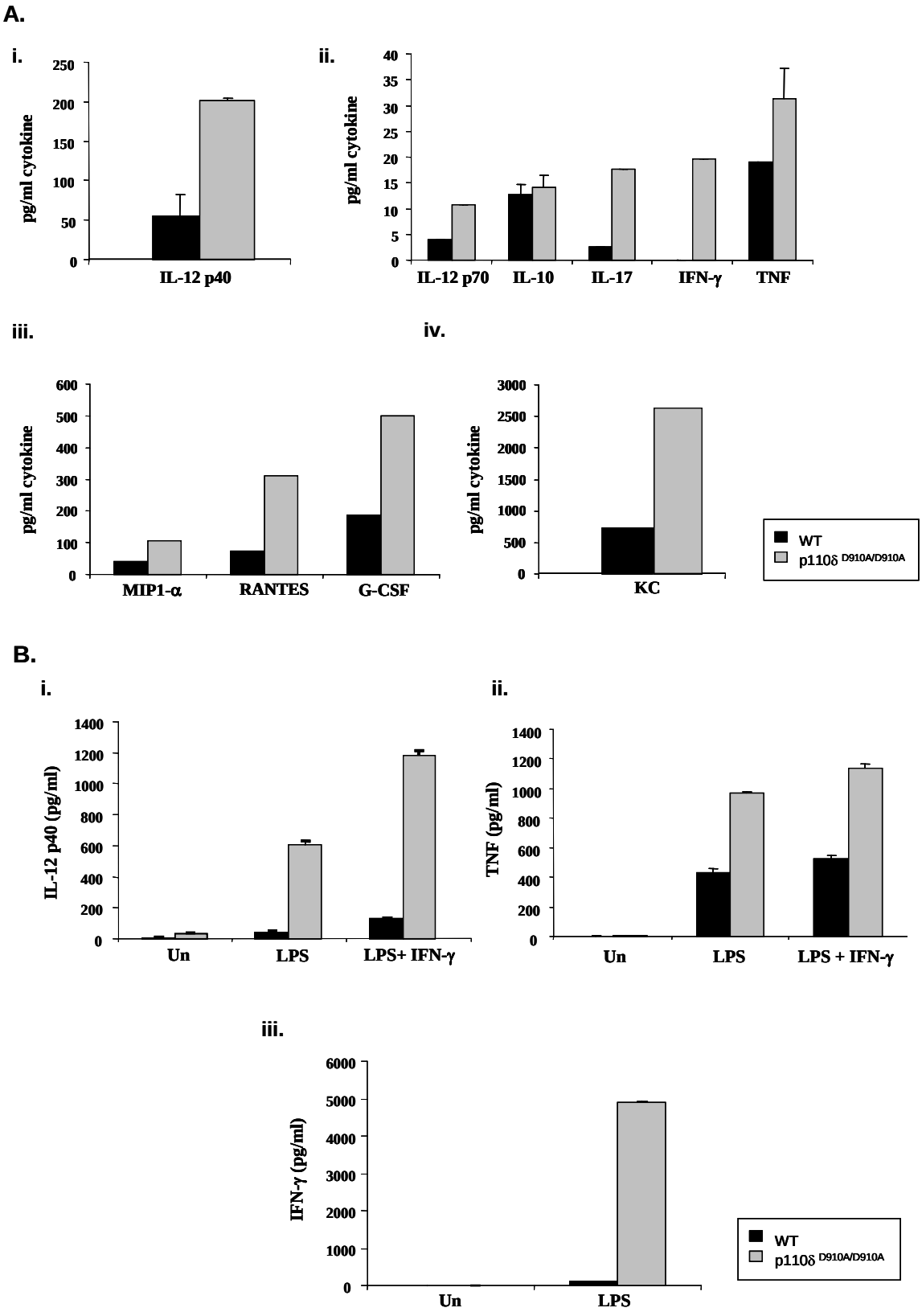

A.
i.
pg/ml cytokine
250
200
150
100
50
0
IL-12 p40
ii.
pg/ml cytokine
40
35
30
25
20
15
10
5
0
IL-12 p70
IL-10
IL-17
IFN-γ
TNF
iii.
pg/ml cytokine
600
500
400
300
200
100
0
MIP1-α
RANTES
G-CSF
iv.
pg/ml cytokine
3000
2500
2000
1500
1000
500
0
KC
WT
p110δ D910A/D910A
B.
i.
IL-12 p40 (pg/ml)
1400
1200
1000
800
600
400
200
0
Un
LPS
LPS+ IFN-γ
ii.
TNF (pg/ml)
1400
1200
1000
800
600
400
200
0
Un
LPS
LPS + IFN-γ
iii.
IFN-γ (pg/ml)
6000
5000
4000
3000
2000
1000
0
Un
LPS
WT
p110δ D910A/D910A

Figure 20. PI3K mutant mice display a proinflammatory mucosal and systemic cytokine profile.

(A.) Intestinal explants from WT (black bars) and PI3K mutant (grey bars) mice were cultured for 24h and cell free supernatants were assayed for spontaneous secretion of the indicated cytokines (i and ii) and chemokines (iii and iv) by ELISA. (B.) Splenocytes from both WT (black bars) and PI3K mutant (grey bars) mice were left untreated (un) or stimulated with LPS (1 μg/ml) alone or LPS and IFN-γ (10 ng/ml) for 24h. IL-12 p40 (i.), TNF (ii) and IFN-γ (iii.) levels in the cell-free supernatants were measured by ELISA. A proinflammatory cytokine profile was observed in both gut explant and splenocyte cultures. Each result represents the mean $\pm$ sd of duplicate assays and is representative of three independent experiments.

3.3.3 IL-12 p40 expression is dysregulated in PI3K mutant macrophages

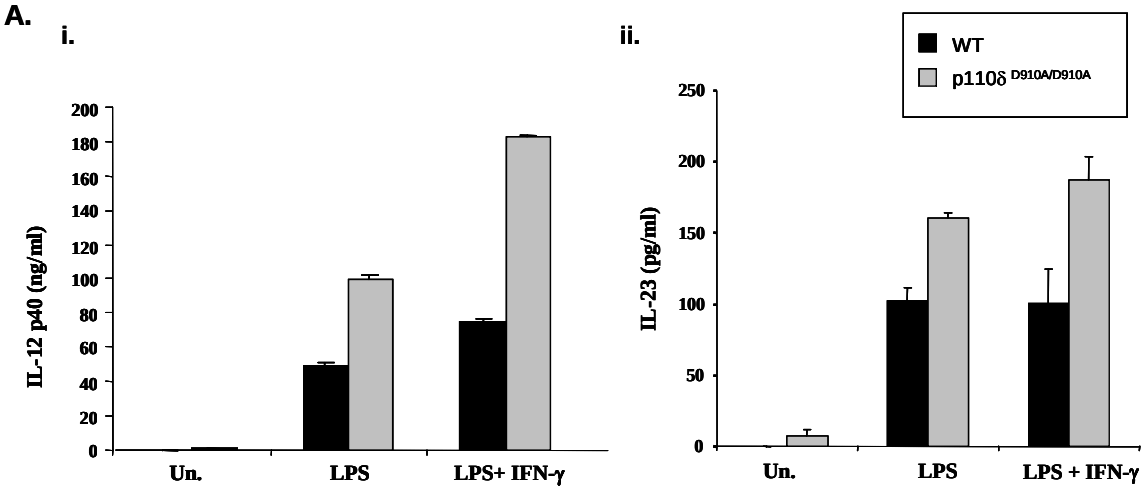
Having shown that PI3K p110δ mutant mice demonstrate mucosal and systemic chronic inflammation, we sought to investigate macrophage specific defects in these mice. The IL-12 family of cytokines (IL-12 p70 and IL-23) serves crucial roles in bridging innate and adaptive immune responses (3) and PI3K has been shown to negatively regulate IL-12 (220). Since IL-12 p40 is a common subunit for both IL-12 p70 and IL-23; and is expressed specifically in macrophages and dendritic cells (DCs) as opposed to the ubiquitously expressed p19 and p35 subunits, we sought to investigate the role of PI3-kinase p110δ in the regulation of IL-12 p40 gene expression. Bone marrow derived macrophages (BMDMs) from PI3K mutant mice were found to secrete significantly greater amounts of IL-12 p40 protein upon LPS or LPS and IFN-γ stimulation compared to those from WT mice (Fig. 21 A-i). IL-23 levels were also elevated in PI3K mutant macrophages. (Fig. 21 A-ii). To better understand the regulation of IL-12 p40 in the mutant macrophages, we performed a time-course analysis of p40 expression both at the protein and the mRNA level. IL-12 p40 protein secretion was detectable as early as 3 to 6 h after stimulation with LPS alone or with LPS and IFN-γ (Fig. 21 B) and increased consistently with time. IFN-γ which has been shown to prime macrophages for IL-12 production (86) further

induced IL-12 p40 secretion without affecting the kinetics. Although the kinetics of p40 induction between WT and PI3K mutant macrophages were not very different, the magnitude of induction at each time point was significantly greater in the latter. Since IL-12 p40 is highly regulated at the transcriptional level, we assessed the kinetics of p40 mRNA induction in response to LPS and LPS+IFN-γ (Fig. 21 C). We observed that with either treatment, IL-12 p40 mRNA peaked between 2 to 8 h and was significantly attenuated by 24h. As with IL-12 p40 protein, PI3K mutant macrophages did not reveal any differences in the kinetics of p40 mRNA induction except for a difference in magnitude. IL-12 p35 and p19 mRNAs were also overexpressed in the mutant macrophages (data not shown). These results confirm that PI3K p110δ plays an important role in the regulation of IL-12 family of proteins.

IL-12 p40 is transcriptionally regulated by numerous cis-acting elements and is synergistically induced by IFN-γ (124, 154). Our group has previously shown that the NFAT/IRF8 site is an important IFN-γ responsive site at the p40 promoter (154). We hypothesized that the enhanced magnitude of IL-12 p40 mRNA expression in PI3K mutant macrophages could be a result of aberrant regulation of a key transcription factor. In order to test this, we focused on IRF8 which has been shown to be critical for IL-12 p40 expression (91). Similar to IL-12 p40, we performed a kinetic analysis of IRF8 mRNA. It was observed that the expression pattern of IRF8 and p40 mRNA was superimposable and PI3K mutant macrophages overexpressed IRF8 mRNA (Fig. 21 D) in response to LPS or LPS and IFN-γ stimulation. This suggests that PI3K p110δ plays a negative regulatory role in IRF8 expression.

3.3.4 PI3K mutant macrophages display a difference in the kinetics and magnitude of MAPK phosphorylation.

IL-12 p40 is shown to be positively regulated by the MAP kinases p38 and JNK (125, 226, 227); and negatively regulated by the ERK MAP kinase pathway (227, 228). We hypothesized that enhanced activation of the MAP kinase pathway could account for the over expression of IL-12 p40 by PI3K mutant macrophages. A kinetic analysis of MAP kinase activation was performed using LPS activated WT and PI3K mutant macrophages. We observed that the mutant macrophages displayed a difference in the kinetics of activation of JNK and p38 MAP kinases (Fig. 21 E). Both these MAP kinases were activated early in the mutant macrophages. We also observed enhanced phosphorylation of the p38 MAP kinase. There were no significant differences in the phosphorylation of ERK. These results suggest that enhanced p38 and JNK activation in PI3K p110δ mutant macrophages could be responsible for the increased IL-12 p40 expression in these cells.



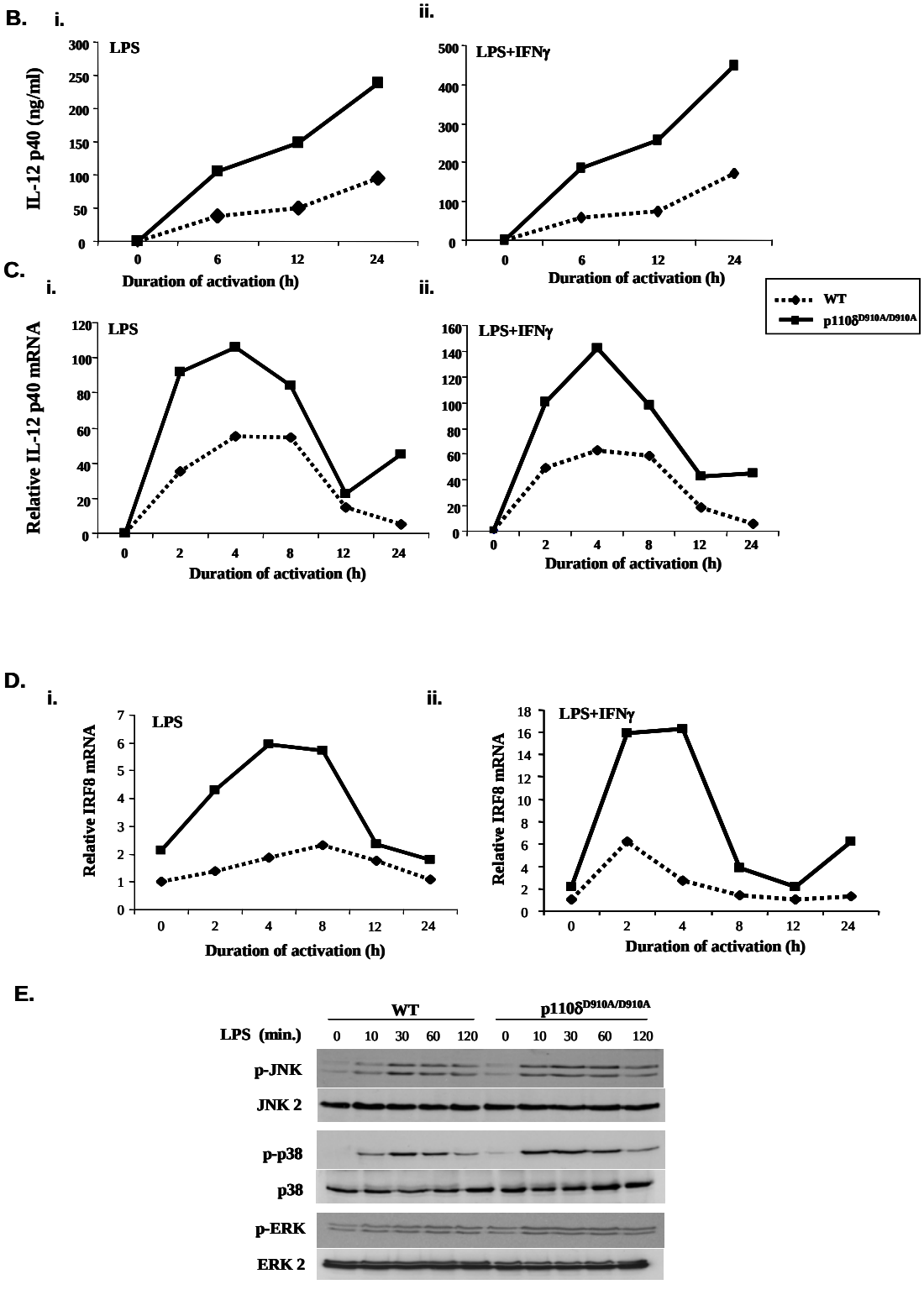

B.
i.
LPS
IL-12 p40 (ng/ml)
Duration of activation (h)
ii.
LPS+IFNγ
Duration of activation (h)
C.
i.
LPS
Relative IL-12 p40 mRNA
Duration of activation (h)
ii.
LPS+IFNγ
Duration of activation (h)
WT
p110δD910A/D910A
D.
i.
LPS
Relative IRF8 mRNA
Duration of activation (h)
ii.
LPS+IFNγ
Relative IRF8 mRNA
Duration of activation (h)
E.
WT
p110δD910A/D910A
LPS (min.)
0 10 30 60 120 0 10 30 60 120
p-JNK
JNK 2
p-p38
p38
p-ERK
ERK 2

Figure 21. IL-12 p40 expression is dysregulated in PI3K mutant macrophages.

(A.) Bone-marrow derived macrophages (BMDMs) from WT (black bars) and PI3K mutant (grey bars) mice were either unstimulated (Un.) or stimulated with LPS alone (1 μg/ml) or LPS and IFN-γ (10 ng/ml) for 24 h. Cell free supernatants were analyzed for IL-12 p40 (i) and IL-23 (ii) levels by ELISA. PI3K mutant BMDMs produced enhanced levels of IL-12 p40 at each time point. Each result represents the mean ± sd of duplicate assays and is representative of three independent experiments.

(B.) Bone-marrow derived macrophages cells from WT (dotted line) and PI3K mutant mice (solid line) mice were stimulated with LPS (1 μg/ml) alone (i) or LPS and IFN-γ (10 ng/ml, ii) for the indicated periods of time. IL-12 p40 levels in the cell-free supernatants were measured by ELISA. (C and D). Bone marrow derived macrophages from WT (dotted line) and PI3K (solid line) mutant mice were treated with LPS (1 μg/ml) alone (i) for the indicated periods of time or pre-treated with IFN-γ (10 ng/ml) for 1h followed by LPS (ii). Cells were harvested at each time point and p40 RNA (C.) or IRF8 mRNA (D.) levels were assessed by real-time RT-PCR. Enhanced magnitude of p40 and IRF8 mRNA was observed for PI3K mutant BMDMs. Each result represents the mean ± sd of duplicate assays and is representative of three independent experiments.

(E.) Bone-marrow derived macrophages from WT and PI3K mutant mice were grown in AIM-V medium overnight and stimulated with LPS (1 μg/ml) for the indicated periods of time. Cells were harvested and whole cell extracts were analyzed for phosphorylation of MAPK by western blotting. The kinetics and magnitude of p-38 and JNK phosphorylation were enhanced in PI3K mutant BMDM extracts. Results are representative of three independent experiments.

3.3.5 PI3K mutant macrophages are hyperresponsive to TLR signaling

The above results suggest that negative regulation of TLR4 (LPS) signaling is impaired in macrophages from PI3K p110δ mutant mice. In order to test whether p110δ is important for regulation of responses to other TLR ligands, BMDMs from WT and mutant mice were stimulated with TLR 9 (CpG) and TLR 2 (synthetic bacterial lipoprotein-sBLP) ligands. We assessed proinflammatory mediators and cytokine production, specifically IL-12 p40, IL-12 p70, IL-23 and nitric oxide (NO). Consistent with the recent literature (215, 229, 230), we observed that PI3-kinase mutant macrophages secrete enhanced amounts of proinflammatory cytokines and nitric oxide in response to TLR ligands (Fig. 22). There was no significant difference in IL-

12 p40 induction by CpG, but IL-12 p70 levels were augmented in CpG stimulated PI3K mutant macrophages. Stimulation with the TLR2 ligand sBLP revealed marked difference in IL-12 p40 and p70 induction between WT and mutant macrophages. This suggests that PI3K may play a greater role in the negative regulation of TLR2 signaling. IL-23 levels were enhanced in the mutant macrophages in response to CpG stimulation. While IL-23 was undetectable in WT macrophages in response to sBLP, mutant macrophages displayed a pronounced upregulation of IL-23. Thus, PI3K mutant macrophages display elevated levels of IL-12 family of cytokines upon stimulation with TLR ligands. In corroboration with reports that PI3K negatively regulates NO production by macrophages (230, 231), we observed increased NO secretion by PI3K mutant macrophages stimulated with all the TLR ligands tested. Collectively, the above results suggest that PI3K mutant macrophages demonstrate a heightened sensitivity to stimulation by TLR ligands underscoring the importance of this molecule in negative regulation of TLR signaling. In addition, our results also suggest that aberrant regulation of innate immune responses could be responsible for the development of colitis in PI3K mutant mice.

3.3.6 Defective inhibition of IL-12 p40 by IL-10 and C5a in PI3K p110δ mutant macrophages

Our results so far have corroborated the existing literature that PI3K is an important signaling molecule that limits inflammation in macrophages and regulates the expression of IL-12 p40 expression. We hypothesized that PI3K p110δ could be involved in the pathways that inhibit IL-12 p40. We first tested this hypothesis in the IL-10 pathway of p40 inhibition. IL-10 is a potent inhibitor of IL-12 p40 transcription and the mechanisms of this inhibition are largely unknown (97, 101). Some reports have shown that IL-10 activates PI3K (232-234) and that PI3K is not important for the anti-inflammatory functions of IL-10 (232). We readdressed this question first by using chemical inhibitors of PI3K – wortmannin and LY294002.

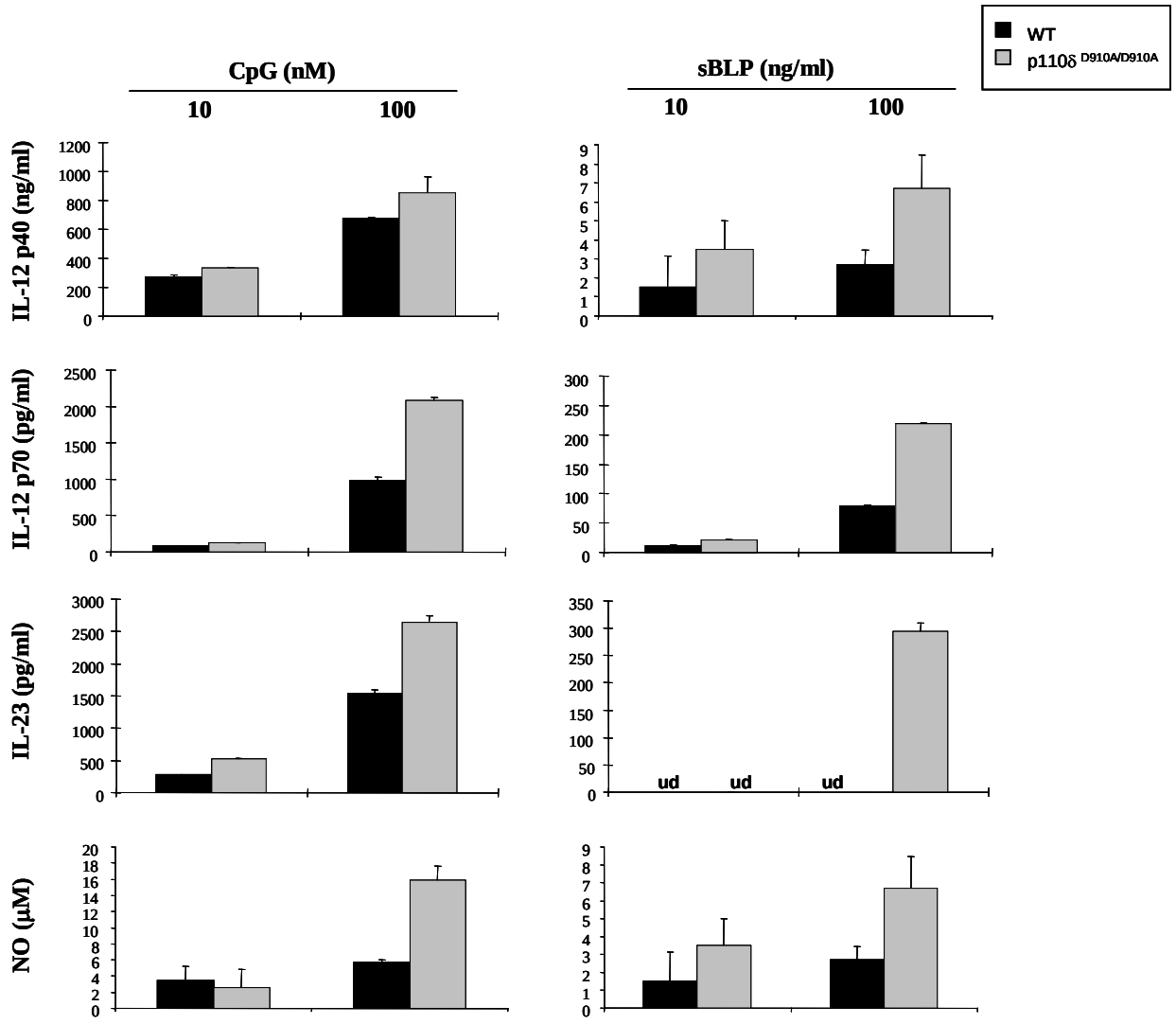


Figure 22. PI3K mutant macrophages demonstrate heightened sensitivity to TLR stimulation.

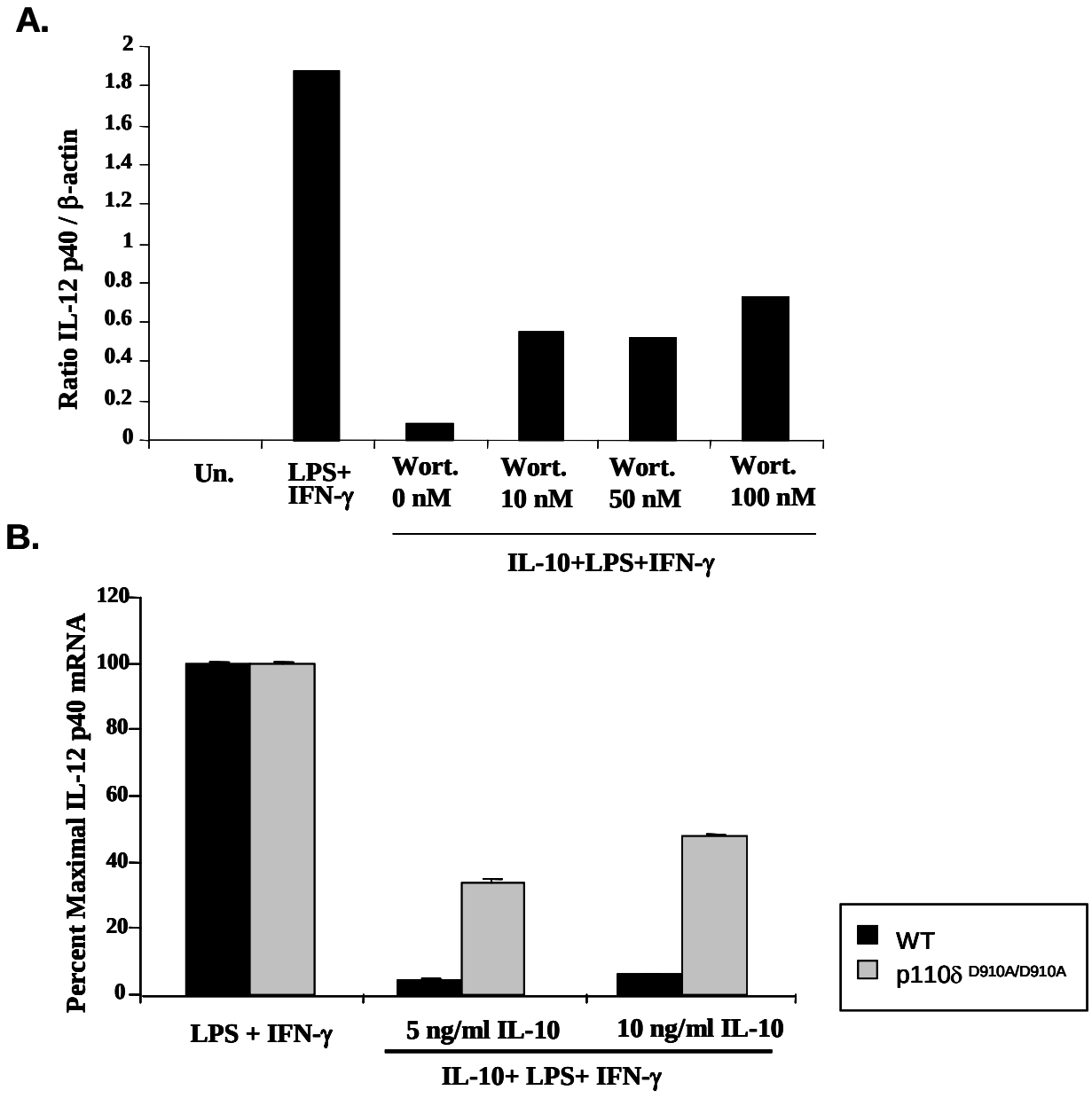
Bone marrow derived macrophages from WT (black bars) and PI3K mutant (grey bars) mice were stimulated with TLR9 (CpG) or TLR2 (sBLP) ligands at the indicated concentrations for 24h. Cell free supernatants were analyzed for IL-12 p40, IL-12 p70 or IL-23 secretion by ELISA and nitric oxide secretion by Greiss' Reaction. PI3K mutant BMDMs demonstrate an overall hypersensitivity to TLR stimulation, especially the TLR2 ligand sBLP. Each result represents the mean $\pm$ sd of duplicate assays and is representative of three independent experiments.

Peritoneal macrophages obtained from C57Bl/6 mice were treated as indicated (Fig. 23 A). IL-10 was found to profoundly inhibit LPS and IFN-γ stimulated IL-12 p40 mRNA levels and treatment of cells with wortmannin partially abrogated this inhibition. Similar results were

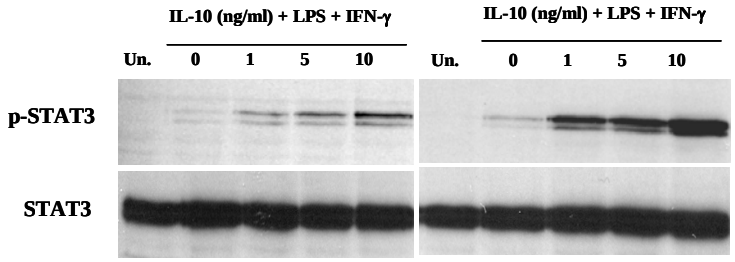
obtained with LY294002 (data not shown). To confirm the role of PI3K in IL-10 mediated inhibition of IL-12 p40, bone marrow-derived macrophages from WT and PI3K mutant macrophages were treated with IL-10 and IL-12 p40 mRNA levels were measured (Fig. 23 B). Consistent with the results obtained using chemical inhibitors, the inhibition of IL-12 p40 mRNA by IL-10 was partially abrogated in PI3K mutant macrophages. In order to verify that the partial abrogation of IL-10 inhibition was not due to impaired IL-10 signaling, we measured the phosphorylation status of STAT3, an important downstream mediator of IL-10 (182). Interestingly, we observed that phosphorylation of STAT3 was enhanced in the PI3K mutant macrophages (Fig. 23 C). To confirm that IL-10 activates PI3K in our system, we employed a cell-based ELISA system to detect phosphorylation of the downstream target of PI3K, Akt. We observed that IL-10 phosphorylates Akt over time, with a peak at 30 min (Fig. 23 D). Stimulation of cells with IL-10, LPS and IFN-γ resulted in a synergistic induction of Akt phosphorylation.

We have previously demonstrated that the anti-inflammatory molecule carbon monoxide exerts its effects on IL-12 p40 by inhibiting IRF8 (155), suggesting that IRF8 could be a downstream target of anti-inflammatory pathways. In addition, previous observations from our group have shown that IL-10-/- macrophages overexpress IRF8 similar to PI3K mutant macrophages (KR, SP, unpublished data). We therefore hypothesized that IRF8 is a target of IL-10 and that PI3K could be mediating this effect. We tested this by measuring IRF8 levels in IL-10, LPS and IFN-γ treated WT and mutant macrophages and observed that while in WT macrophages, IL-10 inhibits IRF8 protein, this inhibition is reduced in PI3K mutant macrophages (Fig. 23 E). This effect was also observed at the level of IRF8 mRNA (Fig. 23 F). This suggests that IL-10 inhibits IRF8 in a PI3K dependent manner. This inhibition of IRF8

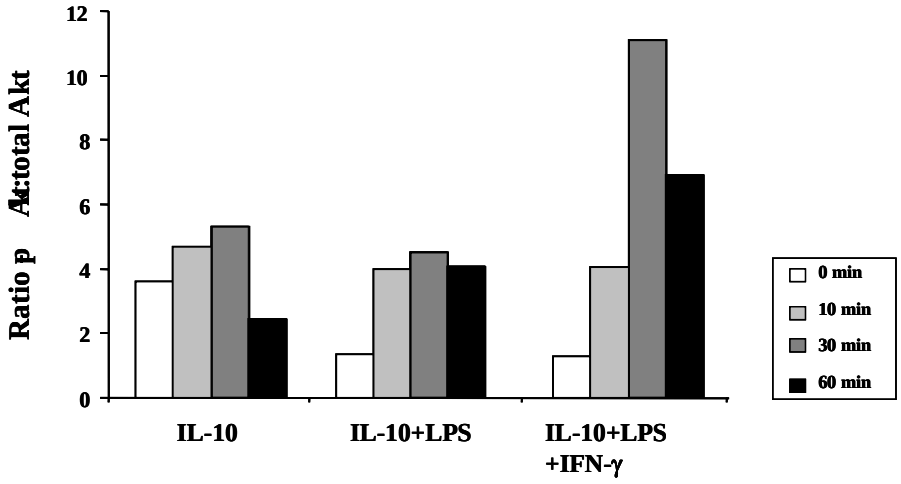
could possibly be one of the mechanisms by which IL-10 exerts its inhibitory effects on IL-12 p40. Thus, the above experiments suggest that the anti-inflammatory effects of IL-10 on IL-12 p40 are ineffective in PI3K mutant macrophages.



C.


IL-10 (ng/ml) + LPS + IFN-γ
IL-10 (ng/ml) + LPS + IFN-γ
Un.
0
1
5
10
Un.
0
1
5
10
p-STAT3
STAT3

D.


Ratio p-Akt:total Akt
12
10
8
6
4
2
0
IL-10
IL-10+LPS
IL-10+LPS +IFN-γ
0 min
10 min
30 min
60 min

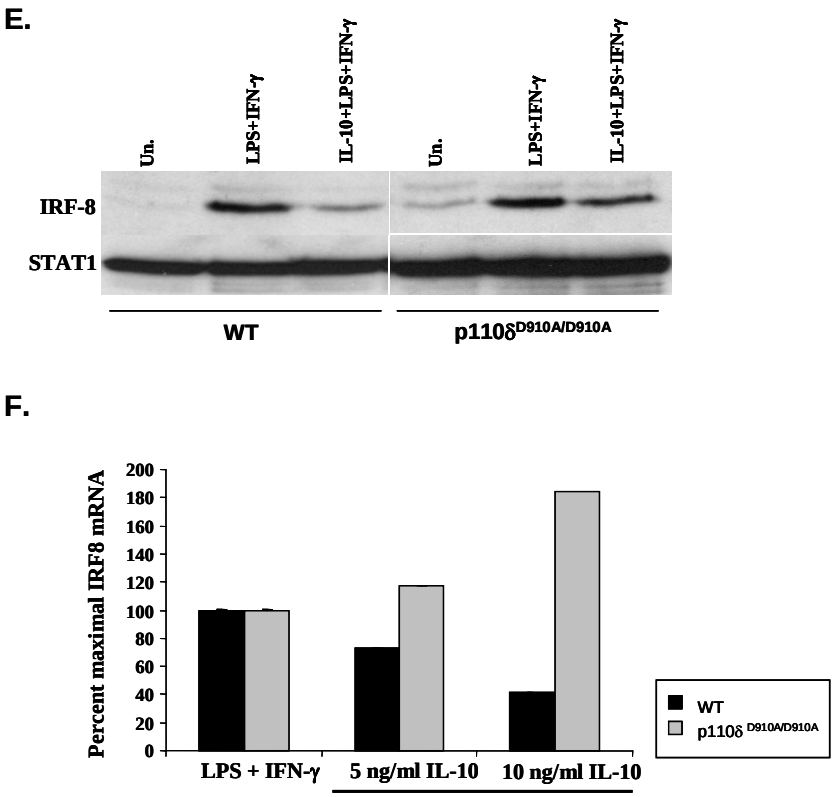


Figure 23. Impaired inhibition of IL-12 p40 by IL-10 in PI3K mutant macrophages.

(A.) Peritoneal macrophages from WT mice were either untreated (Un.) or stimulated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) or pretreated with the indicated concentrations of wortmannin and IL-10 where indicated. IL-12 p40 mRNA levels were assessed by RT-PCR and results represented quantitatively. Inhibition of PI3K by wortmannin resulted in partial restoration of IL-12 p40 mRNA levels inhibited by IL-10. Results are representative of three independent experiments. (B and F.) BMDMs from WT (black bars) and PI3K mutant (grey bars) mice were stimulated with IFN-γ (10 ng/ml, 1 h) and LPS (1 μg/ml, 4 h) and where indicated also pretreated with varying amounts of IL-10 (1 h). IL-12 p40 mRNA (B.) and IRF8 mRNA (F.) levels were measured by real-time RT-PCR and results represented as percent of LPS/IFN-γ induced mRNA. PI3K mutant BMDMs display ineffective inhibition of IL-12 p40 and IRF8 mRNA by IL-10. Each result is mean $\pm$ sd of duplicate assays and is a representative of three independent experiments. (C.) Nuclear extracts from BMDMs stimulated with LPS, IFN-γ and the indicated concentrations of IL-10 were analyzed for p-STAT3 by western blot. Enhanced phosphorylation of STAT3 was observed in mutant cells in response to IL-10 stimulation. Shown here is a representative of three independent experiments. (D.) Phosphorylation of Akt was measured by ELISA in WT BMDMs stimulated with IL-10 alone (10 ng/ml), LPS alone (1 μg/ml) or IL-10, LPS and IFN-γ (10 ng/ml) and results represented as a ratio of p-Akt to total Akt. IL-10 phosphorylates Akt in a time dependent manner. Each result is mean $\pm$ sd of duplicate assays and is a representative of three independent experiments. (E.) IRF8 protein levels were assessed in nuclear extracts of BMDMs treated with LPS (1 μg/ml) and IFN-γ (10 ng/ml) with or without IL-10 (10 ng/ml). Inhibition

of IRF8 mRNA by IL-10 is impaired in PI3K mutant macrophages. Results are representative of three independent experiments.

Recent reports have illustrated that the complement component C5a inhibits TLR responses and IL-12 in particular, by activating PI3K (107, 235, 236). We first confirmed this in peritoneal macrophages obtained from WT mice using wortmannin (50nM) to inhibit PI3K. Consistent with the above reports, we found that treatment of macrophages with wortmannin abrogated the inhibition of IL-12 p40 (Fig. 24 A). We further studied this pathway of IL-12 p40 inhibition in PI3K mutant macrophages. It was found that while C5a inhibits IL-12 p40 protein secretion (Fig. 24 C and D) and mRNA accumulation (Fig. 24 B) in WT macrophages, this inhibition is almost absent in PI3K mutant macrophages. This corroborates the finding by others and also indicates that yet another pathway of IL-12 p40 inhibition is ineffective in PI3K mutant macrophages. PGE2 has also been shown to inhibit IL-12 p40 (127), but this inhibition was found to be intact in PI3K mutant macrophages (data not shown). Taken together, our results suggest that macrophages from PI3K mutant mice lack efficient downregulation of IL-12 p40 by inhibitory pathways.

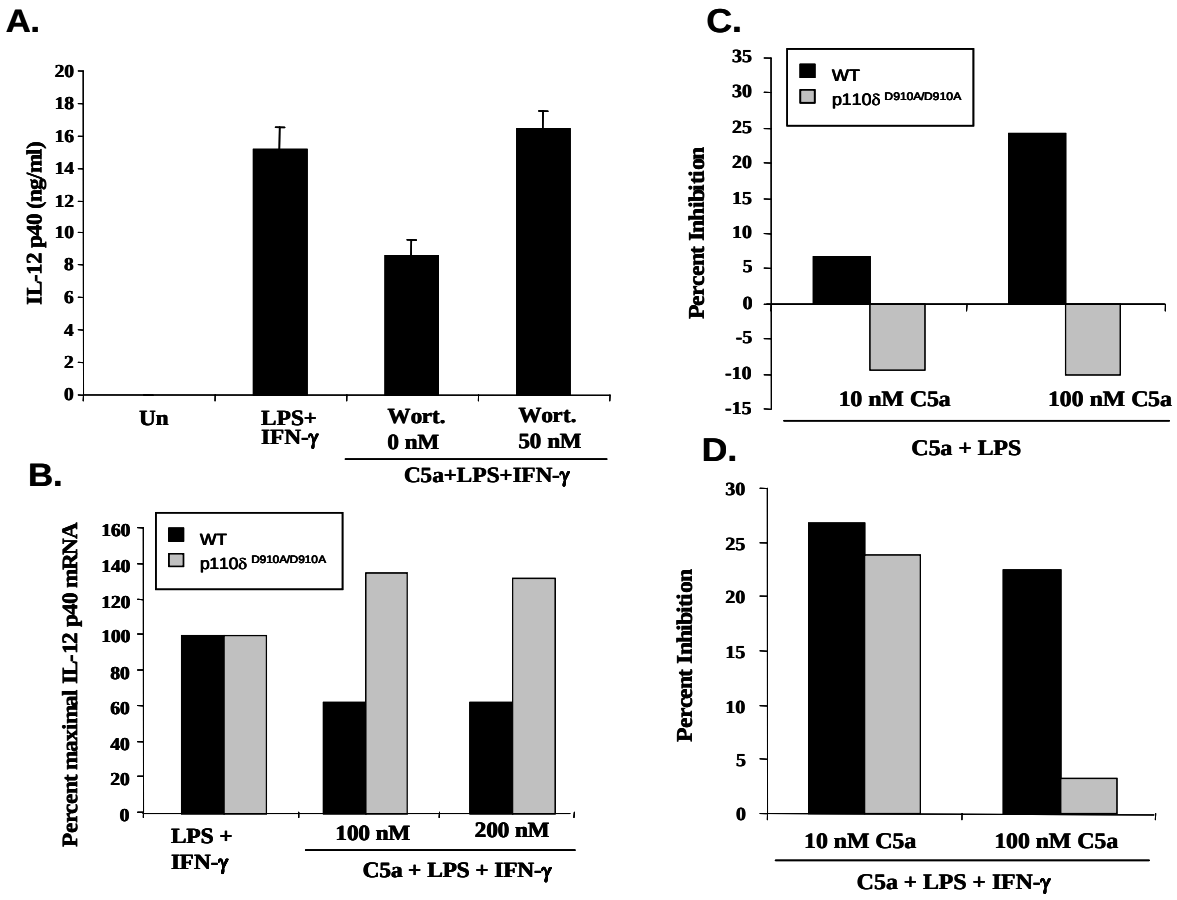


Figure 24. Impaired inhibition of IL-12 p40 by C5a in PI3K mutant macrophages.

(A.) Peritoneal macrophages from WT mice were either untreated (un.) or stimulated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) in the presence or absence of wortmannin (50 nM) and C5a (200 nM) where indicated, for 24 h. IL-12 p40 secretion was assayed in supernatants by ELISA. Inhibition of IL-12 p40 by C5a was abrogated by wortmannin treatment. (B.) BMDMs from WT (black bars) and PI3K mutant (grey bars) mice were stimulated with IFN-γ (10 ng/ml, 1 h) and LPS (1 μg/ml, 4 h) and where indicated with varying amounts of C5a (1h). IL-12 p40 mRNA levels were measured by real-time RT-PCR and results represented as percent of LPS/IFN-γ induced mRNA. Inhibition of IL-12 p40 mRNA by C5a is abrogated in PI3K mutant mice (C and D.) BMDMs were stimulated with LPS alone or LPS and IFN-γ with the indicated amounts of C5a for 24 h. IL-12 p40 levels were measured by ELISA and results represented as percent inhibition of LPS (C.) or LPS and IFN-γ (D.) induced IL-12 p40. Inhibition of IL-12 p40 protein secretion is abrogated in PI3K mutant macrophages. Each result represents the mean $\pm$ sd of duplicate assays and is representative of three independent experiments.

3.3.7 PI3K mutant macrophages display impaired responses to MDP

NOD2 functions as an intracellular pattern-recognition receptor which binds to a component of the bacterial cell wall peptidoglycan – MDP (muramyl di-peptide) (79). Mutations in the NOD2 gene have been associated with Crohn's disease (80, 81) and DCs obtained from Crohn's disease patients have been shown to produce reduced amounts of IL-12 in response to the NOD2 ligand MDP (237). We were interested in studying if this important pathway of bacterial recognition is affected in the PI3K mutant macrophages. WT and mutant bone marrow derived macrophages were stimulated with MDP in the presence or absence of IFN-γ. Interestingly we observed that PI3K mutant macrophages produce significantly less IL-12 p40 protein (Fig. 25 A) and mRNA (Fig. 25 B) in response to MDP. In addition to IL-12 p40, TNF production in response to MDP stimulation was also impaired in PI3K mutant macrophages (Fig. 25 C). In order to test if PI3K is an intermediate in the NOD2 signaling pathway, we monitored Akt phosphorylation. We observed that MDP phosphorylates akt in a time dependent manner in WT bone marrow derived macrophages and treatment with IFN-γ further induces this (Fig. 25 D-i). PI3K mutant macrophages demonstrated severely reduced levels of Akt phosphorylation in response to MDP. Phosphorylation of Akt by TLR4 and TLR2 ligand –LPS and sBLP respectively, was also reduced; although the reduction was greater with sBLP (Fig. 25 D-ii and iii). The expression of NOD2 mRNA was equivalent in WT and mutant macrophages (data not shown). These results demonstrate that PI3-kinase mutant macrophages display a selective impairment of inflammatory cytokine production in response to the NOD2 ligand MDP. This also uncovers a novel intermediate in the NOD2 signaling pathway – p110δ PI3K.

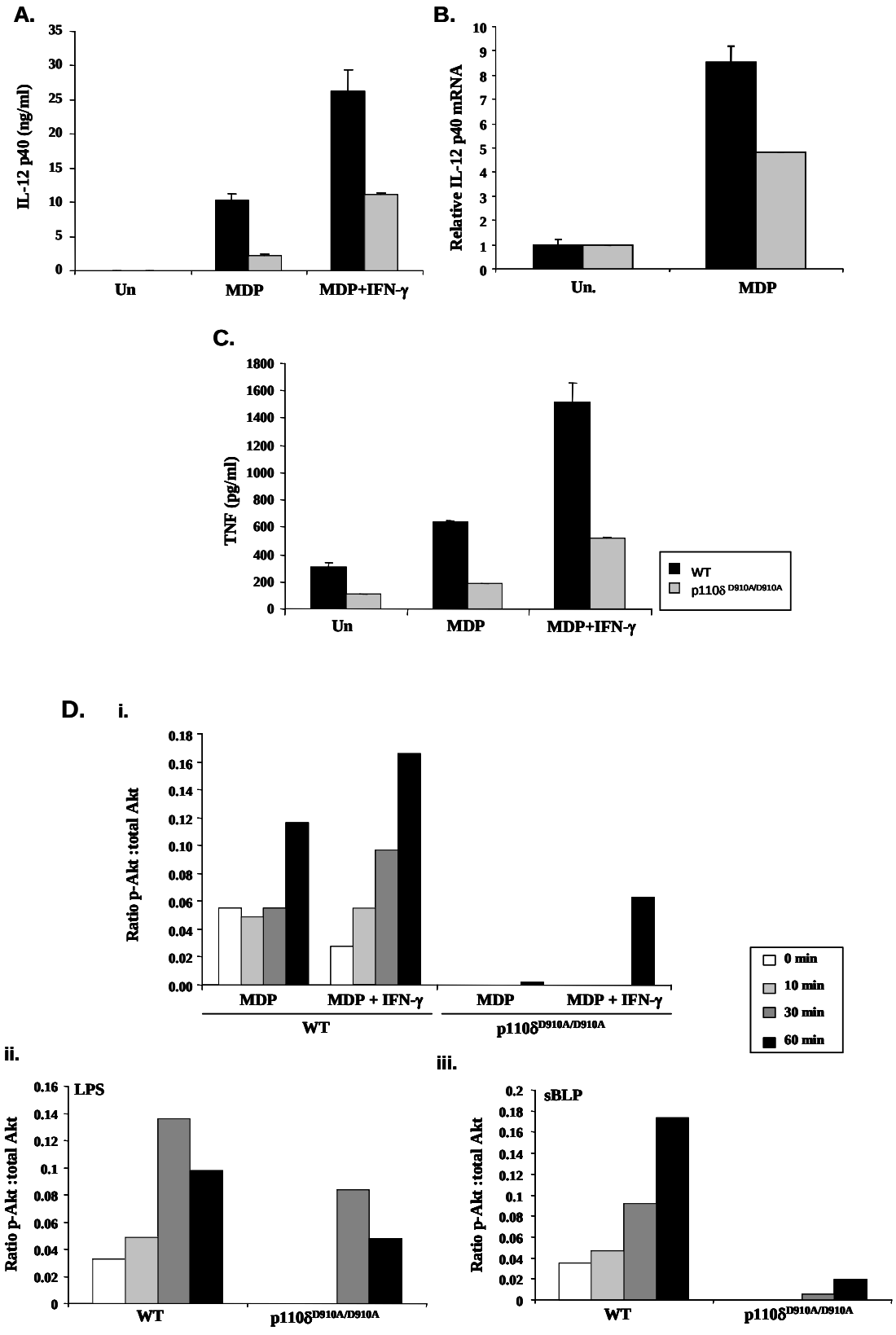

A.
IL-12 p40 (ng/ml)
Un
MDP
MDP+IFN-γ
B.
Relative IL-12 p40 mRNA
Un.
MDP
C.
TNF (pg/ml)
Un
MDP
MDP+IFN-γ
WT
p110δ D910A/D910A
D.
i.
Ratio p-Akt :total Akt
MDP
MDP + IFN-γ
MDP
MDP + IFN-γ
WT
p110δD910A/D910A
0 min
10 min
30 min
60 min
ii.
LPS
Ratio p-Akt :total Akt
WT
p110δD910A/D910A
iii.
sBLP
Ratio p-Akt :total Akt
WT
p110δD910A/D910A

Figure 25. PI3K mutant macrophages display an impaired response to the NOD2 ligand – MDP.

(A. and C.) BMDMs from WT (black bars) and PI3K mutant (grey bars) mice were either unstimulated (un.) or stimulated with MDP alone (10 μg/ml) or MDP and IFN-γ (10 ng/ml) for 24 h. Cell free supernatants were analyzed for IL-12 p40 (A.) and TNF (C.) levels by ELISA. PI3K mutant macrophages produced markedly less IL-12 p40 and TNF in response to MDP stimulation. (B.) IL-12 p40 mRNA levels in BMDMs in response to MDP stimulation (10 μg/ml for 4 h) were assessed by real-time RT PCR. Diminished levels of IL-12 p40 mRNA were observed in PI3K mutant cells. (D.) BMDMs from WT or PI3K mutant mice were stimulated with either MDP (10 μg/ml) or MDP and IFN-γ (10 ng/ml) (i.) ; LPS (100 ng/ml, ii.) or sBLP (100 ng/ml, iii.) for the indicated periods of time and phosphorylation of Akt was assayed by ELISA. Results are presented as a ratio of p-Akt to total Akt. Phosphorylation of Akt by MDP, LPS or sBLP was diminished in PI3K mutant BMDMs. Each result is mean $\pm$ sd of duplicate assays and is representative of three independent experiments.

3.4 DISCUSSION

In summary, the present study reinforces the role of PI3K in the regulation of TLR signaling and innate immune function. We characterize chronic inflammation and IBD in PI3K p110δ mutant mice and report a novel role for PI3K in NOD2 signaling. Histologically, a marked increase in the infiltration of IELs was observed in colonic crypts of mutant mice. A few areas of crypt abscess were also observed. The lamina propria of mutant mice displayed infiltration of lymphocytes and neutrophils, and an increase in apoptotic and mitotic bodies. Immunologically, a proinflammatory cytokine profile tending towards a Th1 response was observed both mucosally and systemically. Excess proinflammatory cytokine secretion was also observed in bone marrow derived macrophages form PI3K mutant mice stimulated with TLR ligands. We observed defects in IL-10 and C5a mediated regulation of IL-12 p40. Finally, we observed impaired responses of PI3K mutant macrophages to stimulation by the NOD2 ligand- MDP.

One of the key histological features of PI3K p110δ mutant colonic sections was the pronounced increase in the infiltration of IELs. Intraepithelial lymphocytes play an important role in maintaining homeostasis at the mucosal epithelium (238). They secrete various effector cytokines including IFN-γ and TNF (239). This suggests that the tremendous increase in IFN-γ secretion by PI3K mutant gut explant cultures could possibly be a result of enhanced IEL migration into the colons of these mice. IFN-γ produced by IELs can in turn alter epithelial cell function (240). IFN-γ has been shown to upregulate MHC-I, MHC-II and intracellular adhesion molecule-1 (ICAM-1) expression on epithelial cells. IEL homing has also been shown to affect epithelial permeability and upregulate chemokine production by epithelial cells; which could in turn augment migration of other proinflammatory cells (240). This correlates with our observation of increased RANTES, KC (IL-8 homolog) and MIP1α secretion by PI3K gut explant cultures. Furthermore, a proinflammatory environment has been shown to induce chemokine secretion by epithelial cells (241), suggesting that the dysregulated innate immune responses at the PI3K mutant mucosal epithelium could alter intestinal homeostasis in these mice. It would be interesting to further characterize the role of IELs in the development of IBD in these mice.

The proinflammatory cytokine IL-17 has been shown to play a role in autoimmune and chronic inflammatory diseases such as rheumatoid arthritis, psoriasis and multiple sclerosis (60, 66, 71, 242-244). Recently, it was reported that a distinct lineage of T cells, Th17 cells, produce IL-17 (62, 63) and that these cells are critical for the pathogenesis of chronic inflammatory diseases, including IBD (72). Furthermore, IL-17 has been shown to be upregulated in IBD patients (145, 146). In gut explant cultures, PI3K mutant mucosal tissue produced more IL-17 than that of WT mice. This implicates a possible role for IL-17 in the pathogenesis of IBD in

PI3K mutant mice. Future studies warrant evaluation of Th17 cells in the development of IBD in these mice. Although IL-23 is not required for the development of Th17 cells, it is reported to be involved in the survival and expansion of these cells (64). We observed that bone marrow derived macrophages from PI3K mutant mice produced elevated levels of IL-23 compared to those from WT mice. This further implicates innate immune dysfunction and the IL-23/IL-17 axis (69) in disease pathogenesis in these mice.

Initial studies have shown PI3K to function as a "gate-keeping system" to limit innate immune responses *in vitro* (monocytes) (245) and *in vivo* (endotoxic shock) (246) and accumulating evidence over the past few years have established the role of PI3K in the attenuation of TLR signaling (215, 216, 220, 229, 230, 247, 248). PI3K is activated upon signaling through various stimuli including LPS (TLR4), peptidoglycan (TLR2), CpG (TLR9), CD-40L and RANKL (receptor activator of NF-κB ligand), suggesting that both positive and negative regulatory pathways are simultaneously activated in innate immune cells (215). Mice with genetic deletion of the p85α subunit of PI3K display altered balance of Th1-Th2 responses (220). These mice demonstrate impaired immunity to the prototypical Th2-inducing pathogen, the intestinal nematode *Strongyloides venezuelensis* and show resistance to the Th1-inducing pathogen *Leishmania major*. Dendritic cells from these mice produce enhanced levels of IL-12 in response to TLR2 (PGN), TLR4 (LPS) and TLR9 (CpG) ligands. However, these mice have not been reported to develop chronic inflammation and IBD. This could be because mice deficient in PI3K subunits demonstrate compensatory changes in expression and availability of regulatory subunits which could affect the phenotype and functional analyses (169). In this regard, the results from our study using PI3K p110δ mutant mice are more biologically relevant. The data presented here corroborate the findings by Fukao et. al., (220) in that mutation of p110δ

enhances secretion of proinflammatory cytokines including IL-12 in macrophages leading to a skewed Th1 response *in vivo*. The enhanced magnitude of IL-12 p40 expression supports the current theory that PI3K serves to regulate the magnitude of induction in the early-phase of the innate immune response. In essence, PI3K functions as the 'volume-control knob' of innate immunity.

In addition to regulating TLR signaling, we propose that PI3K is involved in pathways that inhibit IL-12 p40. IL-10 is a potent anti-inflammatory cytokine which inhibits macrophage activation and proinflammatory response (182) and has been shown to activate PI3K (232-234). IL-10 inhibits IL-12 p40 transcription, the mechanisms of which are elusive (97, 101). Here, we demonstrate that PI3K at least partially mediates the anti-inflammatory effect of IL-10. In addition, we are the first to show that IL-10 inhibits IRF8, which is a critical transcription factor for IL-12 p40. We observed that this inhibition is partially abrogated in PI3K mutant mice. This suggests that IL-10 by activating PI3K inhibits IRF8. This raises questions such as the involvement of STAT3 in the inhibition of IRF8 and activation of PI3K, which are under investigation. A recent report showed that STAT3 induces PI3K subunits (249).

Ligation of Gi-protein coupled receptors (GPCR) has been shown to inhibit IL-12 (104) and mice with a deficiency of Gi-2α develop colitis (106). Receptors for complement components are GPCRs which have been shown to inhibit IL-12 in a PI3K dependent manner (107, 235, 236). However, these studies were performed using chemical inhibitors of PI3K. Here for the first time we demonstrate using PI3K p110δ mutant mice that the complement component C5a mediated inhibition of IL-12 is indeed abrogated in PI3K mutant mice.

NOD1 and NOD2 are cytosolic proteins offering a second line of defense in innate immune cells beyond the membrane-bound TLRs (79). These proteins belong to a family of

proteins with leucine-rich repeats (LRR) in the ligand recognizing C-terminal domain, a nucleotide binding domain and N-terminal caspase activating and recruitment domains (CARD). Mutations in *NOD2* (*CARD15*) are linked to CD (80, 81) and polymorphisms in *NOD1* (*CARD4*) are linked to IBD (250) and asthma (251), which underscore the importance of these proteins in innate immunity. Despite a lot of advances in understanding the biology of NOD proteins, our knowledge of NOD1/2 signaling pathways is still limited. Stimulation of both NOD1 and 2 leads to NF-κB activation mediated by homophilic CARD-CARD interactions between NODs and the molecule RIP2 (also called RICK or CARDIAK) (252). CD associated mutations in NOD2 generate proteins that are defective in PGN and MDP-induced NF-κB activation (253, 254). In addition, it has been shown that DCs from CD patients with the 3020insC frameshift mutation in NOD2 are defective in their response to MDP induced TNF and IL-12 (237). This corroborates our finding that bone marrow derived macrophages from PI3K mutant mice secrete markedly reduced levels of TNF and IL-12 in response to MDP. NOD2 deficient mice and mice expressing a truncated form of NOD2 do not develop spontaneous intestinal pathology (255-257). Here, we report a mouse model with defects in NOD2 signaling which spontaneously develop IBD. Thus, our study uncovers PI3K as a novel intermediate in the NOD2 signaling pathway and provides us a model for further understanding the role of NOD2 in chronic inflammatory diseases.

Our data support the recent study which demonstrated that NOD2 negatively regulates TLR2 responses mediated by PGN (255). In our study, mutation of p110δ PI3K seemed to have a greater effect on TLR2 responses. This result combined with the observation of diminished responses to NOD2 stimulation lead us to propose that PI3K is involved in the negative regulation of TLR2 signaling by NOD2. Similar to RICK, PI3K could be a common

intermediate of both TLR2 and NOD2 signaling (258). Whether PI3K is upstream or downstream of RICK needs to be determined.

The present data suggest that rendering p110δ inactive causes defects at several levels in innate immune cells. Firstly, the negative regulation of TLR signaling by PI3K being lifted, macrophages now become hyperresponsive to stimulation by TLR ligands and secrete proinflammatory cytokines in excess. Secondly, we propose that PI3K is an important downstream modulator in anti-inflammatory/inhibitory pathways and functions as a ‘molecular brake’ to limit inflammation. Such dysregulation of innate immune function could alter the balance of Th1 versus Th2 and lead to chronic inflammatory diseases such as IBD. Thus, PI3K p110δ mutant mice provide a unique mouse model to better understand disease pathogenesis.

4.0 CONCLUSIONS

Inflammation is the first response of our body to injury or infection. The discovery of inflammation dates back to around 30 BC to 38 AD when the Roman writer Cornelius Celsus first documented the four cardinal signs of inflammation as "*rubor et tumor cum calore et dolore*" which means "redness and swelling with heat and pain" (259). A scientific explanation for these features was attempted only in the 19th century by Rudolph Virchow and his pupil Julius Cohnheim, who performed microscopic observations and described that the redness is a result of increased blood flow and the swelling occurs due to accumulation of cells and fluid leading to pain (259). An important milestone in the history of inflammation was the discovery of macrophages by Eli Metchnikoff, who described these cells as 'big eaters' which phagocytose dying cells. Metchnikoff defined inflammation as "…a local reaction often beneficial, of living tissue against an irritant substance. This reaction is mainly produced by phagocytic activity of the mesodermic cells. In this reaction, however, may participate not only changes of the vascular system, but also the chemical action of the blood plasma and the tissue fluids…" (259).

Our understanding of inflammation has come a long way since its discovery more than 2000 years ago and a recurrent theme that has emerged is that inflammation is a double edged sword. We now know that inflammation is a well coordinated sequence of events initiating with the extravasation of leukocytes from the blood stream into the site of injury, followed by an effector phase involving clearance of the pathogen and finally the resolution phase where cell

debris is cleared and the inflammatory cells themselves are removed and tissue repair is initiated. We also know that every stage of inflammation is tightly regulated and that dysregulation at any step, especially the resolution phase, could be potentially detrimental to the host. Inappropriate dampening of inflammation could lead to persistent stimulation of the immune system and a state of chronic inflammation. Our knowledge about the regulation of initiation and the effector phases of inflammation is far greater than that of the resolution phase. However, over the last few years, resolution of inflammation has gained tremendous interest (260, 261). This could be fuelled by the fact that inflammation has recently been associated with several diseases like atherosclerosis, asthma, alzheimer's, diabetes and cancer. In fact, the Time magazine recently coined inflammation as "the secret killer" and ran a cover page article on the links between inflammation and various diseases (262).

Until recently, it was thought that resolution of inflammation is a passive process. However, recent findings indicate that it is an actively orchestrated sequence of events involving prevention of leukocyte migration into inflamed tissue and elimination of inflammatory cells. Resolution also occurs at an intracellular level; the signaling pathways leading to the expression of proinflammatory mediators are dampened at multiple points. Understanding the molecular details of dampening inflammation could reveal novel molecular targets for therapeutic intervention. *This dissertation attempts to explore such intracellular mechanisms of resolving inflammation; both naturally occurring inhibitory pathways and a novel way of inhibiting proinflammatory gene expression, in the context of inflammatory bowel disease (IBD).*

Crohn's disease (CD) and ulcerative colitis (UC), the two forms of IBD, together affect as many as 1.4 million people in the United States and 2.2 million people in Europe and their incidence is rising in areas such as Southern Europe and Asia, which were earlier thought to be

of low-incidence (263). For a long time, pharmacological agents such as 5-aminosalicylic (ASA) compounds, steroids, azathioprine/6-mercaptopurine were the only available treatment options for IBD. Infliximab (Remicade; Centocor), a chimeric monoclonal antibody against TNF-α, was the first biologic agent to be approved for CD treatment in 1998. Since then, tremendous advancement of our understanding of inflammatory responses and mucosal immunology has fuelled the development of new biological therapeutic approaches for the treatment of both forms of IBD (264). A majority of these new agents target either the function or the synthesis of proinflammatory cytokines, underscoring the critical role of cytokines in IBD (264, 265). Several cytokines have been implicated in the pathogenesis of both CD and UC. The proinflammatory cytokines IL-12, IFN-γ and TNF-α have been shown to be elevated in the intestinal mucosa of CD patients; and UC has been associated with an increased secretion of IL-5 and TNF-α. In addition, IL-6, IL-8 and IL-1 levels were also increased in the inflamed mucosa of CD and UC patients (265). Hence, better ways of targeting cytokines has been a constant quest.

In order to efficiently inhibit cytokines, it is important to understand their precise role in IBD, the molecular mechanisms of their synthesis and the molecules and pathways involved in their regulation. Research using animal models of IBD has provided a wealth of information about IBD pathogenesis and has helped develop novel therapeutics, which are currently under clinical trail. The monoclonal antibody directed against IL-12 p40 is a classic example of 'bench to bedside' research. The antibody, designated ABT-874 (Abbott Laboratories) has shown promising results in initial clinical trials in CD patients, in that treatment with ABT-874 donwregulated the proinflammatory cytokines IL-12, IL-23 and IL-6. Secretion of TNF-α and IFN-γ were also decreased (147, 148). IL-12 p40 was first established as a target in animal models of IBD. Neutralizing IL-12 p40 proved therapeutic in mouse models and IL-12 p40 -/-

mice were shown to be resistant to disease (149-151, 266). A crucial prerequisite for such pre-clinical studies in animals and subsequent clinical trials in humans is extensive background basic research about the molecule being targeted. In this case, decades of research have established the role of IL-12 in immune responses (3). The discovery of IL-23 as a novel heterodimer of IL-12 p40 and as a cytokine driving chronic inflammation has rendered IL-12 p40 more attractive as a therapeutic target (23). Furthermore, thorough analysis of IL-12 p40 gene expression and the proximal signaling molecules that impinge on p40 transcription have helped develop specific therapeutic agents. For example, inhibitors for p38/JNK MAP kinases, which play an important role in IL-12 p40 transcription; semapimod (Cytokine PharmaSciences) and RDP 58 have been promising in initial clinical trials for CD and UC respectively (264).

Our group has been instrumental in studying IL-12 p40 gene expression and applying the findings originating thereof in pre-clinical studies in mouse models of IBD. We have identified several cis-acting elements in the IL-12 p40 promoter, the latest of which is the composite NFAT/IRF8 element (154). The initial part of this dissertation study helped complete the characterization of this site, as part of a collaborative study. In a subsequent collaboration, IRF8 was identified as a target for the anti-inflammatory pathway of CO and HO-1. This suggests that inhibition of IRF8 could be a potential therapeutic approach in IBD. However, IRF8 has been shown to be crucial for IL-12; and IRF8 deficient mice demonstrate severe immunodeficiency and also develop a chronic myelogenous leukemia-like syndrome (91, 267). Hence, future studies are required towards generating approaches to inhibit IRF8 functions, while preserving the balance between therapy and disease.

Herein, we target NFAT in macrophages using a novel cell permeable peptide – TAT-VIVIT. In our study VIVIT was found to inhibit expression of both IL-12 p40 protein and

mRNA. In addition, we describe a general role for NFAT in macrophage specific proinflammatory gene expression. TNF-α, IL-12 p70, IL-23 and NO secretion were inhibited by VIVIT. In order to study the global role of NFAT in macrophage gene expression, high-throughput analyses such as microarray or chromatin immunoprecipitation (ChIP)-chip analysis would be required. The latter is a technique for identifying novel targets for a specific transcription factor.

Although macrophages express all the family members of NFAT, our study did not address the differential functions of individual NFAT members in macrophages. In addition, each NFAT member has several isoforms, each probably having unique regulatory functions. Since VIVIT peptide inhibits the interaction of calcineurin with NFAT, nuclear translocation of NFAT 1-4 would be inhibited (176). Understanding the biology of NFAT family of transcription factors could facilitate the generation of member/isoform specific inhibitors. This study provides the groundwork for testing NFAT inhibition in disease models. However, the diverse functions and the complex nature of the NFAT family make it challenging for interventional approaches in animal models. A recent study using NFATc2 -/- and recombinase activating gene-2 (RAG-2) -/- double knock out mice reported the development of spontaneous UC-like syndrome in these mice (268). In addition, in a mouse model for arthritis, NFATc2 -/- mice developed severe, inflammatory arthritis (269). This suggests that NFATc2 has a negative regulatory role in innate immune functions and IBD. Thus, dissecting the functions of each NFAT member would be critical for further studies.

Although VIVIT peptide is more specific than its pharmacological counterparts – FK506 and CsA – it might still have non-specific effects. VIVIT could inhibit the interaction of calcineurin with other substrates which use similar binding motifs as NFAT (270). Recently, a

new generation of small molecule and organic NFAT inhibitors has been generated (271-273). These inhibitors claim to be highly specific inhibitors of NFAT-calcineurin interaction. In addition, use of these inhibitors could also circumvent delivery and stability problems associated with the use of peptide inhibitors.

Taken together, NFAT-IRF8 interactions could serve critical functions in proinflammatory gene expression. Further characterization of specific interaction domains and residues by x-ray crystallographic analysis could facilitate specific inhibition of this interaction and might provide new therapeutic approaches for IBD.

We next investigated intracellular mechanisms that negatively regulate IL-12 p40 expression. Several proteins have been described that negatively regulate TLR signaling and IL-12 expression. Although mice deficient in these proteins are hypotolerant to LPS induced endotoxic shock, they have not been reported to develop spontaneous IBD (214). PI3K is one such intracellular signaling pathway which inhibits TLR signaling and IL-12 expression (215). We were extremely intrigued by the report that PI3K p110δ mutant mice spontaneously develop IBD (169) and we sought to explore macrophage defects in these mice.

Our study revealed interesting facets of immune regulation and IBD in these mice. Histological features of colitis, which comprised of leukocytic infiltration, apoptotic and mitotic bodies and a marked increase in intraepithelial lymphocytes, were observed as early as 8 wks of age. A proinflammatory mucosal cytokine profile was observed with excessive production of IL-12, IL-23, TNF-α, IL-17 and IFN-γ. Macrophages from these mice were hyperresponsive to TLR signaling, especially TLR2 and TLR4. In addition to its role in negative regulation of TLR signaling, PI3K was found to be involved in the inhibition of IL-12 p40 by C5a (as reported earlier (235)) and IL-10. Interestingly, similar to DCs from CD patients, macrophages from PI3K

mutant mice responded poorly to the NOD2 ligand MDP. Thus, PI3K p110δ mutant mice represent a unique mouse model for IBD.

Furthermore, our study reinforces a negative regulatory role for PI3K in TLR signaling. It was earlier thought that Class IA PI3K alone inhibits TLR signaling. A recent study analyzed the role of individual classes of PI3K in TLR signaling and reports a distinct role for Class III PI3K in TLR9 signaling (247). Thus, PI3K is emerging as a class of enzymes involved in keeping the innate immune functions under control. The mechanisms by which PI3K inhibits TLR signaling is unknown. In a study by Kagan and Medzhitov, phosphatidylinositol 4,5-bisphosphate (PIP2) was necessary for recruitment of the TLR adaptor protein TIRAP in order to initiate signaling (274). It is possible that PI3K, by means of phosphorylating PIP2 to its tri-phosphate form – PIP3 (phosphatidylinositol 3,4,5-triphosphate) inhibits the recruitment of TIRAP to TLR. In this study, only TLR2 and 4 required PIP2 for signaling; which also explains our observation that TLR2 and 4 signaling were most affected by inactivation of PI3K p110δ.

Glycogen synthase kinase (GSK) 3, a downstream target for PI3K has been suggested to mediate the inhibitory effect of PI3K (275). GSK3 is constitutively active in resting cells and is known to regulate transcription factors NF-κB, AP-1, NFAT and CREB (cAMP response element-binding protein) (275, 276). GSK3 is inhibited through phosphorylation by kinases such as Akt (also protein kinase B), which is induced by PI3K. Inhibition of GSK3 led to reduced nuclear amounts of NF-κB and the co-activator CBP (CREB binding protein) and attenuated proinflammatory cytokine production. Thus, it is possible that PI3K directly affects recruitment of TLR adaptor proteins and also inhibits the downstream kinase GSK3, in order to inhibit TLR signaling. It would be interesting to study the activation status of GSK3 in PI3K mutant cells.

The gene encoding NOD2 has been extensively as a susceptibility gene for human IBD (80, 81). DCs from CD patients demonstrate impaired responses to the NOD2 ligand MDP (237). However, NOD2 knock out and mutant mice do not develop IBD spontaneously and whether NOD2 mutation is a gain-of-function or a loss-of-function mutation is controversial (255-257, 277). The findings from our analysis of PI3K mutant mice support the notion that NOD2 is a negative regulator of TLR2 signaling and resemble the phenotype of CD patients with NOD2 mutations. Our study also suggests that PI3K is a downstream component of NOD2 signaling and may mediate its negative regulatory effect. Furthermore, the human gene for p110δ (*PIK3CD*) maps to the IBD7 susceptibility region on chromosome 1p36 (169). Further investigations of *PIK3CD* as an IBD susceptibility gene are required to better understand the complex interplay between PI3K, TLR and NOD2 signaling..

It has been proposed that macrophages, similar to Th1 and Th2 subsets, differentiate into polarized subsets (called M1 and M2) depending on the stimuli and influence T-helper responses (278, 279). M1 macrophages produce high amounts of IL-12, IL-23 and other proinflammatory cytokines and participate in Th1 responses. M2 macrophages produce high levels of IL-10 and promote Th2 responses. In a recent study, it was reported that macrophages from mice deficient for SHIP (src homology 2-containing inositol-5'-phosphatase) demonstrate a pronounced skewing towards the M2 phenotype (280). SHIP is a potent negative regulator of PI3K and SHIP-/- mice would have enhanced PI3K activity. The M2 skewing of macrophages in SHIP-/- mice suggests that PI3K has a role in M2 macrophage differentiation. Furthermore, M2 macrophages are thought to be involved in the resolution of inflammation due to their debris scavenging, tissue remodeling and wound healing properties (278). This is consistent with a role for PI3K in dampening inflammation. Hence, it would be interesting to study macrophage

differentiation in PI3K mutant mice; the hypothesis being – PI3K mutant mice show a skewed M1 macrophage phenotype.

Although we studied macrophage specific defects in our study, it is possible that a defect in other immune cell compartments is also contributing to IBD in the PI3K p110δ mutant mice. In order to identify the cell type responsible for IBD, adoptive transfer experiments into immunodeficient animals need to be performed.

Another interesting feature of these mice was the profound increase in the number of IELs in the colonic mucosa which is reminiscent of human lymphocytic colitis and celiac disease (225). Characterizing the role of IELs in the induction and propagation of colitis in the PI3K mutant mice would provide valuable insight into the pathogenesis of the above forms of colitis in humans, which are much less studied compared to UC and CD.

Thus, the PI3K p110δ mutant mice present several interesting phenotypic features which could further our understanding of both human IBD and basic mechanisms of regulating inflammation and immunity. Manipulation of the PI3K pathway could be a potential therapeutic approach for treating IBD.

This dissertation has explored mechanisms for inhibition of inflammation in IBD, both by external manipulation of proinflammatory gene expression or by naturally occurring negative regulatory pathways; which are summarized in figure. 26.

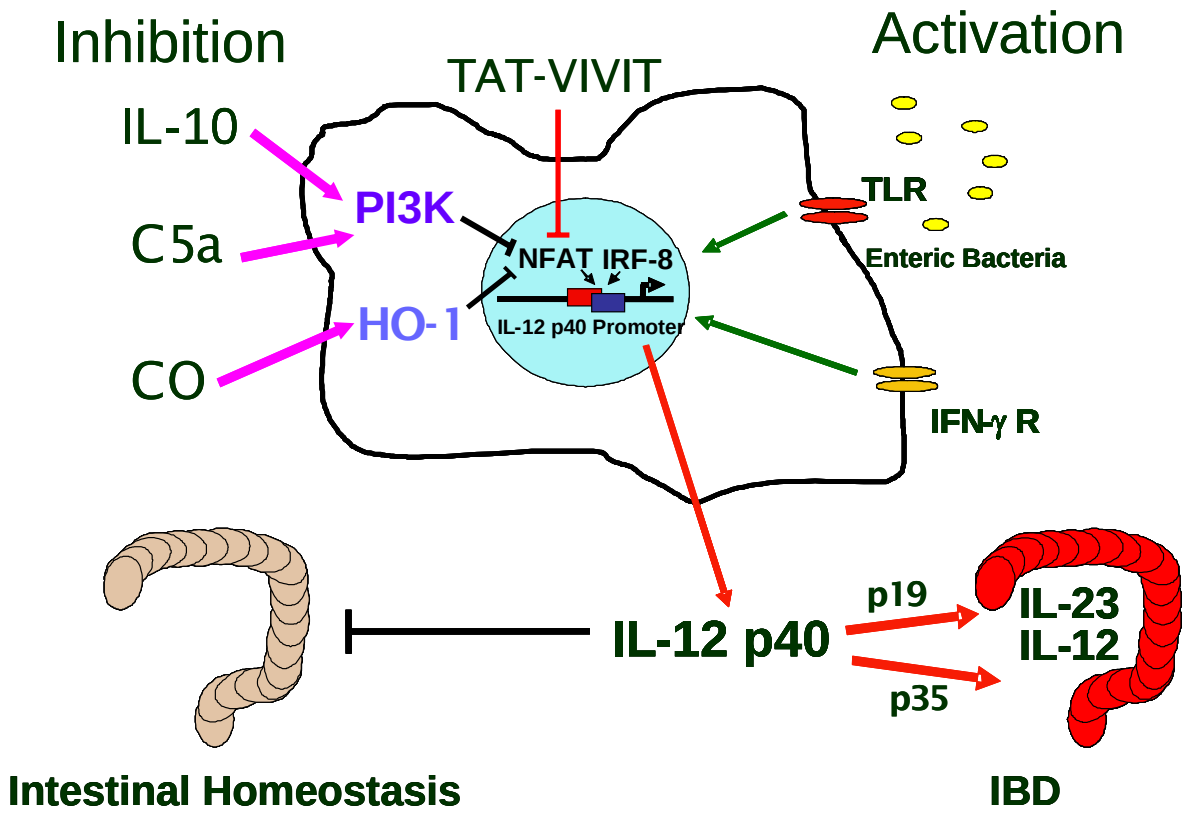


Figure 26. IL-12 p40 regulation in macrophages : A central event in intestinal inflammation.

The IL-12 p40 promoter is activated in macrophages are activated through ligation of TLRs and synergistically induced by IFN-γ. The NFAT/IRF8 element is important for this induction. IL-12 p40 thus produced heterodimerises with either p35 to form IL-12 or p19 to form IL-23. Both these cytokines are critical for Th1 responses. However, inappropriate regulation of these cytokines can lead to a chronic inflammatory disorders such as IBD. Inhibitory pathways in macrophages such as those mediated by IL-10, CO and complement components are crucial for negative regulation of IL-12 p40 and help maintain intestinal homeostasis. Data from this dissertation suggests that PI3K serves as an important 'molecular brake' to limit inflammation. Targeting of transcription factors such as NFAT also inhibits IL-12 p40, providing us new approaches for treating IBD.

BIBLIOGRAPHY

1. Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-2357.
2. Kobayashi, M., L. Fitz, M. Ryan, R.M. Hewick, S.C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med* 170:827-845.
3. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 3:133-146.
4. Merberg, D.M., S.F. Wolf, and S.C. Clark. 1992. Sequence similarity between NKSF and the IL-6/G-CSF family. *Immunol Today* 13:77-78.
5. Gearing, D.P., and D. Cosman. 1991. Homology of the p40 subunit of natural killer cell stimulatory factor (NKSF) with the extracellular domain of the interleukin-6 receptor. *Cell* 66:9-10.
6. Wolf, S.F., P.A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R.M. Hewick, and et al. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J Immunol* 146:3074-3081.
7. Sieburth, D., E.W. Jabs, J.A. Warrington, X. Li, J. Lasota, S. LaForgia, K. Kelleher, K. Huebner, J.J. Wasmuth, and S.F. Wolf. 1992. Assignment of genes encoding a unique cytokine (IL12) composed of two unrelated subunits to chromosomes 3 and 5. *Genomics* 14:59-62.
8. Schoenhaut, D.S., A.O. Chua, A.G. Wolitzky, P.M. Quinn, C.M. Dwyer, W. McComas, P.C. Familletti, M.K. Gately, and U. Gubler. 1992. Cloning and expression of murine IL-12. *J Immunol* 148:3433-3440.
9. Gubler, U., A.O. Chua, D.S. Schoenhaut, C.M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A.G. Wolitzky, P.M. Quinn, P.C. Familletti, and et al. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci U S A* 88:4143-4147.
10. Wysocka, M., M. Kubin, L.Q. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri. 1995. Interleukin-12 is required for interferon-gamma production and lethality in lipopolysaccharide-induced shock in mice. *Eur J Immunol* 25:672-676.
11. Stern, A.S., F.J. Podlaski, J.D. Hulmes, Y.C. Pan, P.M. Quinn, A.G. Wolitzky, P.C. Familletti, D.L. Stremlo, T. Truitt, R. Chizzonite, and et al. 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc Natl Acad Sci U S A* 87:6808-6812.

12. D'Andrea, A., M. Rengaraju, N.M. Valiante, J. Chehimi, M. Kubin, M. Aste, S.H. Chan, M. Kobayashi, D. Young, E. Nickbarg, and et al. 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J Exp Med* 176:1387-1398.
13. Heinzel, F.P., A.M. Hujer, F.N. Ahmed, and R.M. Rerko. 1997. In vivo production and function of IL-12 p40 homodimers. *J Immunol* 158:4381-4388.
14. Gillessen, S., D. Carvajal, P. Ling, F.J. Podlaski, D.L. Stremlo, P.C. Familletti, U. Gubler, D.H. Presky, A.S. Stern, and M.K. Gately. 1995. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *Eur J Immunol* 25:200-206.
15. Piccotti, J.R., S.Y. Chan, K. Li, E.J. Eichwald, and D.K. Bishop. 1997. Differential effects of IL-12 receptor blockade with IL-12 p40 homodimer on the induction of CD4+ and CD8+ IFN-gamma-producing cells. *J Immunol* 158:643-648.
16. Piccotti, J.R., K. Li, S.Y. Chan, J. Ferrante, J. Magram, E.J. Eichwald, and D.K. Bishop. 1998. Alloantigen-reactive Th1 development in IL-12-deficient mice. *J Immunol* 160:1132-1138.
17. Carra, G., F. Gerosa, and G. Trinchieri. 2000. Biosynthesis and posttranslational regulation of human IL-12. *J Immunol* 164:4752-4761.
18. Pflanz, S., J.C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W.M. Blumenschein, J.D. Mattson, J.L. Wagner, W. To, S. Zurawski, T.K. McClanahan, D.M. Gorman, J.F. Bazan, R. de Waal Malefyt, D. Rennick, and R.A. Kastelein. 2002. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity* 16:779-790.
19. Oppmann, B., R. Lesley, B. Blom, J.C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J.S. Abrams, K.W. Moore, D. Rennick, R. de Waal-Malefyt, C. Hannum, J.F. Bazan, and R.A. Kastelein. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13:715-725.
20. Devergne, O., M. Birkenbach, and E. Kieff. 1997. Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. *Proc Natl Acad Sci U S A* 94:12041-12046.
21. Devergne, O., M. Hummel, H. Koeppen, M.M. Le Beau, E.C. Nathanson, E. Kieff, and M. Birkenbach. 1996. A novel interleukin-12 p40-related protein induced by latent Epstein-Barr virus infection in B lymphocytes. *J Virol* 70:1143-1153.
22. Brombacher, F., R.A. Kastelein, and G. Alber. 2003. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol* 24:207-212.
23. Hunter, C.A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* 5:521-531.
24. Rock, F.L., G. Hardiman, J.C. Timans, R.A. Kastelein, and J.F. Bazan. 1998. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A* 95:588-593.
25. Janeway, C.A., Jr. 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* 13:11-16.
26. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388:394-397.

27. Chan, S.H., B. Perussia, J.W. Gupta, M. Kobayashi, M. Pospisil, H.A. Young, S.F. Wolf, D. Young, S.C. Clark, and G. Trinchieri. 1991. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J Exp Med* 173:869-879.
28. Kubin, M., M. Kamoun, and G. Trinchieri. 1994. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J Exp Med* 180:211-222.
29. Watford, W.T., M. Moriguchi, A. Morinobu, and J.J. O'Shea. 2003. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev* 14:361-368.
30. Chan, S.H., M. Kobayashi, D. Santoli, B. Perussia, and G. Trinchieri. 1992. Mechanisms of IFN-gamma induction by natural killer cell stimulatory factor (NKSF/IL-12). Role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. *J Immunol* 148:92-98.
31. Robinson, D., K. Shibuya, A. Mui, F. Zonin, E. Murphy, T. Sana, S.B. Hartley, S. Menon, R. Kastelein, F. Bazan, and A. O'Garra. 1997. IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. *Immunity* 7:571-581.
32. Micallef, M.J., T. Ohtsuki, K. Kohno, F. Tanabe, S. Ushio, M. Namba, T. Tanimoto, K. Torigoe, M. Fujii, M. Ikeda, S. Fukuda, and M. Kurimoto. 1996. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. *Eur J Immunol* 26:1647-1651.
33. Lauwerys, B.R., J.C. Renauld, and F.A. Houssiau. 1999. Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. *Cytokine* 11:822-830.
34. Yoshimoto, T., H. Okamura, Y.I. Tagawa, Y. Iwakura, and K. Nakanishi. 1997. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-gamma production from activated B cells. *Proc Natl Acad Sci U S A* 94:3948-3953.
35. Airoldi, I., G. Gri, J.D. Marshall, A. Corcione, P. Facchetti, R. Guglielmino, G. Trinchieri, and V. Pistoia. 2000. Expression and function of IL-12 and IL-18 receptors on human tonsillar B cells. *J Immunol* 165:6880-6888.
36. Frucht, D.M., T. Fukao, C. Bogdan, H. Schindler, J.J. O'Shea, and S. Koyasu. 2001. IFN-gamma production by antigen-presenting cells: mechanisms emerge. *Trends Immunol* 22:556-560.
37. Hsieh, C.S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* 260:547-549.
38. Manetti, R., P. Parronchi, M.G. Giudizi, M.P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* 177:1199-1204.
39. Park, A.Y., and P. Scott. 2001. Il-12: keeping cell-mediated immunity alive. *Scand J Immunol* 53:529-532.

40. O'Shea, J.J., and W.E. Paul. 2002. Regulation of T(H)1 differentiation--controlling the controllers. *Nat Immunol* 3:506-508.
41. Glimcher, L.H. 2001. Lineage commitment in lymphocytes: controlling the immune response. *J Clin Invest* 108:s25-s30.
42. Dong, C., and R.A. Flavell. 2001. Th1 and Th2 cells. *Curr Opin Hematol* 8:47-51.
43. Presky, D.H., H. Yang, L.J. Minetti, A.O. Chua, N. Nabavi, C.Y. Wu, M.K. Gately, and U. Gubler. 1996. A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc Natl Acad Sci U S A* 93:14002-14007.
44. Chua, A.O., R. Chizzonite, B.B. Desai, T.P. Truitt, P. Nunes, L.J. Minetti, R.R. Warrier, D.H. Presky, J.F. Levine, M.K. Gately, and et al. 1994. Expression cloning of a human IL-12 receptor component. A new member of the cytokine receptor superfamily with strong homology to gp130. *J Immunol* 153:128-136.
45. Grohmann, U., M.L. Belladonna, R. Bianchi, C. Orabona, E. Ayroldi, M.C. Fioretti, and P. Puccetti. 1998. IL-12 acts directly on DC to promote nuclear localization of NF-kappaB and primes DC for IL-12 production. *Immunity* 9:315-323.
46. Szabo, S.J., A.S. Dighe, U. Gubler, and K.M. Murphy. 1997. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 185:817-824.
47. Rogge, L., L. Barberis-Maino, M. Biffi, N. Passini, D.H. Presky, U. Gubler, and F. Sinigaglia. 1997. Selective expression of an interleukin-12 receptor component by human T helper 1 cells. *J Exp Med* 185:825-831.
48. Sinigaglia, F., D. D'Ambrosio, P. Panina-Bordignon, and L. Rogge. 1999. Regulation of the IL-12/IL-12R axis: a critical step in T-helper cell differentiation and effector function. *Immunol Rev* 170:65-72.
49. Gorham, J.D., M.L. Guler, D. Fenoglio, U. Gubler, and K.M. Murphy. 1998. Low dose TGF-beta attenuates IL-12 responsiveness in murine Th cells. *J Immunol* 161:1664-1670.
50. Wu, C., R.R. Warrier, X. Wang, D.H. Presky, and M.K. Gately. 1997. Regulation of interleukin-12 receptor beta1 chain expression and interleukin-12 binding by human peripheral blood mononuclear cells. *Eur J Immunol* 27:147-154.
51. Wu, C., X. Wang, M. Gadina, J.J. O'Shea, D.H. Presky, and J. Magram. 2000. IL-12 receptor beta 2 (IL-12R beta 2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. *J Immunol* 165:6221-6228.
52. Becker, C., S. Wirtz, and M.F. Neurath. 2005. Stepwise regulation of TH1 responses in autoimmunity: IL-12-related cytokines and their receptors. *Inflamm Bowel Dis* 11:755-764.
53. Kaplan, M.H., Y.L. Sun, T. Hoey, and M.J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382:174-177.
54. Thierfelder, W.E., J.M. van Deursen, K. Yamamoto, R.A. Tripp, S.R. Sarawar, R.T. Carson, M.Y. Sangster, D.A. Vignali, P.C. Doherty, G.C. Grosveld, and J.N. Ihle. 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382:171-174.
55. Langrish, C.L., B.S. McKenzie, N.J. Wilson, R. de Waal Malefyt, R.A. Kastelein, and D.J. Cua. 2004. IL-12 and IL-23: master regulators of innate and adaptive immunity. *Immunol Rev* 202:96-105.
56. Smits, H.H., A.J. van Beelen, C. Hessle, R. Westland, E. de Jong, E. Soeteman, A. Wold, E.A. Wierenga, and M.L. Kapsenberg. 2004. Commensal Gram-negative bacteria prime

human dendritic cells for enhanced IL-23 and IL-27 expression and enhanced Th1 development. *Eur J Immunol* 34:1371-1380.
57. Re, F., and J.L. Strominger. 2001. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *J Biol Chem* 276:37692-37699.
58. Verreck, F.A., T. de Boer, D.M. Langenberg, M.A. Hoeve, M. Kramer, E. Vaisberg, R. Kastelein, A. Kolk, R. de Waal-Malefyt, and T.H. Ottenhoff. 2004. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci U S A* 101:4560-4565.
59. Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S.A. Lira, D. Gorman, R.A. Kastelein, and J.D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.
60. Murphy, C.A., C.L. Langrish, Y. Chen, W. Blumenschein, T. McClanahan, R.A. Kastelein, J.D. Sedgwick, and D.J. Cua. 2003. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 198:1951-1957.
61. Wiekowski, M.T., M.W. Leach, E.W. Evans, L. Sullivan, S.C. Chen, G. Vassileva, J.F. Bazan, D.M. Gorman, R.A. Kastelein, S. Narula, and S.A. Lira. 2001. Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J Immunol* 166:7563-7570.
62. Harrington, L.E., R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, and C.T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132.
63. Park, H., Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.
64. Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-189.
65. Happel, K.I., P.J. Dubin, M. Zheng, N. Ghilardi, C. Lockhart, L.J. Quinton, A.R. Odden, J.E. Shellito, G.J. Bagby, S. Nelson, and J.K. Kolls. 2005. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. *J Exp Med* 202:761-769.
66. Kolls, J.K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity* 21:467-476.
67. Stark, M.A., Y. Huo, T.L. Burcin, M.A. Morris, T.S. Olson, and K. Ley. 2005. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* 22:285-294.
68. McKenzie, B.S., R.A. Kastelein, and D.J. Cua. 2006. Understanding the IL-23-IL-17 immune pathway. *Trends Immunol* 27:17-23.
69. Iwakura, Y., and H. Ishigame. 2006. The IL-23/IL-17 axis in inflammation. *J Clin Invest* 116:1218-1222.
70. Chen, Y., C.L. Langrish, B. McKenzie, B. Joyce-Shaikh, J.S. Stumhofer, T. McClanahan, W. Blumenschein, T. Churakovsa, J. Low, L. Presta, C.A. Hunter, R.A. Kastelein, and D.J. Cua. 2006. Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. *J Clin Invest* 116:1317-1326.

71. 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.
72. Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M.A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D.J. Cua, R.A. Kastelein, and D. Rennick. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 116:1310-1316.
73. Zahn, S., S. Wirtz, M. Birkenbach, R.S. Blumberg, M.F. Neurath, and E. von Stebut. 2005. Impaired Th1 responses in mice deficient in Epstein-Barr virus-induced gene 3 and challenged with physiological doses of Leishmania major. *Eur J Immunol* 35:1106-1112.
74. Yoshida, H., S. Hamano, G. Senaldi, T. Covey, R. Faggioni, S. Mu, M. Xia, A.C. Wakeham, H. Nishina, J. Potter, C.J. Saris, and T.W. Mak. 2001. WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection. *Immunity* 15:569-578.
75. Chen, Q., N. Ghilardi, H. Wang, T. Baker, M.H. Xie, A. Gurney, I.S. Grewal, and F.J. de Sauvage. 2000. Development of Th1-type immune responses requires the type I cytokine receptor TCCR. *Nature* 407:916-920.
76. Villarino, A., L. Hibbert, L. Lieberman, E. Wilson, T. Mak, H. Yoshida, R.A. Kastelein, C. Saris, and C.A. Hunter. 2003. The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. *Immunity* 19:645-655.
77. Hamano, S., K. Himeno, Y. Miyazaki, K. Ishii, A. Yamanaka, A. Takeda, M. Zhang, H. Hisaeda, T.W. Mak, A. Yoshimura, and H. Yoshida. 2003. WSX-1 is required for resistance to Trypanosoma cruzi infection by regulation of proinflammatory cytokine production. *Immunity* 19:657-667.
78. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat Rev Immunol* 4:499-511.
79. Inohara, N., and G. Nunez. 2003. NODs: intracellular proteins involved in inflammation and apoptosis. *Nat Rev Immunol* 3:371-382.
80. Ogura, Y., D.K. Bonen, N. Inohara, D.L. Nicolae, F.F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R.H. Duerr, J.P. Achkar, S.R. Brant, T.M. Bayless, B.S. Kirschner, S.B. Hanauer, G. Nunez, and J.H. Cho. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411:603-606.
81. Hugot, J.P., M. Chamaillard, H. Zouali, S. Lesage, J.P. Cezard, J. Belaiche, S. Almer, C. Tysk, C.A. O'Morain, M. Gassull, V. Binder, Y. Finkel, A. Cortot, R. Modigliani, P. Laurent-Puig, C. Gower-Rousseau, J. Macry, J.F. Colombel, M. Sahbatou, and G. Thomas. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411:599-603.
82. Hayes, M.P., J. Wang, and M.A. Norcross. 1995. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes. *Blood* 86:646-650.
83. Liu, J., S. Cao, L.M. Herman, and X. Ma. 2003. Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. *J Exp Med* 198:1265-1276.
84. Liu, J., X. Guan, T. Tamura, K. Ozato, and X. Ma. 2004. Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein. *J Biol Chem* 279:55609-55617.

85. Ma, X., G. Gri, and G. Trinchieri. 1996. A novel ets-2-related nuclear factor is involved in transcriptional activation of the human interleukin-12 p40 gene promoter in response to interferon-gamma and LPS stimulation of monocytic cells. *Ann N Y Acad Sci* 795:357-360.
86. Ma, X., J.M. Chow, G. Gri, G. Carra, F. Gerosa, S.F. Wolf, R. Dzialo, and G. Trinchieri. 1996. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *J Exp Med* 183:147-157.
87. Maruyama, S., K. Sumita, H. Shen, M. Kanoh, X. Xu, M. Sato, M. Matsumoto, H. Shinomiya, and Y. Asano. 2003. Identification of IFN regulatory factor-1 binding site in IL-12 p40 gene promoter. *J Immunol* 170:997-1001.
88. Masumi, A., S. Tamaoki, I.M. Wang, K. Ozato, and K. Komuro. 2002. IRF-8/ICSBP and IRF-1 cooperatively stimulate mouse IL-12 promoter activity in macrophages. *FEBS Lett* 531:348-353.
89. Goriely, S., C. Molle, M. Nguyen, V. Albarani, N.O. Haddou, R. Lin, D. De Wit, V. Flamand, F. Willems, and M. Goldman. 2006. Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. *Blood* 107:1078-1084.
90. Lohoff, M., G.S. Duncan, D. Ferrick, H.W. Mittrucker, S. Bischof, S. Prechtl, M. Rollinghoff, E. Schmitt, A. Pahl, and T.W. Mak. 2000. Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1 cells. *J Exp Med* 192:325-336.
91. Scharton-Kersten, T., C. Contursi, A. Masumi, A. Sher, and K. Ozato. 1997. Interferon consensus sequence binding protein-deficient mice display impaired resistance to intracellular infection due to a primary defect in interleukin 12 p40 induction. *J Exp Med* 186:1523-1534.
92. Taki, S., T. Sato, K. Ogasawara, T. Fukuda, M. Sato, S. Hida, G. Suzuki, M. Mitsuyama, E.H. Shin, S. Kojima, T. Taniguchi, and Y. Asano. 1997. Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. *Immunity* 6:673-679.
93. Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med* 184:747-752.
94. Shu, U., M. Kiniwa, C.Y. Wu, C. Maliszewski, N. Vezzio, J. Hakimi, M. Gately, and G. Delespesse. 1995. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. *Eur J Immunol* 25:1125-1128.
95. Schulz, O., A.D. Edwards, M. Schito, J. Aliberti, S. Manickasingham, A. Sher, and C. Reis e Sousa. 2000. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. *Immunity* 13:453-462.
96. D'Andrea, A., M. Aste-Amezaga, N.M. Valiante, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* 178:1041-1048.
97. Aste-Amezaga, M., X. Ma, A. Sartori, and G. Trinchieri. 1998. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *J Immunol* 160:5936-5944.

98. Driessler, F., K. Venstrom, R. Sabat, K. Asadullah, and A.J. Schottelius. 2004. Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. *Clin Exp Immunol* 135:64-73.
99. Rahim, S.S., N. Khan, C.S. Boddupalli, S.E. Hasnain, and S. Mukhopadhyay. 2005. Interleukin-10 (IL-10) mediated suppression of IL-12 production in RAW 264.7 cells also involves c-rel transcription factor. *Immunology* 114:313-321.
100. Schottelius, A.J., M.W. Mayo, R.B. Sartor, and A.S. Baldwin, Jr. 1999. Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *J Biol Chem* 274:31868-31874.
101. Zhou, L., A.A. Nazarian, and S.T. Smale. 2004. Interleukin-10 inhibits interleukin-12 p40 gene transcription by targeting a late event in the activation pathway. *Mol Cell Biol* 24:2385-2396.
102. Gazzinelli, R.T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *J Immunol* 157:798-805.
103. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75:263-274.
104. Braun, M.C., and B.L. Kelsall. 2001. Regulation of interleukin-12 production by G-protein-coupled receptors. *Microbes Infect* 3:99-107.
105. van der Pouw Kraan, T.C., L.C. Boeije, R.J. Smeenk, J. Wijdenes, and L.A. Aarden. 1995. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. *J Exp Med* 181:775-779.
106. He, J., S. Gurunathan, A. Iwasaki, B. Ash-Shaheed, and B.L. Kelsall. 2000. Primary role for Gi protein signaling in the regulation of interleukin 12 production and the induction of T helper cell type 1 responses. *J Exp Med* 191:1605-1610.
107. Karp, C.L., and M. Wills-Karp. 2001. Complement and IL-12: yin and yang. *Microbes Infect* 3:109-119.
108. Rizzo, G., and S. Fiorucci. 2006. PPARs and other nuclear receptors in inflammation. *Curr Opin Pharmacol*.
109. 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-kappaB activation by nitric oxide in murine macrophages and dendritic cells. *J Biol Chem* 279:10776-10783.
110. Ma, X., and G. Trinchieri. 2001. Regulation of interleukin-12 production in antigen-presenting cells. *Adv Immunol* 79:55-92.
111. Snijders, A., C.M. Hilkens, T.C. van der Pouw Kraan, M. Engel, L.A. Aarden, and M.L. Kapsenberg. 1996. Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. *J Immunol* 156:1207-1212.
112. Yoshimoto, T., K. Kojima, T. Funakoshi, Y. Endo, T. Fujita, and H. Nariuchi. 1996. Molecular cloning and characterization of murine IL-12 genes. *J Immunol* 156:1082-1088.
113. Tone, Y., S.A. Thompson, J.M. Babik, K.F. Nolan, M. Tone, C. Raven, and H. Waldmann. 1996. Structure and chromosomal location of the mouse interleukin-12 p35 and p40 subunit genes. *Eur J Immunol* 26:1222-1227.

114. Hayes, M.P., F.J. Murphy, and P.R. Burd. 1998. Interferon-gamma-dependent inducible expression of the human interleukin-12 p35 gene in monocytes initiates from a TATA-containing promoter distinct from the CpG-rich promoter active in Epstein-Barr virus-transformed lymphoblastoid cells. *Blood* 91:4645-4651.
115. Babik, J.M., E. Adams, Y. Tone, P.J. Fairchild, M. Tone, and H. Waldmann. 1999. Expression of murine IL-12 is regulated by translational control of the p35 subunit. *J Immunol* 162:4069-4078.
116. Grumont, R., H. Hochrein, M. O'Keeffe, R. Gugasyan, C. White, I. Caminschi, W. Cook, and S. Gerondakis. 2001. c-Rel regulates interleukin 12 p70 expression in CD8(+) dendritic cells by specifically inducing p35 gene transcription. *J Exp Med* 194:1021-1032.
117. Goriely, S., D. Demonte, S. Nizet, D. De Wit, F. Willems, M. Goldman, and C. Van Lint. 2003. Human IL-12(p35) gene activation involves selective remodeling of a single nucleosome within a region of the promoter containing critical Sp1-binding sites. *Blood* 101:4894-4902.
118. Murphy, F.J., M.P. Hayes, and P.R. Burd. 2000. Disparate intracellular processing of human IL-12 preprotein subunits: atypical processing of the P35 signal peptide. *J Immunol* 164:839-847.
119. Murphy, T.L., M.G. Cleveland, P. Kulesza, J. Magram, and K.M. Murphy. 1995. Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. *Mol Cell Biol* 15:5258-5267.
120. Gri, G., D. Savio, G. Trinchieri, and X. Ma. 1998. Synergistic regulation of the human interleukin-12 p40 promoter by NFkappaB and Ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. *J Biol Chem* 273:6431-6438.
121. Wang, I.M., C. Contursi, A. Masumi, X. Ma, G. Trinchieri, and K. Ozato. 2000. An IFN-gamma-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. *J Immunol* 165:271-279.
122. Salkowski, C.A., K. Kopydlowski, J. Blanco, M.J. Cody, R. McNally, and S.N. Vogel. 1999. IL-12 is dysregulated in macrophages from IRF-1 and IRF-2 knockout mice. *J Immunol* 163:1529-1536.
123. Sanjabi, S., A. Hoffmann, H.C. Liou, D. Baltimore, and S.T. Smale. 2000. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc Natl Acad Sci U S A* 97:12705-12710.
124. Plevy, S.E., J.H. Gemberling, S. Hsu, A.J. Dorner, and S.T. Smale. 1997. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol Cell Biol* 17:4572-4588.
125. Zhu, C., K. Gagnidze, J.H. Gemberling, and S.E. Plevy. 2001. Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach. *J Biol Chem* 276:18519-18528.
126. Zhu, C., H. Xiong, K. Gagnidze, K. Rao, C. Horvath, and S. Plevy. Activation of the IL-12 p40 promoter by functional interactions between NFAT and ICSBP. *Manuscript in revision.*
127. Becker, C., S. Wirtz, X. Ma, M. Blessing, P.R. Galle, and M.F. Neurath. 2001. Regulation of IL-12 p40 promoter activity in primary human monocytes: roles of NF-

kappaB, CCAAT/enhancer-binding protein beta, and PU.1 and identification of a novel repressor element (GA-12) that responds to IL-4 and prostaglandin E(2). *J Immunol* 167:2608-2618.
128. Weinmann, A.S., S.E. Plevy, and S.T. Smale. 1999. Rapid and selective remodeling of a positioned nucleosome during the induction of IL-12 p40 transcription. *Immunity* 11:665-675.
129. Weinmann, A.S., D.M. Mitchell, S. Sanjabi, M.N. Bradley, A. Hoffmann, H.C. Liou, and S.T. Smale. 2001. Nucleosome remodeling at the IL-12 p40 promoter is a TLR-dependent, Rel-independent event. *Nat Immunol* 2:51-57.
130. Delgado, M., and D. Ganea. 1999. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit interleukin-12 transcription by regulating nuclear factor kappaB and Ets activation. *J Biol Chem* 274:31930-31940.
131. D'Ambrosio, D., M. Cippitelli, M.G. Cocciolo, D. Mazzeo, P. Di Lucia, R. Lang, F. Sinigaglia, and P. Panina-Bordignon. 1998. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. *J Clin Invest* 101:252-262.
132. Grazia Cappiello, M., F.S. Sutterwala, G. Trinchieri, D.M. Mosser, and X. Ma. 2001. Suppression of Il-12 transcription in macrophages following Fc gamma receptor ligation. *J Immunol* 166:4498-4506.
133. Bouma, G., and W. Strober. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 3:521-533.
134. Autschbach, F., T. Giese, N. Gassler, B. Sido, G. Heuschen, U. Heuschen, I. Zuna, P. Schulz, H. Weckauf, I. Berger, H.F. Otto, and S.C. Meuer. 2002. Cytokine/chemokine messenger-RNA expression profiles in ulcerative colitis and Crohn's disease. *Virchows Arch* 441:500-513.
135. Komatsu, M., D. Kobayashi, K. Saito, D. Furuya, A. Yagihashi, H. Araake, N. Tsuji, S. Sakamaki, Y. Niitsu, and N. Watanabe. 2001. Tumor necrosis factor-alpha in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR. *Clin Chem* 47:1297-1301.
136. McCormack, G., D. Moriarty, D.P. O'Donoghue, P.A. McCormick, K. Sheahan, and A.W. Baird. 2001. Tissue cytokine and chemokine expression in inflammatory bowel disease. *Inflamm Res* 50:491-495.
137. Cominelli, F. 2004. Cytokine-based therapies for Crohn's disease--new paradigms. *N Engl J Med* 351:2045-2048.
138. Byrne, F.R., and J.L. Viney. 2006. Mouse models of inflammatory bowel disease. *Curr Opin Drug Discov Devel* 9:207-217.
139. Berrebi, D., M. Besnard, G. Fromont-Hankard, R. Paris, J.F. Mougenot, P. De Lagausie, D. Emilie, J.P. Cezard, J. Navarro, and M. Peuchmaur. 1998. Interleukin-12 expression is focally enhanced in the gastric mucosa of pediatric patients with Crohn's disease. *Am J Pathol* 152:667-672.
140. Monteleone, G., L. Biancone, R. Marasco, G. Morrone, O. Marasco, F. Luzza, and F. Pallone. 1997. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* 112:1169-1178.
141. Parronchi, P., P. Romagnani, F. Annunziato, S. Sampognaro, A. Becchio, L. Giannarini, E. Maggi, C. Pupilli, F. Tonelli, and S. Romagnani. 1997. Type 1 T-helper cell

predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol* 150:823-832.
142. Stallmach, A., T. Marth, N. Adrian, B.M. Wittig, K.W. Ecker, M. Schilling, and M. Zeitz. 2002. Increased expression of interleukin-12 receptor beta(2) on lamina propria mononuclear cells of patients with active Crohn's disease. *Int J Colorectal Dis* 17:303-310.
143. Parrello, T., G. Monteleone, S. Cucchiara, I. Monteleone, L. Sebkova, P. Doldo, F. Luzza, and F. Pallone. 2000. Up-regulation of the IL-12 receptor beta 2 chain in Crohn's disease. *J Immunol* 165:7234-7239.
144. Schmidt, C., T. Giese, B. Ludwig, I. Mueller-Molaian, T. Marth, S. Zeuzem, S.C. Meuer, and A. Stallmach. 2005. Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. *Inflamm Bowel Dis* 11:16-23.
145. Nielsen, O.H., I. Kirman, N. Rudiger, J. Hendel, and B. Vainer. 2003. Upregulation of interleukin-12 and -17 in active inflammatory bowel disease. *Scand J Gastroenterol* 38:180-185.
146. Fujino, S., A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba, and Y. Fujiyama. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52:65-70.
147. Mannon, P.J., I.J. Fuss, L. Mayer, C.O. Elson, W.J. Sandborn, D. Present, B. Dolin, N. Goodman, C. Groden, R.L. Hornung, M. Quezado, Z. Yang, M.F. Neurath, J. Salfeld, G.M. Veldman, U. Schwertschlag, and W. Strober. 2004. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 351:2069-2079.
148. Fuss, I.J., C. Becker, Z. Yang, C. Groden, R.L. Hornung, F. Heller, M.F. Neurath, W. Strober, and P.J. Mannon. 2006. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. *Inflamm Bowel Dis* 12:9-15.
149. Tozawa, K., H. Hanai, K. Sugimoto, S. Baba, H. Sugimura, T. Aoshi, M. Uchijima, T. Nagata, and Y. Koide. 2003. Evidence for the critical role of interleukin-12 but not interferon-gamma in the pathogenesis of experimental colitis in mice. *J Gastroenterol Hepatol* 18:578-587.
150. Davidson, N.J., S.A. Hudak, R.E. Lesley, S. Menon, M.W. Leach, and D.M. Rennick. 1998. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J Immunol* 161:3143-3149.
151. Neurath, M.F., I. Fuss, B.L. Kelsall, E. Stuber, and W. Strober. 1995. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med* 182:1281-1290.
152. Becker, C., S. Wirtz, M. Blessing, J. Pirhonen, D. Strand, O. Bechthold, J. Frick, P.R. Galle, I. Autenrieth, and M.F. Neurath. 2003. Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. *J Clin Invest* 112:693-706.
153. Rao, A., C. Luo, and P.G. Hogan. 1997. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 15:707-747.
154. Zhu, C., K. Rao, H. Xiong, K. Gagnidze, F. Li, C. Horvath, and S. Plevy. 2003. Activation of the murine interleukin-12 p40 promoter by functional interactions between NFAT and ICSBP. *J Biol Chem* 278:39372-39382.

155. 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.
156. Snijders, A., P. Kalinski, C.M. Hilkens, and M.L. Kapsenberg. 1998. High-level IL-12 production by human dendritic cells requires two signals. *Int Immunol* 10:1593-1598.
157. Conboy, I.M., D. Manoli, V. Mhaiskar, and P.P. Jones. 1999. Calcineurin and vacuolar-type H+-ATPase modulate macrophage effector functions. *Proc Natl Acad Sci U S A* 96:6324-6329.
158. Boyko, E.J., T.D. Koepsell, D.R. Perera, and T.S. Inui. 1987. Risk of ulcerative colitis among former and current cigarette smokers. *N Engl J Med* 316:707-710.
159. Jick, H., and A.M. Walker. 1983. Cigarette smoking and ulcerative colitis. *N Engl J Med* 308:261-263.
160. Lashner, B.A., S.B. Hanauer, and M.D. Silverstein. 1990. Testing nicotine gum for ulcerative colitis patients. Experience with single-patient trials. *Dig Dis Sci* 35:827-832.
161. Pullan, R.D., J. Rhodes, S. Ganesh, V. Mani, J.S. Morris, G.T. Williams, R.G. Newcombe, M.A. Russell, C. Feyerabend, G.A. Thomas, and et al. 1994. Transdermal nicotine for active ulcerative colitis. *N Engl J Med* 330:811-815.
162. Tenhunen, R., H.S. Marver, and R. Schmid. 1970. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. *J Lab Clin Med* 75:410-421.
163. McCoubrey, W.K., Jr., T.J. Huang, and M.D. Maines. 1997. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem* 247:725-732.
164. Otterbein, L.E., M.P. Soares, K. Yamashita, and F.H. Bach. 2003. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 24:449-455.
165. Soares, M.P., Y. Lin, J. Anrather, E. Csizmadia, K. Takigami, K. Sato, S.T. Grey, R.B. Colvin, A.M. Choi, K.D. Poss, and F.H. Bach. 1998. Expression of heme oxygenase-1 can determine cardiac xenograft survival. *Nat Med* 4:1073-1077.
166. Otterbein, L.E., P.J. Lee, B.Y. Chin, I. Petrache, S.L. Camhi, J. Alam, and A.M. Choi. 1999. Protective effects of heme oxygenase-1 in acute lung injury. *Chest* 116:61S-63S.
167. Otterbein, L.E., J.K. Kolls, L.L. Mantell, J.L. Cook, J. Alam, and A.M. Choi. 1999. Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. *J Clin Invest* 103:1047-1054.
168. Otterbein, L.E., F.H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R.J. Davis, R.A. Flavell, and A.M. Choi. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 6:422-428.
169. 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 p110delta PI 3-kinase mutant mice. *Science* 297:1031-1034.
170. Han, J., and R.J. Ulevitch. 2005. Limiting inflammatory responses during activation of innate immunity. *Nat Immunol* 6:1198-1205.
171. Costa, G.L., M.R. Sandora, A. Nakajima, E.V. Nguyen, C. Taylor-Edwards, A.J. Slavin, C.H. Contag, C.G. Fathman, and J.M. Benson. 2001. Adoptive immunotherapy of experimental autoimmune encephalomyelitis via T cell delivery of the IL-12 p40 subunit. *J Immunol* 167:2379-2387.

172. Honda, K., K. Nakamura, N. Matsui, M. Takahashi, Y. Kitamura, T. Mizutani, N. Harada, H. Nawata, S. Hamano, and H. Yoshida. 2005. T helper 1-inducing property of IL-27/WSX-1 signaling is required for the induction of experimental colitis. *Inflamm Bowel Dis* 11:1044-1052.
173. Hong, K., A. Chu, B.R. Ludviksson, E.L. Berg, and R.O. Ehrhardt. 1999. IL-12, independently of IFN-gamma, plays a crucial role in the pathogenesis of a murine psoriasis-like skin disorder. *J Immunol* 162:7480-7491.
174. Trinchieri, G. 1997. Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma). *Curr Opin Immunol* 9:17-23.
175. Macian, F. 2005. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* 5:472-484.
176. Aramburu, J., M.B. Yaffe, C. Lopez-Rodriguez, L.C. Cantley, P.G. Hogan, and A. Rao. 1999. Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science* 285:2129-2133.
177. Kaibori, M., K. Sakitani, M. Oda, Y. Kamiyama, Y. Masu, M. Nishizawa, S. Ito, and T. Okumura. 1999. Immunosuppressant FK506 inhibits inducible nitric oxide synthase gene expression at a step of NF-kappaB activation in rat hepatocytes. *J Hepatol* 30:1138-1145.
178. Meyer, S., N.G. Kohler, and A. Joly. 1997. Cyclosporine A is an uncompetitive inhibitor of proteasome activity and prevents NF-kappaB activation. *FEBS Lett* 413:354-358.
179. Andres, D., C. Diez-Fernandez, A. Zaragoza, A. Alvarez, and M. Cascales. 2001. Induction of cell proliferation by cyclosporine A in primary cultures of rat hepatocytes. *Biochem Pharmacol* 61:427-435.
180. Schwarze, S.R., and S.F. Dowdy. 2000. In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. *Trends Pharmacol Sci* 21:45-48.
181. Lindgren, M., M. Hallbrink, A. Prochiantz, and U. Langel. 2000. Cell-penetrating peptides. *Trends Pharmacol Sci* 21:99-103.
182. Moore, K.W., R. de Waal Malefyt, R.L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.
183. Hu, X., P.K. Paik, J. Chen, A. Yarilina, L. Kockeritz, T.T. Lu, J.R. Woodgett, and L.B. Ivashkiv. 2006. IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins. *Immunity* 24:563-574.
184. Xiong, H., C. Zhu, H. Li, F. Chen, L. Mayer, K. Ozato, J.C. Unkeless, and S.E. Plevy. 2003. Complex formation of the interferon (IFN) consensus sequence-binding protein with IRF-1 is essential for murine macrophage IFN-gamma-induced iNOS gene expression. *J Biol Chem* 278:2271-2277.
185. Tsai, E.Y., J. Jain, P.A. Pesavento, A. Rao, and A.E. Goldfeld. 1996. Tumor necrosis factor alpha gene regulation in activated T cells involves ATF-2/Jun and NFATp. *Mol Cell Biol* 16:459-467.
186. Winslow, M.M., E.M. Gallo, J.R. Neilson, and G.R. Crabtree. 2006. The calcineurin phosphatase complex modulates immunogenic B cell responses. *Immunity* 24:141-152.
187. Monticelli, S., D.C. Solymar, and A. Rao. 2004. Role of NFAT proteins in IL13 gene transcription in mast cells. *J Biol Chem* 279:36210-36218.
188. Seminario, M.C., J. Guo, B.S. Bochner, L.A. Beck, and S.N. Georas. 2001. Human eosinophils constitutively express nuclear factor of activated T cells p and c. *J Allergy Clin Immunol* 107:143-152.

189. Schroeder, J.T., K. Miura, H.H. Kim, A. Sin, A. Cianferoni, and V. Casolaro. 2002. Selective expression of nuclear factor of activated T cells 2/c1 in human basophils: evidence for involvement in IgE-mediated IL-4 generation. *J Allergy Clin Immunol* 109:507-513.
190. Aramburu, J., L. Azzoni, A. Rao, and B. Perussia. 1995. Activation and expression of the nuclear factors of activated T cells, NFATp and NFATc, in human natural killer cells: regulation upon CD16 ligand binding. *J Exp Med* 182:801-810.
191. Hirotani, H., N.A. Tuohy, J.T. Woo, P.H. Stern, and N.A. Clipstone. 2004. The calcineurin/nuclear factor of activated T cells signaling pathway regulates osteoclastogenesis in RAW264.7 cells. *J Biol Chem* 279:13984-13992.
192. Sutterwala, F.S., G.J. Noel, R. Clynes, and D.M. Mosser. 1997. Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J Exp Med* 185:1977-1985.
193. Delvig, A.A., and J.H. Robinson. 1999. The role of calcium homeostasis and flux during bacterial antigen processing in murine macrophages. *Eur J Immunol* 29:2414-2419.
194. Kim, Y., J.S. Moon, K.S. Lee, S.Y. Park, J. Cheong, H.S. Kang, H.Y. Lee, and H.D. Kim. 2004. Ca2+/calmodulin-dependent protein phosphatase calcineurin mediates the expression of iNOS through IKK and NF-kappaB activity in LPS-stimulated mouse peritoneal macrophages and RAW 264.7 cells. *Biochem Biophys Res Commun* 314:695-703.
195. Watanabe, N., J. Suzuki, and Y. Kobayashi. 1996. Role of calcium in tumor necrosis factor-alpha production by activated macrophages. *J Biochem (Tokyo)* 120:1190-1195.
196. Miskin, J.E., C.C. Abrams, and L.K. Dixon. 2000. African swine fever virus protein A238L interacts with the cellular phosphatase calcineurin via a binding domain similar to that of NFAT. *J Virol* 74:9412-9420.
197. Jeffrey, K.L., T. Brummer, M.S. Rolph, S.M. Liu, N.A. Callejas, R.J. Grumont, C. Gillieron, F. Mackay, S. Grey, M. Camps, C. Rommel, S.D. Gerondakis, and C.R. Mackay. 2006. Positive regulation of immune cell function and inflammatory responses by phosphatase PAC-1. *Nat Immunol* 7:274-283.
198. Peng, S.L., A.J. Gerth, A.M. Ranger, and L.H. Glimcher. 2001. NFATc1 and NFATc2 together control both T and B cell activation and differentiation. *Immunity* 14:13-20.
199. Hodge, M.R., A.M. Ranger, F. Charles de la Brousse, T. Hoey, M.J. Grusby, and L.H. Glimcher. 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity* 4:397-405.
200. Kiani, A., J.P. Viola, A.H. Lichtman, and A. Rao. 1997. Down-regulation of IL-4 gene transcription and control of Th2 cell differentiation by a mechanism involving NFAT1. *Immunity* 7:849-860.
201. Porter, C.M., and N.A. Clipstone. 2002. Sustained NFAT signaling promotes a Th1-like pattern of gene expression in primary murine CD4+ T cells. *J Immunol* 168:4936-4945.
202. Al-Daraji, W.I., K.R. Grant, K. Ryan, A. Saxton, and N.J. Reynolds. 2002. Localization of calcineurin/NFAT in human skin and psoriasis and inhibition of calcineurin/NFAT activation in human keratinocytes by cyclosporin A. *J Invest Dermatol* 118:779-788.
203. Liu, J., J.D. Farmer, Jr., W.S. Lane, J. Friedman, I. Weissman, and S.L. Schreiber. 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66:807-815.
204. Bechstein, W.O. 2000. Neurotoxicity of calcineurin inhibitors: impact and clinical management. *Transpl Int* 13:313-326.

205. Olyaei, A.J., A.M. de Mattos, and W.M. Bennett. 2001. Nephrotoxicity of immunosuppressive drugs: new insight and preventive strategies. *Curr Opin Crit Care* 7:384-389.
206. Noguchi, H., M. Matsushita, T. Okitsu, A. Moriwaki, K. Tomizawa, S. Kang, S.T. Li, N. Kobayashi, S. Matsumoto, K. Tanaka, N. Tanaka, and H. Matsui. 2004. A new cell-permeable peptide allows successful allogeneic islet transplantation in mice. *Nat Med* 10:305-309.
207. Kuriyama, M., M. Matsushita, A. Tateishi, A. Moriwaki, K. Tomizawa, K. Ishino, S. Sano, and H. Matsui. 2006. A cell-permeable NFAT inhibitor peptide prevents pressure-overload cardiac hypertrophy. *Chem Biol Drug Des* 67:238-243.
208. Mueller, T., and D.K. Podolsky. 2005. Nucleotide-binding-oligomerization domain proteins and toll-like receptors: sensors of the inflammatory bowel diseases' microbial environment. *Curr Opin Gastroenterol* 21:419-425.
209. Cario, E. 2005. Bacterial interactions with cells of the intestinal mucosa: Toll-like receptors and NOD2. *Gut* 54:1182-1193.
210. 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.
211. Matsuda, J.L., L. Gapin, B.C. Sydora, F. Byrne, S. Binder, M. Kronenberg, and R. Aranda. 2000. Systemic activation and antigen-driven oligoclonal expansion of T cells in a mouse model of colitis. *J Immunol* 164:2797-2806.
212. Takahashi, I., J. Matsuda, L. Gapin, H. DeWinter, Y. Kai, H. Tamagawa, M. Kronenberg, and H. Kiyono. 2002. Colitis-related public T cells are selected in the colonic lamina propria of IL-10-deficient mice. *Clin Immunol* 102:237-248.
213. Farrell, R.J., and J.T. LaMont. 2002. Microbial factors in inflammatory bowel disease. *Gastroenterol Clin North Am* 31:41-62.
214. 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.
215. Fukao, T., and S. Koyasu. 2003. PI3K and negative regulation of TLR signaling. *Trends Immunol* 24:358-363.
216. Ruse, M., and U.G. Knaus. 2006. New players in TLR-mediated innate immunity: PI3K and small Rho GTPases. *Immunol Res* 34:33-48.
217. 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.
218. Vanhaesebroeck, B., S.J. Leevers, G. Panayotou, and M.D. Waterfield. 1997. Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* 22:267-272.
219. 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. P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc Natl Acad Sci U S A* 94:4330-4335.
220. 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.
221. Cho, J.H., D.L. Nicolae, R. Ramos, C.T. Fields, K. Rabenau, S. Corradino, S.R. Brant, R. Espinosa, M. LeBeau, S.B. Hanauer, J. Bodzin, and D.K. Bonen. 2000. Linkage and

linkage disequilibrium in chromosome band 1p36 in American Chaldeans with inflammatory bowel disease. *Hum Mol Genet* 9:1425-1432.
222. Seki, N., Y. Nimura, M. Ohira, T. Saito, S. Ichimiya, N. Nomura, and A. Nakagawara. 1997. Identification and chromosome assignment of a human gene encoding a novel phosphatidylinositol-3 kinase. *DNA Res* 4:355-358.
223. 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.
224. Xiong, H., I. Kawamura, T. Nishibori, and M. Mitsuyama. 1996. Suppression of IFN-gamma production from Listeria monocytogenes-specific T cells by endogenously produced nitric oxide. *Cell Immunol* 172:118-125.
225. Mosnier, J.F., L. Larvol, J. Barge, S. Dubois, G. De La Bigne, D. Henin, and M. Cerf. 1996. Lymphocytic and collagenous colitis: an immunohistochemical study. *Am J Gastroenterol* 91:709-713.
226. Lu, H.T., D.D. Yang, M. Wysk, E. Gatti, I. Mellman, R.J. Davis, and R.A. Flavell. 1999. Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. *Embo J* 18:1845-1857.
227. 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.
228. Goodridge, H.S., W. Harnett, F.Y. Liew, and M.M. Harnett. 2003. Differential regulation of interleukin-12 p40 and p35 induction via Erk mitogen-activated protein kinase-dependent and -independent mechanisms and the implications for bioactive IL-12 and IL-23 responses. *Immunology* 109:415-425.
229. 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.
230. Diaz-Guerra, M.J., A. Castrillo, P. Martin-Sanz, and L. Bosca. 1999. Negative regulation by phosphatidylinositol 3-kinase of inducible nitric oxide synthase expression in macrophages. *J Immunol* 162:6184-6190.
231. Hazeki, K., S. Kinoshita, T. Matsumura, K. Nigorikawa, H. Kubo, and O. Hazeki. 2006. Opposite effects of wortmannin and 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride on toll-like receptor-mediated nitric oxide production: negative regulation of nuclear factor-{kappa}B by phosphoinositide 3-kinase. *Mol Pharmacol* 69:1717-1724.
232. Crawley, J.B., L.M. Williams, T. Mander, F.M. Brennan, and B.M. Foxwell. 1996. Interleukin-10 stimulation of phosphatidylinositol 3-kinase and p70 S6 kinase is required for the proliferative but not the antiinflammatory effects of the cytokine. *J Biol Chem* 271:16357-16362.
233. Pahan, K., M. Khan, and I. Singh. 2000. Interleukin-10 and interleukin-13 inhibit proinflammatory cytokine-induced ceramide production through the activation of phosphatidylinositol 3-kinase. *J Neurochem* 75:576-582.

234. Zhou, J.H., S.R. Broussard, K. Strle, G.G. Freund, R.W. Johnson, R. Dantzer, and K.W. Kelley. 2001. IL-10 inhibits apoptosis of promyeloid cells by activating insulin receptor substrate-2 and phosphatidylinositol 3'-kinase. *J Immunol* 167:4436-4442.
235. Hawlisch, H., Y. Belkaid, R. Baelder, D. Hildeman, C. Gerard, and J. Kohl. 2005. C5a negatively regulates toll-like receptor 4-induced immune responses. *Immunity* 22:415-426.
236. la Sala, A., M. Gadina, and B.L. Kelsall. 2005. G(i)-protein-dependent inhibition of IL-12 production is mediated by activation of the phosphatidylinositol 3-kinase-protein 3 kinase B/Akt pathway and JNK. *J Immunol* 175:2994-2999.
237. Kramer, M., M.G. Netea, D.J. de Jong, B.J. Kullberg, and G.J. Adema. 2006. Impaired dendritic cell function in Crohn's disease patients with NOD2 3020insC mutation. *J Leukoc Biol* 79:860-866.
238. Cheroutre, H. 2005. IELs: enforcing law and order in the court of the intestinal epithelium. *Immunol Rev* 206:114-131.
239. Lundqvist, C., S. Melgar, M.M. Yeung, S. Hammarstrom, and M.L. Hammarstrom. 1996. Intraepithelial lymphocytes in human gut have lytic potential and a cytokine profile that suggest T helper 1 and cytotoxic functions. *J Immunol* 157:1926-1934.
240. Shibahara, T., K. Miyazaki, D. Sato, H. Matsui, A. Yanaka, A. Nakahara, and N. Tanaka. 2005. Alteration of intestinal epithelial function by intraepithelial lymphocyte homing. *J Gastroenterol* 40:878-886.
241. Shibahara, T., J.N. Wilcox, T. Couse, and J.L. Madara. 2001. Characterization of epithelial chemoattractants for human intestinal intraepithelial lymphocytes. *Gastroenterology* 120:60-70.
242. Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177:566-573.
243. Nakae, S., S. Saijo, R. Horai, K. Sudo, S. Mori, and Y. Iwakura. 2003. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc Natl Acad Sci U S A* 100:5986-5990.
244. Albanesi, C., C. Scarponi, A. Cavani, M. Federici, F. Nasorri, and G. Girolomoni. 2000. Interleukin-17 is produced by both Th1 and Th2 lymphocytes, and modulates interferon-gamma- and interleukin-4-induced activation of human keratinocytes. *J Invest Dermatol* 115:81-87.
245. Guha, M., and N. Mackman. 2002. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem* 277:32124-32132.
246. Williams, D.L., C. Li, T. Ha, T. Ozment-Skelton, J.H. Kalbfleisch, J. Preiszner, L. Brooks, K. Breuel, and J.B. Schweitzer. 2004. Modulation of the phosphoinositide 3-kinase pathway alters innate resistance to polymicrobial sepsis. *J Immunol* 172:449-456.
247. 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.
248. 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-kappa B activation and IFN-beta synthesis downstream of Toll-like receptor 3 and 4. *Eur J Immunol* 35:2200-2209.

249. Abell, K., A. Bilancio, R.W. Clarkson, P.G. Tiffen, A.I. Altaparmakov, T.G. Burdon, T. Asano, B. Vanhaesebroeck, and C.J. Watson. 2005. Stat3-induced apoptosis requires a molecular switch in PI(3)K subunit composition. *Nat Cell Biol* 7:392-398.
250. McGovern, D.P., P. Hysi, T. Ahmad, D.A. van Heel, M.F. Moffatt, A. Carey, W.O. Cookson, and D.P. Jewell. 2005. Association between a complex insertion/deletion polymorphism in NOD1 (CARD4) and susceptibility to inflammatory bowel disease. *Hum Mol Genet* 14:1245-1250.
251. Hysi, P., M. Kabesch, M.F. Moffatt, M. Schedel, D. Carr, Y. Zhang, B. Boardman, E. von Mutius, S.K. Weiland, W. Leupold, C. Fritzsch, N. Klopp, A.W. Musk, A. James, G. Nunez, N. Inohara, and W.O. Cookson. 2005. NOD1 variation, immunoglobulin E and asthma. *Hum Mol Genet* 14:935-941.
252. Strober, W., P.J. Murray, A. Kitani, and T. Watanabe. 2006. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol* 6:9-20.
253. Abbott, D.W., A. Wilkins, J.M. Asara, and L.C. Cantley. 2004. The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitinylation of a novel site on NEMO. *Curr Biol* 14:2217-2227.
254. Inohara, N., Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto, M. Hashimoto, S.J. Foster, A.P. Moran, J.L. Fernandez-Luna, and G. Nunez. 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 278:5509-5512.
255. Watanabe, T., A. Kitani, P.J. Murray, and W. Strober. 2004. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol* 5:800-808.
256. Wehkamp, J., and E.F. Stange. 2005. NOD2 mutation and mice: no Crohn's disease but many lessons to learn. *Trends Mol Med* 11:307-309.
257. Kobayashi, K.S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez, and R.A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307:731-734.
258. Kobayashi, K., N. Inohara, L.D. Hernandez, J.E. Galan, G. Nunez, C.A. Janeway, R. Medzhitov, and R.A. Flavell. 2002. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416:194-199.
259. Plytycz, B., and R. Seljelid. 2003. From inflammation to sickness: historical perspective. *Arch Immunol Ther Exp (Warsz)* 51:105-109.
260. Henson, P.M. 2005. Dampening inflammation. *Nat Immunol* 6:1179-1181.
261. 2005. Get the balance right. *Nat Immunol* 6:1177.
262. Gorman, C., A. Park, and K. Dell. 2004. The fires within. *In* Time.
263. Loftus, E.V., Jr. 2004. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 126:1504-1517.
264. Korzenik, J.R., and D.K. Podolsky. 2006. Evolving knowledge and therapy of inflammatory bowel disease. *Nat Rev Drug Discov* 5:197-209.
265. Papadakis, K.A., and S.R. Targan. 2000. Role of cytokines in the pathogenesis of inflammatory bowel disease. *Annu Rev Med* 51:289-298.
266. Stallmach, A., T. Marth, B. Weiss, B.M. Wittig, A. Hombach, C. Schmidt, M. Neurath, M. Zeitz, S. Zeuzem, and H. Abken. 2004. An interleukin 12 p40-IgG2b fusion protein abrogates T cell mediated inflammation: anti-inflammatory activity in Crohn's disease and experimental colitis in vivo. *Gut* 53:339-345.

267. Holtschke, T., J. Lohler, Y. Kanno, T. Fehr, N. Giese, F. Rosenbauer, J. Lou, K.P. Knobeloch, L. Gabriele, J.F. Waring, M.F. Bachmann, R.M. Zinkernagel, H.C. Morse, 3rd, K. Ozato, and I. Horak. 1996. Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. *Cell* 87:307-317.
268. Gerth, A.J., L. Lin, M.F. Neurath, L.H. Glimcher, and S.L. Peng. 2004. An innate cell-mediated, murine ulcerative colitis-like syndrome in the absence of nuclear factor of activated T cells. *Gastroenterology* 126:1115-1121.
269. Gerth, A.J., C.T. Pham, and S.L. Peng. 2004. Regulation of the symmetry and intensity of immune complex-mediated synovitis by nuclear factor of activated T cells. *Arthritis Rheum* 50:3392-3395.
270. Li, H., A. Rao, and P.G. Hogan. 2004. Structural delineation of the calcineurin-NFAT interaction and its parallels to PP1 targeting interactions. *J Mol Biol* 342:1659-1674.
271. Venkatesh, N., Y. Feng, B. DeDecker, P. Yacono, D. Golan, T. Mitchison, and F. McKeon. 2004. Chemical genetics to identify NFAT inhibitors: potential of targeting calcium mobilization in immunosuppression. *Proc Natl Acad Sci U S A* 101:8969-8974.
272. Roehrl, M.H., S. Kang, J. Aramburu, G. Wagner, A. Rao, and P.G. Hogan. 2004. Selective inhibition of calcineurin-NFAT signaling by blocking protein-protein interaction with small organic molecules. *Proc Natl Acad Sci U S A* 101:7554-7559.
273. Roehrl, M.H., J.Y. Wang, and G. Wagner. 2004. Discovery of small-molecule inhibitors of the NFAT--calcineurin interaction by competitive high-throughput fluorescence polarization screening. *Biochemistry* 43:16067-16075.
274. Kagan, J.C., and R. Medzhitov. 2006. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* 125:943-955.
275. 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.
276. Woodgett, J.R., and P.S. Ohashi. 2005. GSK3: an in-Toll-erant protein kinase? *Nat Immunol* 6:751-752.
277. Ogura, Y., L. Saab, F.F. Chen, A. Benito, N. Inohara, and G. Nunez. 2003. Genetic variation and activity of mouse Nod2, a susceptibility gene for Crohn's disease. *Genomics* 81:369-377.
278. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.
279. Mantovani, A., A. Sica, and M. Locati. 2005. Macrophage polarization comes of age. *Immunity* 23:344-346.
280. Rauh, M.J., V. Ho, C. Pereira, A. Sham, L.M. Sly, V. Lam, L. Huxham, A.I. Minchinton, A. Mui, and G. Krystal. 2005. SHIP represses the generation of alternatively activated macrophages. *Immunity* 23:361-374.